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STARD checklist for the reporting of studies of diagnostic accuracy (Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al., for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Intern Med 2003;138:40-4.) (http://www.stard-statement.org/);

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Conclusion: Should summarize the results of the study, the clinical applicability of the results should be defined, and the favorable and unfavorable aspects should be declared.

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Materials and Methods: The study plan should be clearly described, indicating whether the study is randomized or not, whether it is retrospective or prospective, the number of trials, the characteristics, and the statistical methods used.

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ORIGINAL ARTICLE

Development and Validation of an Ultra Performance Liquid Chromatography Method for the Determination of Dexketoprofen Trometamol, Salicylic Acid and Diclofenac Sodium

Deksketoprofen Trometamol, Salisilik Asit ve Diklofenak Sodyum Etkin Maddeleri için Ultra Performanslı Sıvı Kromatografisi Yönteminin Geliştirilmesi ve Validasyonu

Sibel İLBASMIŞ TAMER

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ABSTRACT

Objectives: A simple, fast, accurate and precise method has been developed for the determination of dexketoprofen trometamol (DKP), salicylic acid (SA) and diclofenac sodium (DIC) in the drug solutions using ultra high performance liquid chromatography (UPLC).

Materials and Methods: UPLC method is highly reliable and sensitive method to quantify the amount of the active ingredient and the method is validated according to ICH guidelines.

Results: The developed method is found to be precise, accurate, specific and selective. The method was also found to be linear and reproducible. The value of limit of dedection (LOD) of DKP, SA, DIC were found 0.00325 µg/mL, 0.0027 µg/mL and 0.0304 µg/mL, respectively. The limit of quantitation (LOQ) of DKP, SA and DIC were found 0.00985 µg/mL, 0.0081 µg/mL and 0.0920 µg/mL, respectively.

Conclusion: Proposed methods can be successfully applicable to the pharmaceutical preparation containing the above mentioned drugs (dexketoprofen trometamol, salicylic acid and diclofenac sodium). Even very small amounts of active substance can be analyzed and validations can be performed easily.

Key words: Dexketoprofen trometamol, salicylic acid, diclofenac sodium, UPLC, validation

ÖZ

Amaç: Deksketoprofen trometamol (DKP), salisilik asit (SA) ve diklofenak sodyumun (DIC) ilaç çözeltisindeki analizi için ultra yüksek basınçlı sıvı kromatografisi (UPLC) kullanılarak basit, hızlı, doğru ve kesin bir yöntem geliştirilmiştir.

Gereç ve Yöntemler: UPLC yöntemi aktif bileşikleri analiz etmek için oldukça hassas bir yöntemdir ve yöntem ICH kurallarına göre valide edilmiştir.

Bulgular: Geliştirilen yöntem, kesin, doğru, spesifik ve seçici bulunmuştur. Yöntem, doğrusal ve tekrarlanabilir bulunmuştur. DKP maddesi için teşhis sınırı-duyarlılık sınırı (LOD) 0.00325 µg/mL ve tayin alt sınırı-saptama sınırı (LOQ) 0.00985 µg/mL olarak bulunmuştur. SA için LOD 0.0027 µg/mL ve LOQ 0.0081 µg/mL olarak bulunmuştur. DIC için LOD 0.0304 µg/mL ve LOQ 0.0920 µg/mL olarak bulunmuştur.

Sonuç: Böylece önerilen yöntemler yukarıda bahsedilen ilaçları içeren (deksketoprofen trometamol, salisilik asit ve diklofenak sodyum) farmasötik preparatlarda başarılı bir şekilde uygulanabilecektir. Aktif bileşikler çok küçük miktarlarda analiz edilebilecek ve kolaylıkla valide edilebilecektir.

Anahtar kelimeler: Deksketoprofen trometamol, salisilik asit, diklofenak sodyum, UPLC, validasyon

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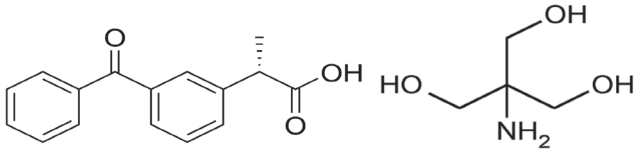
INTRODUCTION

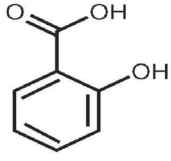
Dexketoprofen trometamol (DKP) chemically, 2-amino-2-(hydroxymethyl) propane-1,3-diol; 2-(3-benzoylphenyl propionic acid is a water-soluble salt of the (S)-(+)- enantiomer of the non-steroidal anti-inflammatory drug (NSAID) ketoprofen.[1] The enantiomer is a relatively new oral NSAID with analgesic, anti-inflammatory and anti-pyretic properties and is one of the most potent *in vitro* inhibitors of prostaglandin synthesis.[2] DKP is a new, quick acting analgesic for the treatment of painful musculoskeletal conditions such as osteoarthritis and low back pain. It is also used as a treatment for post-operative pain, toothache and dysmenorrhea.[3] It is the active optical isomer (eutomer) of ketoprofen, a propionic acid NSAID. The eutomer has been separated to halve the dosage required and halve the metabolic load. The inactive isomer (distomer) has been discarded in the hope of eliminating or reducing potential unnecessary side effects.[4]



Dexketoprofen trometamol[5]

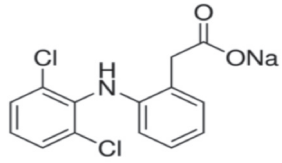
Salicylic acid (SA) is aminohydroxybenzoic acid, a type of phenolic acid and a beta hydroxy acid. It has the formula $C_7H_6O_3$. SA is the most widely consumed analgesic, antipyretic, and anti-inflammatory agent in the World.[6] It is a natural product found in the bark of a willow tree and has been used to relieve fever and pain.[7] SA is a precursor to acetylsalicylic acid, better known as aspirin.[8] SA is used topically for its keratolytic, bacteriostatic, fungicidal, and photoprotective properties. Topical application has been shown to reduce the rate of keratinocyte proliferation. It also inhibits cholesterol sulfotransferase, an enzyme responsible for cholesterol sulfate formation within keratinocytes. SA directly solubilizes the stratum corneum by dissolving the intercellular cement. Through these mechanisms, SA increases the elimination of squames from the stratum corneum.[9] The principal use of topical SA in dermatology is as a keratolytic agent. SA toxicity can occur with topical use of 6% SA over as little as 40% body surface area.[10]

Diclofenac (DIC) sodium is chemically 2-[2-(2,6-dichlorophenylamino)phenyl] acetic acid, a NSAID exhibits anti-inflammatory and analgesic properties. The primary mechanism responsible for its anti-inflammatory, antipyretic, and analgesic action is thought to be inhibition of prostaglandin synthesis by inhibition of cyclooxygenase.



Salicylic acid[8]

It also appears to exhibit bacteriostatic activity by inhibiting bacterial DNA synthesis.[11]



Diclofenac sodium[12]

The chosen data from literature sources are shown in Table 1.[13,14,15] It is well known that DKP is highly soluble in water and class 1 group compound according to the biopharmaceutical classification system. Permeability is also high.[16] SA is class 1 compound, and highly soluble in water.[17] DIC has poor water solubility and high permeability as class 2 compound.[18] The solubility, permeability properties, molecular weights, melting points, ionization constants and octanol-water partition coefficient of active ingredients are different from each other. The ultra performance liquid chromatography (UPLC) methods were developed of these different active ingredients.

Although several high-performance liquid chromatography methods can be found in the literature for DKP, SA and DIC to date, there is a few UPLC methods for aforementioned active ingredients. In the present investigation, a simple, optimized, and validated UPLC methods were proposed for the standardization of DKP, SA, DIC. The aim of this study was to develop and validate an analytical method for DKP, SA, DIC in buffer solution. Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use, to ensure the identity, strength, quality, purity, and potency of the drug substance and drug product.[19,20] Linearity, accuracy, repeatability, specificity, sensitivity and detection limit parameters were validated by examining these parameters.

EXPERIMENTAL

Materials

DKP was purchased Huangshi Shixing Pharmaceutical Co. Ltd. (China). SA was purchased from Botafarma Pharmaceutical Laboratory (Turkey) and DIC was obtained from Fako Pharmaceuticals, Inc. (Düzce, Turkey).

Acetonitrile, methanol, ammonium chloride, monosodium phosphate and disodium phosphate were purchased fom Merck Darmstadt (Germany). Acetone was purchased from Sigma-aldrich, MO, (USA), potassium dihydrogen phosphate was purchased from Reidel-deHaën, (Germany), distilled water (18 MΩ.cm).

Instrumentation

Waters Acquity, UPLC system was used (MA, USA). Inertsil® (ODS-4, GL Sciences, 2 µm, 2.1x50 mm, C/N 5020-81202, S/N OFF50005) was used as a UPLC colon. Diode array dedector was used.

Table 1. Used physicochemical properties of the active ingredients[13,14,15]

	Molecular weight (g/moL)	Topological polar surface area (A2)	Melting point (°C)	Log P (Oct/water)	Pka
Dexketoprofen	375.4	141	104	3.36	3.88
Salicylic acid	138	57.5	159	2.26	2.97
Diclofenac	318	52.2	275	4.40	3.80

Pka: Lonization constant

Ultra performance liquid chromatography assay method and validation of active ingredients

UPLC method was developed to quantify the drug in the saturated drug solutions. Active ingredients were dissolved in 25% pH 7.4 sodium phosphate buffer. To prepare phosphate buffer at pH 7.4; KH_2PO_4 (250 mL, 0.2 M) was prepared, then 0.2 M NaOH (195.5 mL) was mixed and completed to 1 liter with deionized water. Then, dilution was made with deionized water. The purpose of 25% diluting the buffer solution is to reduce ion concentration of solution thereby facilitating the analysis of the active substance. After dilution was checking whether there is change in pH. It was determined that the pH of the environment remains constant.

Ultra performance liquid chromatography assay method and validation of dexketoprofen trometamol

UPLC methods and conditions of DKP was adopted and validated.[2,4,5,21] The method was found to be linear and reproducible. The A solvent was acetonitrile and the B solvent was MeOH/Water (1/1) (v/v). Starting conditions were 98% B, and within two min 30% B was employed. The final condition is 98% B. The temperature was 25˚C. Ultraviolet (UV) absorbance data were collected at 254 nm. The flow rate was 0.25 mL/min. The retention time was 0.734 min. Injection volume was 20 µL.

Ultra performance liquid chromatography assay method and validation of salicylic acid

UPLC method of SA was also adopted from literature and validated.[22,23,24,25] The gradient mobile phase flow was almost same with dexketoprofen analysis. The temperature was 25˚C. UV absorbance data were collected at 292 nm. The flow rate was 0.25 mL/min. The retention time was 0.704 min. Injection volume was 20 µL.

Ultra performance liquid chromatography assay method and validation of diclofenac sodium

UPLC method and conditions for DIC was also adopted.[11,12,26,27] The A solvent was acetonitrile and B solvent was 50 mM acetate buffer (1/1), (v/v) pH 3.1. The column temperature was stable at 25˚C. UV absorbance data were collected at 254 nm. The flow rate was 0.5 mL/min. The retention time was 1.22 min. Injection volume was 20 µL. The method used was found to be reproducible. Injection volume was 20 µL.

Real sample applications

To evaluate the performance of the proposed method, real sample application was performed using commercial tablet, test tablet and commercial eye drop. Test tablets were prepared with SA, lactose, starch 1500, magnesium stearate, aerosil 200. The brand name of commercial DKP tablet is Arveles® 25 mg film tablets. The brand name of commercial DIC eye drop is Inflased® 1%, 5 mL. In this scope, the amounts of DKP, SA and DIC were determined in commercial tablet, test tablet and eye drop, respectively.

Ten tablets (each tablet containing 36.9 mg DKP) were weighed and finally powdered. A portion of powder equivalent to about 36.9 mg DKP was weighed accurately and dissolved completely in exact volume of 369 mL phosphate buffered saline (PBS). Then, solution was stirred for 154 min on a magnetic stirrer. The solution was filtered and diluted with PBS up to mark. 20 µL volume of sample solution was injected into the column.

Ten tablets (each tablet containing 1% SA) were weighed and finally powdered. A portion of powder equivalent to about 1.5 mg SA was weighed accurately and 300 µL dimethyl sulfoxide added, transferred to a 25 mL volumetric flask and stirred with PBS on a magnetic stirrer for 15 min. The solution was filtered and diluted with PBS up to mark. 20 µL volume of sample solution was injected into the column.

Eye drop solution containing 0.1% DIC sodium were used. Total amount of eye drop solution is 5 mL. The solution was filtered and diluted with PBS up to mark. 20 µL volume of sample solution was injected into the column.

RESULTS AND DISCUSSION

The aim of method validation was to confirm that the present method was suitable for its intended purpose as described in International Council for Harmonisation guidelines.[28] The chromatograms of DKP, SA and DIC are given in Figure 1, 2, 3, respectively.

Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample.[5] Calibration curve of DKP was constructed by plotting absorbance versus concentration which showed linearity over the concentration ranges of 0.39-20 µg/mL (Figure 4). Calibration curve of SA was constructed by plotting absorbance versus concentration which showed linearity over the concentration ranges of 0.0061-0.78 µg/mL (Figure 5). Calibration curve of DIC was constructed by plotting absorbance versus concentration which shows linearity over the concentration ranges of 0.0488-100 µg/mL in Figure 6.

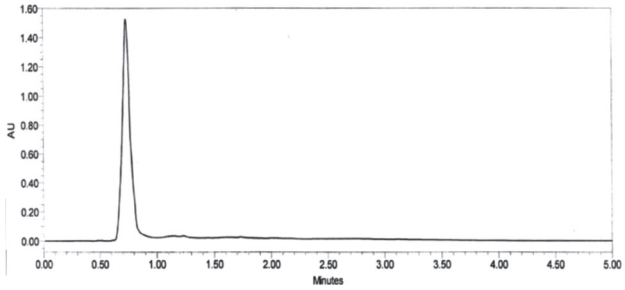


Figure 1. The peak of 1.25 µg/mL concentration of DKP in pH 7.4 phosphate buffer solution, the A solvent was acetonitrile and the B solvent was MeOH/Water (1/1) (v/v), starting conditions were 98% B, and within two min 30% B was employed, the final condition is 98% B, The temperature was 25°C, ultraviolet absorbance data were collected at 254 nm, the flow rate was 0.25 mL/min

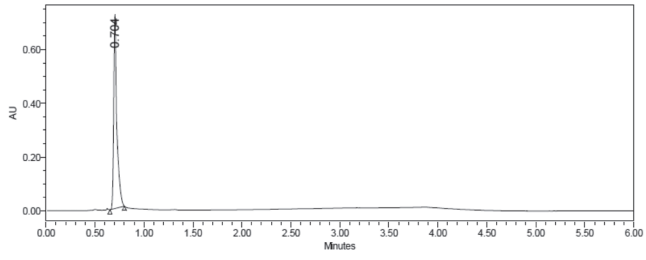


Figure 2. The peak of 1.56 µg/mL concentration of SA in pH 7.4 phosphate buffer solution, the A solvent was acetonitrile and the B solvent was MeOH/Water (1/1) (v/v), starting conditions were 98% B, and within two min 30% B was employed, the final condition is 98% B, the temperature was 25°C, ultraviolet absorbance data were collected at 292 nm, the flow rate was 0.25 mL/min

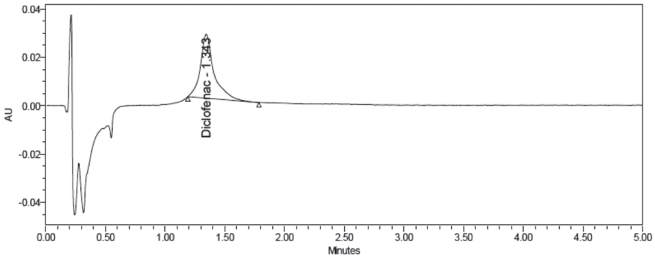


Figure 3. The peak of 3.125 µg/mL concentration of DIC in pH 7.4 phosphate buffer solution, the A solvent was acetonitrile and B solvent was 50 mM acetate buffer (1/1), (v/v) pH 3.1, the column temperature was stable at 25°C, ultraviolet absorbance data were collected at 254 nm

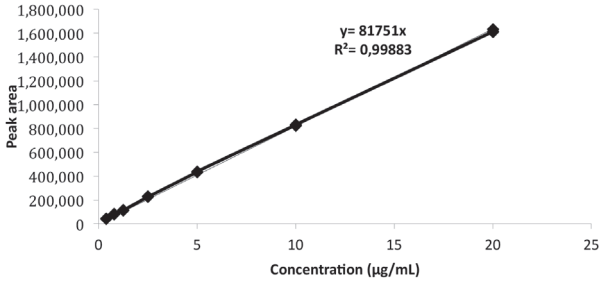


Figure 4. Calibration curve of dexketoprofen trometamol in 25% pH 7.4 phosphate buffer obtained by ultra high performance liquid chromatography method (calibration equations were obtained for a concentration range of 0.39-20 µg/mL)

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value and is defined recovery.[29] As you can see the accuracy results of DKP, SA and DIC in Table 2, 3, 4.

Precision

The precision of an analytical method is the agreement within a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous samples under the same conditions.[19]

Repeatability: In terms of method precision study of our experiment, 15 µg/mL solutions were injected into the system and the percentage of precision was evaluated. Shown in Table 5 shows, the percentage of mean precision value of DKP

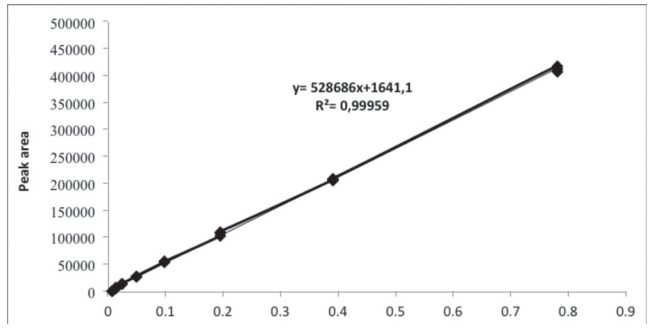


Figure 5. Calibration curve of salicylic acid in 25% pH 7.4 phosphate buffer obtained by ultra high performance liquid chromatography method (calibration equations were obtained for a concentration range of 0.0061-0.78 µg/mL)

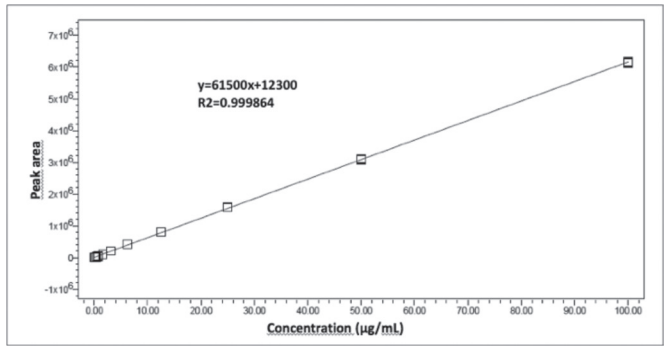


Figure 6. Calibration curve of diclofenac in 25% pH 7.4 phosphate buffer obtained by ultra high performance liquid chromatography method (calibration equations were obtained for a concentration range of 0.0488-100 µg/mL)

Table 2. The calculated recovery amount of DKP from the solution in phosphate buffer

Percentage of DKP in solution	Concentration of DKP in solution (µg/mL)	Recovery of DKP (µg/mL) ± C.I.	Coefficient of variation %
80	2.50	3.01±0.02	0.56
100	3.13	3.85±1.37	1.16
120	3.75	4.56±0.56	0.48

DKP: Dexketoprofen trometamol

concentration level was 108.5 with standard deviation of 1.42. As Table 6 shows, the percentage of mean precision value of SA concentration level was 90.1 with standard deviation of 1.48. As Table 7, 8 shows, the percentage of mean precision value of high DIC concentration and less DIC concentration level were 110, 101 with standard deviation of 1.74, 1.31, respectively. Since the percentage of precision has been found almost 100 and the standard deviation less than the acceptance criteria which is 2%, the analysis system for the determination of assay is verified. Low values of standard deviation denoted very good repeatability of the measurement. Thus it was showing that the equipment used for the study was correct and hence the developed analytical method is highly repetitive.

Reproducibility: To evaluated the reproducibility parameters of DKP stock solution was prepared. Different concentrations of solution were also prepared from the stock solution by dilution. These solutions were measured by UPLC 6 times in 3 consecutive days (Table 9, 10, 11). The obtained to the average of peak heights were calculated standard deviation and standard error of the mean (SEM) values. The average of the concentration, standard deviation and SEM values were calculated. The recovery values from the commercial tablets, test tablets and eye drop, were found to be 103, 114%, 134, 118% and 111, 104% for DKP, SA and DIC respectively. The precision of the chromatographic analysis in tablets and eye drop was determined at two concentrations of each active substance. The coefficients of variation were obtained by repeating the procedure three times for each sample as shown in Table 12, 13, 14.

Table 3. The calculated recovery amount of SA from the solution in phosphate buffer

Percentage of SA in solution	Concentration of SA in solution (µg/mL)	Recovery of SA (µg/mL) ± C.I.	Coefficient of variation %
80	0.31	0.29±1.74	1.96
100	0.39	0.39±0.98	1.03
120	0.47	0.42±1.58	1.84

SA: Salicylic acid

Table 4. The calculated recovery amount of DIC from the solution in phosphate buffer

Percentage of DIC in solution	Concentration of DIC in solution (µg/mL)	Recovery of DIC (µg/mL) ± C.I.	Coefficient of variation %
80	5.00	4.78±1.42	1.85
100	6.25	6.39±1.32	1.78
120	7.50	7.90±1.27	1.46

DIC: Diclofenac

Table 5. The results of precision study for 15.00 µg/mL DKP

Injection number of test solution	Concentration (µg/mL)	Percent value
1	16.60	110.65
2	16.35	109.00
3	16.21	108.09
4	16.08	107.21
5	16.10	107.33
Mean		**108.46**
SD		**1.42**
RSD %		**1.31**

DKP: Dexketoprofen trometamol, RSD: Relative standard deviation, SD: Standard deviation

Specificity/Selectivity

The specificity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample.[19,30]

Specificity was observed that the diluents did not interfere for the detection of DKP, SA or DIC.

Table 6. The results of precision study for 0.47 µg/mL SA

Injection number of test solution	Concentration (µg/mL)	Percent value
1	0.42	90.06
2	0.41	87.31
3	0.42	90.53
4	0.43	90.95
5	0.43	91.61
6	0.42	90.01
Mean		**90.08**
SD		**1.48**
RSD %		**1.64**

SA: Salicylic acid, RSD: Relative standard deviation, SD: Standard deviation

Table 7. The results of precision study for 15.00 µg/mL DIC (obtained in high DIC concentration)

Injection number of test solution	Concentration (µg/mL)	Percent value
1	16.31	108.72
2	16.41	109.37
3	16.57	110.49
4	16.12	107.47
5	16.83	112.23
6	16.69	111.24
Mean		**109.92**
SD		**1.74**
RSD %		**1.58**

DIC: Diclofenac, RSD: Relative standard deviation, SD: Standard deviation

Table 8. The results of precision study for 3.75 µg/mL DIC (obtained in less DIC concentration)

Injection number of test solution	Concentration (µg/mL)	Percent value
1	3.75	99.88
2	3.76	100.33
3	3.85	102.61
4	3.71	98.95
5	3.81	101.53
6	3.80	101.36
Mean		**100.78**
SD		**1.31**
RSD %		**1.30**

DIC: Diclofenac, RSD: Relative standard deviation, SD: Standard deviation

Table 9. The results of reproducibility of DKP solutions

Concentration (µg/mL)	First day	Second day	Third day	Mean	SD	SEM
1.25	1.55	1.44	1.38	1.46	0.09	0.04
2.50	2.46	2.37	2.32	2.38	0.07	0.03
5.00	5.38	5.28	4.75	5.14	0.34	0.15
10.00	10.49	10.06	9.30	9.95	0.61	0.27
20.00	19.20	21.00	19.50	19.90	0.95	0.43

DKP: Dexketoprofen trometamol, SD: Standard deviation, SEM: Standard error of the mean

Table 10. The results of reproducibility of SA solutions

Concentration (µg/mL)	First day	Second day	Third day	Mean	SD	SEM
0.08	0.07	0.10	0.07	0.08	0.02	0.01
0.16	0.14	0.16	0.14	0.15	0.01	0.00
0.31	0.28	0.26	0.28	0.27	0.01	0.01
0.63	0.54	0.54	0.55	0.54	0.00	0.00

SA: Salicylic acid, SD: Standard deviation, SEM: Standard error of the mean

Table 11. The results of reproducibility of DIC solutions

Concentration (µg/mL)	First day	Second day	Third day	Mean	SD	SEM
2.50	2.50	2.50	2.57	2.53	0.04	0.02
10.00	11.60	11.31	10.52	11.14	0.56	0.25
20.00	23.53	23.04	21.10	22.56	1.28	0.57
40.00	47.16	46.16	42.57	45.30	2.41	1.08
80.00	93.95	91.26	85.89	90.37	4.10	1.84

DIC: Diclofenac, SD: Standard deviation, SEM: Standard error of the mean

The limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyses. LOD is defined as the lowest amount of analyze that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyze which can be reproducibly quantitated above the baseline noise.[19,31] LOD was found 0.00325 µg/mL and LOQ was found 0.00985 µg/mL for DKP. LOD was found 0.0027 µg/mL and LOQ was found 0.0081 µg/mL for SA. LOD was found

Table 12. The recovery results of DKP from tablet formulation

The commercial tablet concentration of DKP (µg/mL)	Injection time of test solution	Found concentration of DKP (µg/mL)	% Recovery
1.56	1	1.63	104.49
	2	1.58	101.28
	3	1.59	101.92
	Mean		102.56
	SD		1.70
	RSD %		1.65
12.50	1	14.20	113.60
	2	14.10	112.80
	3	14.30	114.40
	Mean		113.60
	SD		0.80
	RSD %		0.70

DKP: Dexketoprofen trometamol, SD: Standard deviation, RSD: Relative standard deviation

Table 13. The recovery results of SA from tablet formulation

The test tablet concentration of SA (µg/mL)	Injection time of test solution	Found concentration of SA (µg/mL)	% Recovery
0.03	1	0.04	136.52
	2	0.04	133.11
	3	0.04	133.11
	Mean		134.24
	SD		1.97
	RSD %		1.47
0.47	1	0.57	121.60
	2	0.58	123.09
	3	0.52	110.29
	Mean		118.33
	SD		7.00
	RSD %		5.91

SA: Salicylic acid, SD: Standard deviation, RSD: Relative standard deviation

Table 14. The recovery results of DIC sodium from eye drop formulation

The commercial eye drop concentration of DIC (µg/mL)	Injection number of test solution	Found concentration of DIC (µg/mL)	% Recovery
1.56	1	1.75	112.18
	2	1.72	110.26
	3	1.73	110.90
	Mean		111.11
	SD		0.98
	RSD %		0.88
50.00	1	51.50	103.00
	2	52.40	104.80
	3	51.80	103.60
	Mean		103.80
	SD		0.92
	RSD %		0.88

DIC: Diclofenac, SD: Standard deviation, RSD: Relative standard deviation

0.0304 µg/mL and LOQ was found 0.0920 µg/mL for DIC.

LOD= (3.3xσ)/S

LOQ= (10xσ)/S

σ: The standard deviation of the lowest concentration in the calibration range,

S: The slope of the calibration curve (to find the slope of the calibration curve has equation common calibration and taken his slope).

CONCLUSION

A simple, precise, accurate, reproducible, highly sensitive and effective stability indicating UPLC method was developed and validated for quantitative determination of DKP, SA and DIC. The method was validated for accuracy, precision, specificity, and linearity. The developed method has LOD and LOQ values are 0.00325 µg/mL and 0.00985 µg/mL for DKP, respectively. The LOD and LOQ values are 0.0027 µg/mL and 0.0081 µg/mL for SA, respectively. The LOD and LOQ values are 0.0304 µg/mL and 0.0920 µg/mL for DIC, respectively. In this study, the high recovery and low relative standard deviation confirm the suitability of the method for determination of DKP, SA and DIC in pharmaceutical dosage forms. In conclusion, this method can be used for the routine determination of DKP, SA and DIC in pure and pharmaceutical formulations.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

In Vivo Effects of Naringenin and Lead on Rat Erythrocyte Carbonic Anhydrase Enzyme

Naringenin ve Kurşunun Sıçan Eritrosit Karbonik Anhidraz Enzimi Üzerine *In Vivo* Etkileri

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ABSTRACT

Objectives: Carbonic anhydrase (CA) enzyme catalyses the reversible reactions of CO_2 with water and takes part in metabolically important events such as systemic acid-base regulation and respiration. In this study, *in vivo* effects of lead, which is a heavy metal and to which living beings are exposed by different ways, with naringenin, a flavanone, were investigated.
Materials and Methods: For this purpose, four different rat groups were established and one of them was chosen as the control group. The other three groups were given lead, naringenin and lead+naringenin substances to analyze the changes in the CA enzyme of rat erythrocytes.
Results: The research findings showed that the enzyme activity in the control group was higher than that in the other groups. The naringenin group showed the highest inhibition effect, while the lead group showed the lowest inhibition.
Conclusion: Therefore, it can be said that naringenin is a strong inhibitor of the CA enzyme.
Key words: Carbonic anhydrase, inhibition, lead, naringenin

ÖZ

Amaç: Karbonik anhidraz (CA) enzimi CO_2'nin su ile tersinir reaksiyonunu katalizler ve sistemik asit baz dengesi, solunum gibi metabolik açıdan oldukça önemli olaylarda görev alır. Bu çalışmada canlıların farklı yollarla maruz kaldığı bir ağır metal olan kurşun ile bir flavanon olan naringeninin enzim üzerine *in vivo* etkisi araştırıldı.
Gereç ve Yöntemler: Bu amaçla, dört farklı sıçan grubu oluşturuldu ve bunlardan biri kontrol grubu olarak belirlendi. Diğer üç gruba ise kurşun, naringenin ve kurşun+naringenin maddeleri verildi ve eritrositlerdeki CA enzim aktivitesindeki değişimler incelendi.
Bulgular: Araştırma bulguları kontrol grubundaki enzim aktivitesinin diğer gruplardan daha fazla olduğunu gösterdi. Kurşun grubu en düşük inhibisyon etkisini gösterirken, narigenin grubu en yüksek inhibisyon etkisini gösterdi.
Sonuç: Bu nedenle naringeninin CA enzimi için kuvvetli bir inhibitör olduğu söylenebilir.
Anahtar kelimeler: Karbonik anhidraz, inhibisyon, kurşun, naringenin

INTRODUCTION

Carbonic anhydrase (CA) is a metalloenzyme found in all organisms and it contains a Zn^{+2} ion in the active site (carbonate hydrolase E.C.4.2.1.1). Discovered first from cattle erythrocytes, CA is a significant enzyme which reversibly catalyzes CO_2 hydration and HCO_3- dehydration reactions in living beings.[1] This reaction is involved in imported physiological and pathological processes such as respiration, transport of CO_2, pH and CO_2 homeostasis, some biosynthetic reactions, calcification and tumorigenicity.[2]

Sixteen isozymes of this zinc-attached enzyme family are defined according to inhibitor differences, catalytic activities and their places in the cell. Some of these isoenzymes are cytosolic (CA I, CA II, CA III, CA VII and CA XIII), some are attached to membrane (CA IV, CA IX, CA XII and CA XIV), two are mitochondrial (CA VA and CA VB) and one is secretory (CA VI). CA XV isoform is not expressed in humans and other anthropoids, while expression is very commonly found in rats and other higher vertebrates.[3,4] Many of this CA isozymes are therapeutic targets to fight some disorders like oedema, glaucoma, obesity, cancer, epilepsy and

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osteoporosis. Also, it is emphasized that CA inhibitors may be used for treatment of infections caused by protozoa, fungi and bacteria.[2]

Flavanone compounds are antioxidant substances which are widely used. Naringenin is a flavanone compound, and can be found in nature in the leaves, flowers and stems of plants used as herbal teas in various fields of medicine, food chemistry and biochemistry.[5] As a natural flavonone, naringenin (4',5,7-trihydroxy-flavanone-7-ramnoglucosidet) is found in rich amounts in citrus, tomato, fruits, strawberry, grapefruit and cacao. There is a great deal of research on the antioxidant, anti-tumor, anti-inflammatory and hepato-protective effects of naringenin. Naringenin's structural similarity to silymarin, and quercetin improves its use as an antioxidant against arsenic toxicity and also as a chelate agent.[6,7] Naringenin is pharmacologically accepted as a potential antioxidant, and its anticarcinogenic, antiaterogenic, hepatoprotective, nephroprotective, and antimutagenic activities have been reported.[8,9]

Lead is a metal found in the air, in many foods, drinking water, soil and thus, also in humans. The increase of lead levels in the body inhibits certain physiological processes and leads to toxicity of the cardiovascular and hematopoietic systems.[10,11]

Although there are studies concerning *in vitro* inhibition effects of heavy metals on CAs, there are limited data *in vivo* effects of them. Also, no data on the *in vivo* effect of naringenin on CA activity has been detected in the literature. Therefore, the aim of this study was to determine the effects of naringenin, lead, and naringenin+lead combination on the activity of rat erythrocyte CA.

EXPERIMENTAL

Chemicals

Protein assay reagents and 4-nitrophenylacetate (NPA) were obtained from Sigma-Aldrich Co. The other chemicals were of analytical grade and obtained from Merck.

Preparation of the hemolysate

Fresh rat blood samles were collected in tubes containing ethylenediamine tetraacetic acid, then centrifuged (15 min, 2.500xg) and plasma and buffy coat (leucocytes) were removed. The packed red cells were washed three times with physiological serum, homolyzed with 5 volume of ice-cold water and then centrifuged (10.000xg, for 30 min) to remove the ghost and intact cells.

Animals and treatment

Twenty eight healthy adult male Wistar albino rats (240±40 g body weight) were used in this study. The animals were obtained from Fırat University Experimental Research Centre, Elazığ, Turkey. They have been kept at 21±1°C with a 12-h light/dark cycle and have been given a commercial pellet diet (Elazığ Food Company, Elazığ, Turkey) and fresh drinking water ad libitum. Animal use protocol was approved by the National Institute of Health and Local Committee on Animal Research (Number: 03.03.2011-59). The rats were randomly divided into four groups with each group containing six rats.

First group: Control (C),

Second group: Naringenin alone administered group (N),

Third group: Lead acetate alone administered group (L),

Fourth group: Naringenin+Lead acetate (N+L) administered group.

Naringenin was dissolved in corn oil and administered to animals by gavage at the dose of 50 mg/kg body weight. Wistar male rats were treated with lead acetate (500 ppm) through drinking water for a period of 4 weeks. The last dose was administered 12 h before the operation.[7,12]

Protein determination

Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method[13], with bovine serum albumin being used as a standard.

Esterase activity assay

The esterase activity was assayed by following the change in absorbance of 4-NPA to 4-nitrophenylate ion at 348 nm over a period of 3 min at 25°C using a spectrophotometer (BECKMAN COULTER UV-VIS) according to the method described by Verpoorte et al.[14] The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1.0 mL 3 mM 4-NPA, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

Statistical analysis

Statistical analysis data were analyzed using one-way analysis of variance followed by a post hoc Tukey's test. All results were presented as mean ± standard error mean (SEM). For all analyses, $p<0.05$ was accepted as statistically significant.

RESULTS

In this study, we determined CA enzyme average specific activity as 0.006±0.00023, 0.0035±0.00013, 0.0051±0.00028 and 0.0038±0.00013 in C, N, L, N+L groups respectively.

Values are expressed as mean ± SEM; n=6 for each treatment group A (a: $p<0.01$), (b: $p<0.001$). Statistical significancy compared to the C group (c: $p<0.001$). Statistical significancy compared to the L group.

Naringenin and lead acetate decreased CA enzymes activity in erythrocyte ($p<0.001$, $p<0.01$) when compared to the C group. No statistically significant difference was observed in CA enzymes activity among N and L+N groups ($p>0.05$) (Figure 1).

DISCUSSION

The administration of naringenin to experimental animals grants a protection against illnesses. For example, naringenin antioxidant is known to have protective effects against inflammation, thrombosis, tumorogenesis, atherosclerosis and hypercholesterolemia.[6] Another study shows that naringenin which is extracted from citrus peels crosses the blood-brain

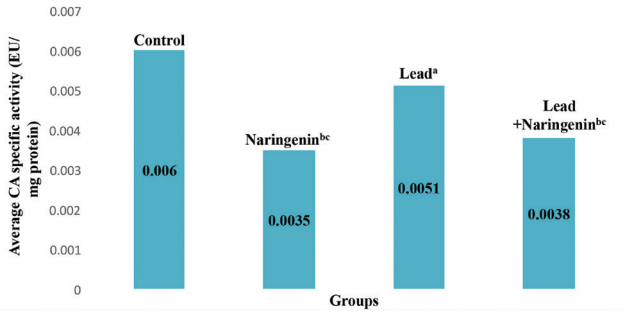

Figure 1. Specific carbonic anhydrase activity of groups

Values are expressed as means ± standard error; n=6 for each treatment group, Statistical significancy compared to the control group. a: p<0.01, b: p<0.001, Statistical significancy compared to the lead acetate group. c: p<0.001

CA: Carbonic anhydrase

barrier in rats. Due to this property, naringenin can be used to repair the nervous system.[15] Moreover, it is found that naringenin inhibits liver cell leakage, lipid peroxidation and protection oxidation, increases the level of enzymatic and non-enzymatic antioxidants and also inhibits DNA damage.[6] Naringenin can inhibit iron-dependent Fenton reaction by chelating with iron ions, there by reducing the formation of hydroxyl radicals. Because of the electron providing and radical-binding property of the 4'-hydroxyl group on its ring, naringenin is proven to remove free radicals effectively. This protects the cell against free radical attacks, and inhibits lipid peroxidation.[8] However, many studies show that various flavonoids strongly inhibit the CA isozymes under *in vitro* conditions.[16,17] In the previous study conducted by Ekinci et al.[17] and co-workers, it was determined that quercetin, apigenin, luteolin and morin which are a flavanone inhibited human CA I and II isozyme with K_i values 3.6-2.4 μM; 4.1-2.7 μM; 2.2-0,74 μM and 12.8-4.4 μM, respectively.

Due to the fact that metals reduce the amounts of phosphate, calcium, glycogen and protein in kidneys, fatal results may occur in mammals.[18] Heavy metals affect humans and particularly the brains of babies during the post-natal stage. Compared to other organs, the forming of the brain and its growth takes longer. Brain growth continues during the post-natal stage.[19]

Many *in vitro* and *in vivo* studies show that reactive oxygen species arising out of lead, namely hydroperoxides, lead to an increase in singlet oxygen, hydrogen peroxide and superoxide radicals. Many studies show that lead changes the composition of fatty acids in the cell membrane structure. It is also known to increase the level of malondialdehyde (MDA) in the liver and MDA is a significant oxidative stress indicator. It is found that puerarin, a flavonoid derivative, reduces the growth of radicals and MDA levels in liver tissues of rats administered lead.[20] Moreover, it is observed that lipid peroxidation products and oxidative stress increased by a considerable amount in the brain tissues of rats administered 500 ppm lead.[12] In addition, researchers have frequently reported the effects of heavy metal ions on different enzymes in previous years and inhibition effect of heavy metals on metabolically imported enzyme such as CA[21], glucose 6-phopsphate dehydrogenase[22], cytochrome P450 reductase[23], glutathione S-transferase[24] have been determined. For example, Ekinci et al.[17] and co-workers mentions the inhibition effects of lead, copper, cobalt heavy metals on cytosolic human CA I and II enzyme activities.[21] Heavy metals have enzyme inhibition property by establishing a bond with the sulfhydryl groups of proteins.[25]

Literature shows that many substances like medical drugs, various metals, anions and pesticides have inhibitory effects on CAs.[21,26,27,28] In this study, the effects of lead and naringenin were examined. A comparison of the enzyme activity results of the groups shows that both lead and naringenin inhibit the CA enzyme in erythrocytes. This study shows that, compared with CA activity in control group, naringenin is an effective inhibitor. Previous studies also indicate that flavonoids similar to naringenin perform a powerful inhibition on CA. Similarly, lead is also found to inhibit the CA enzyme. It is also evident in the literature that lead and other metal ions also inhibit the CA enzyme. However, the inhibition value of naringenin is found to be higher than that of lead and L+N group. In our previous study, inductively coupled plasma-mass spectrometry device measurements in four different groups show that the lead levels of the control, naringenin, lead and L+N groups are 44.88, 36.706, 172.36 and 95.076 ppm respectively.[29] Because a portion of naringenin reacts with lead in a L+N application, the observed inhibition value of the L+N group was found to be lower than that of naringenin.

CONCLUSION

Consequently, the results obtained from the study showed that lead and naringenin inhibited significantly the CA activity in erythrocytes. In this study, we observed that the interaction between lead and naringenin has no significant impact about inhibition effect of naringenin on CA activity. According to results, it may be said that investigation of naringenin on other CA isozymes and synthesis naringenin derivatives are important to improve new and stronger CA inhibitors.

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Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Antibiotic Resistance Profiles and Genotypes of *Acinetobacter baumannii* Isolates and *In Vitro* Interactions of Various Antibiotics in Combination with Tigecycline and Colistin

Acinetobacter baumannii İzolatlarının Antibiyotik Direnç Profili ve Genotipleri ile Tigesiklin ve Kolistinin Çeşitli Antibiyotiklerle Kombinasyonlarının *İn Vitro* Etkileşimleri

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ABSTRACT

Objectives: The aim of this study was to determine the antibiotic resistance profile, clonal relation and efficacy of antibiotic combinations in nosocomial multidrug resistant (MDR) *Acinetobacter baumannii*.

Materials and Methods: Antibiotic susceptibilities of 84 MDR *A. baumannii* against tigecycline (TGC), colistin (CL), amikacin (AK), ciprofloxacin (CIP), meropenem (MR), moxifloxacin (MXF), rifampicin (RF) were determined by microdilution method. Clonal relationship was investigated by genotyping using AP-PCR and antibiotyping. Interactions of antibiotic combinations were tested against clonally unrelated strains by the checkerboard (CB) method. The efficacy of the best combinations was also assesed on a selected isolate by the time-kill (TK) method.

Results: CIP, RF, MXF, MR, AK resistance was found as 90.47%; 47.62%; 22.62%; 58.33%; 50% respectively; however; CL and TGC were not ascertained. The isolates were distinguished as 25 different antibiotypes and 15 varied molecular patterns. The best synergistic effect was detected in combinations of CL with RF (100%) and MR (100%), in combinations of TGC with RF (53%) against clonally unrelated 15 MDR *A. baumannii* isolates by the CB method. While CL-RF and CL-MR showed synergy by TK method like CB, on the other hand TGC-RF indicated additive interactions by TK.

Conclusion: In this study, both synergy tests showed that CL in combination with RF would be a good option in MDR *A. baumannii*.

Key words: *Acinetobacter*, AP-PCR, checkerboard, time-kill, tigecycline, colistin

ÖZ

Amaç: Bu çalışmada nozokomiyal çoklu ilaç dirençli (ÇİD) *Acinetobacter baumannii* izolatlarının antibiyotik direnç profilinin belirlenmesi, moleküler düzeyde tiplendirmelerinin yapılması ve dirençli izolatlarda antibiyotik kombinasyonlarının aktivitesinin araştırılması amaçlandı.

Gereç ve Yöntemler: Seksen dört ÇİD *A. baumannii* izolatına karşı tigesiklin (TGC), kolistin (CL), amikasin (AK), siprofloksasin (CIP), meropenem (MR), moksifloksasin (MXF) ve rifampisinin (RF) minimum inhibitör konsantrasyon (MİK) değerleri sıvı mikrodilüsyon yöntemi ile belirlendi. Epidemiyolojik ilişki, AP-PZR ve antibiyotiplendirme ile saptandı. Klonal ilişkisiz kökenlere antibiyotik kombinasyonlarının etkinliği dama tahtası (CB) yöntemi ile belirlendi. Dama tahtası yöntemi sonucunda, en etkin gözlenen kombinasyonun etkinliği, seçilmiş bir kökene karşı zamana bağlı öldürme eğrisi (TK) yöntemi ile de araştırıldı.

Bulgular: CIP, RF, MXF, MR, AK'nin direnç oranları sırasıyla; %90.47; %47.62; %22.62; %58.33; %50 olarak belirlendi. TGC ve CL direnci görülmedi. Antibiyotik direnç profillerine göre 25 antibiyotip grubu belirlenirken, 15 farklı patern ayırt edildi. Klonal ilişkisiz 15 ÇİD *A. baumannii* izolatında CB yöntemiyle en iyi sinerjistik etki CL-RF (%100), CL-MR (%100) ve TGC-RF (%53) kombinasyonlarında gözlendi. Seçilmiş bir kökende TK yöntemiyle CL-RF ve CL-MR ile sinerji gözlendi, TGC-RF ile ise aditif etki saptandı.

Sonuç: Bu çalışmada her iki sinerji testi de ÇİD *A. baumannii* izolatlarına karşı CL ile RF kombinasyonunun tedavide iyi bir seçim olacağını işaret etmiştir.

Anahtar kelimeler: *Acinetobacter*, AP-PZR, dama tahtası, zamana bağlı öldürme eğrisi, tigesiklin, kolistin

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INTRODUCTION

Acinetobacter baumannii is an opportunistic pathogen and causes hospital acquired infections such as bacteremia, septicemia, and ventilator associated pneumonia.[1,2,3] Before the 1970s, *A. baumannii* was susceptible to most traditional antibiotics such as broad spectrum beta-lactams, cephalosporins and tetracyclines. In recent years *Acinetobacter* related infections have been eradicated with difficulty in general by using single antimicrobial agent because of the ability of the bacteria to develop resistance. Especially multidrug resistant (MDR) *A. baumannii* has been continuously reported as nosocomial pathogen which causes significant morbidity and mortality in critically ill patients.[4,5]

Carbapenems are considered the drugs of choice for the treatment of serious infections caused by MDR *A. baumannii*. However carbapenem-resistant *A. baumannii* has been reported worldwide.[6] As a result of this increasing carbapenem resistance, alternative antibiotic classes have become part of treatment.[1] Of these alternative classes, polymyxin and tigecycline (TGC) remain the most active treatments *in vitro* against MDR *A. baumannii* but resistance against these antibiotics is also reported.[4,6,7,8] Combination therapy is often used in the treatment of MDR *A. baumannii* infections to prevent the emergence of resistance and obtain a synergistic effect.[1,3,8]

The aim of this study was to determine the antimicrobial resistance profile, clonal relation and efficacy of antimicrobial combinations on nosocomial MDR *A. baumannii*.

EXPERIMENTAL

Microorganisms

Identifications and antibiotic susceptibilities of *Acinetobacter* spp. isolates were elicited by Phoenix TM 100 (BD, United States) at the Clinical Microbiology Laboratory of İzmir Katip Çelebi University Atatürk Training and Research Hospital, between 2009-2010, and 84 MDR *Acinetobacter* spp. isolates were selected for the study. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

Determination of minimum inhibitory concentrations and antibiotypes

Minimum inhibitory concentrations (MICs) of TGC (Pfizer, İstanbul, Turkey), colistin (CL) (Sigma-Aldrich, USA), amikacin (AK) (Eczacıbaşı, İstanbul, Turkey), ciprofloxacin (CIP) (Koçak, İstanbul, Turkey), meropenem (MR) (Astra Zeneca, İstanbul, Turkey), moxifloxacin (MXF) (Bayer, İstanbul, Turkey) and rifampicin (RF) (Koçak, İstanbul, Turkey) antibiotics against the isolates were indicated by broth microdilution method according to "Clinical and Laboratory Standards Institute (CLSI)".[9] Because, CLSI has not suggested available breakpoints, the United States Food and Drug Administration (2005) susceptibility breakpoints for TGC, and the recommendations of Principe et al.[7] for MXF and RF were utilized. By considering MIC values, the isolates were classified into different antibiotype groups.

Molecular typing

Epidemiologic relations were investigated genetically with M13 universal primers by Arbitrarily-primed polymerase chain reaction (AP-PCR).[10,11] To obtain crude DNA extracts, freshly cultured bacterial colony suspensions in sterile distilled water were heated for 10 min at 95°C, cooled on ice, and centrifuged at 12.000 g. For totally 50 µL volume PCR mix, 2 mM primers, 200 µM dNTP's together with 1 U of *Taq* polymerase (Fermentas) were used. M13 primers [5'-GTA AAA CGA CGG CCA GTG AA-3' (forward amplification primer) and 5'-GGA AAC AGC TAT GAC CAT GA-3' (reverse amplification primer)] were purchased from Fermentas. The PCR conditions were as follows: 2 cycles of 94°C for 5 min, 40°C for 5 min, 72°C for 5 min followed by 40 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min. PCR end products were examined by a ultraviolet transilluminator after electrophoresis for 60 min on 1.5 percent (wt/vol) agarose gels. The genotypes were interpreted according to the band patterns of DNA marker.

Efficacy of the antibiotic combinations

Chequerboard (CB) method: Activity of TGC and CL combination, and combinations of these antibiotics individually with AK, CIP, MR, MXF, RF against clonally unrelated 15 MDR *A. baumannii* isolates were tested. *In vitro* interactions by fractional inhibitory concentration index (FICI) of each agent was calculated as a ratio of MIC when used in combination and MIC when used alone. For each antibiotic, seven concentrations (8X MIC, 4X MIC, 2X MIC, MIC, MIC/2, MIC/4 ve MIC/8) were investigated. FICI were interpreted as synergistic, indifference, additivity and antagonism. FICI was interpreted as follows: synergy FICI≤0.5, additivity/indifference 0.5<FICI≤4 and antagonism FICI>4.0.[7,12]

Time-kill (TK) method: Effective combinations found by the CB method were also approved by TK method against one of the test isolates. TK studies were performed in flasks containing Mueller Hinton Broth (Merck, Darmstadt, Germany) at 37°C. Samples were removed at 0th, 6th, and 24th h of incubation from the test and growth-control cultures and appropriately diluted and inoculated onto Mueller-Hinton Agar (Merck, Darmstadt, Germany) plates. After incubation at 37°C for 24-48 h, bacterial colonies were counted. All TK studies were performed twice. Synergy or antagonism was defined as an increase or decrease of at least 100-fold compared to the effect of the most active agent singly and an increase of 100 times less than additive interaction.[12]

RESULTS

While 25 different antibiotypes were observed according to antibiotic resistance profiles of 84 MDR *Acinetobacter* spp. isolates, 15 different patterns were distinguished by AP-PCR with M13 primers. Thirty six (43%) isolates showed similar genetic pattern and 22 of these isolates were found in the same resistance pattern according to antibiotype groups and the band patterns of 50 bp DNA marker (Table 1) (Figure 1).

Resistance rates in 84 MDR *Acinetobacter* spp. isolates against CIP, RF, MXF, MR, AK were found as 90.47%; 47.62%; 22.62%; 58.33%; 50% respectively. CL and TGC resistance were not found.

CL in combination with MR and RF demonstrated higher levels of synergy than the other antibiotics according to the CB method. As shown in Table 2, the best synergistic effect was detected in the CL combinations for CL-RF (100%), CL-MR (100%), in the TGC combinations for TGC-RF (53%) combinations. The lowest synergy was seen in the CL combinations for CL-AK (47%) and seen in the TGC combinations for TGC-CIP (20%). Generally, the combinations with TGC demonstrated a higher rate of additive interaction in compared with the CL combinations. Antagonistic interaction was observed between TGC-CL (20%), TGC-AK (6%), TGC-MXF (6%) and CL-CIP (6%) (Table 2).

TGC-RF combination (0.015-2 µg/mL) indicated synergy according to the CB method, but it indicated additive interactions by the TK method. Besides, CL-RF (0.06-0.25 µg/mL) and CL-MR (0.03-0.12 µg/mL) combinations showed a synergistic effect when considering both CB and TK methods. TGC-CL combination (0.03-0.5 µg/mL) was found as additive according to the CB method, but that combination demonstrated synergistic effect at 3th and 6th hours and an additive effect at 24 h as to the TK method. The synergistic effect of CL-RF (0.06-0.25 µg/mL) combination by the TK method was demonstrated in the Figure 2.

Table 1. The distribution of *A. baumannii* isolates according to antibiotypes and genotypes

	Genotypes														
Antibiotypes	P_A	P_B	P_C	P_D	P_E	P_F	P_M	P_N	P_O	P_P	P_Q	P_S	P_X	P_Y	P_Z
A_A	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-
A_B	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-
A_C	3	-	-	2	1	-	-	-	-	-	-	-	-	-	-
A_D	-	1	-	-	-	-	-	-	-	2	-	-	-	-	-
A_E	22	-	-	-	-	3	-	-	1	-	-	-	-	-	-
A_F	1	1	-	-	-	1	-	-	-	-	-	-	-	-	-
A_G	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A_H	-	-	-	-	-	-	-	-	1	-	1	2	-	-	1
A_I	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-
A_J	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
A_K	-	1	-	-	-	-	1	1	-	-	-	-	-	-	-
A_L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A_M	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
A_N	6	-	-	2	-	-	-	-	-	-	-	1	-	-	-
A_O	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A_P	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
A_Q	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
A_R	-	-	-	2	-	1	-	-	-	-	-	-	-	-	1
A_S	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A_T	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A_X	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A_U	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-
A_V	-	-	-	-	-	-	1	1	-	-	-	-	-	1	-
A_Y	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
A_Z	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Total	35	10	1	9	1	7	3	3	4	2	1	3	1	2	2

-: There is no isolate, A: Antibiotypes, P: Genotypes

DISCUSSION

Antibiotic susceptibility testing and molecular typing are necessary for the monitoring and treatment of infections caused by *Acinetobacter* species that become resistant against many antibiotics easily through more than one mechanism. AP-PCR is a quite common PCR-based genotyping method currently because it is easily applicable, is easily distinguishable and provides quick results. It is reported in some studies that the use of M13 universal primers in the AP-PCR generates more distinct band patterns, gives good results and has quite good distinguishing ability is quite good.[13,14] In this study 15 different band patterns were detected according to AP-PCR method by using M13 universal primers. Almost 8-15 bands were obtained after the gel electrophoresis of the PCR products. The number of the bands was found sufficient to determine clonally associated strains. To see the reproducibility of the results, the trials were triplicated. Similar to recent studies,[10,15] our results showed intense clonal spread of resistant *A. baumannii* strains in the intensive care units (80%), particularly. The isolates in the same or close antibiotic resistance patterns were often observed in the same clonal group according to molecular classification with AP-PCR. It was considered that isolation dates and locations in the hospital of *A. baumannii* isolates resulted in different molecular clones.

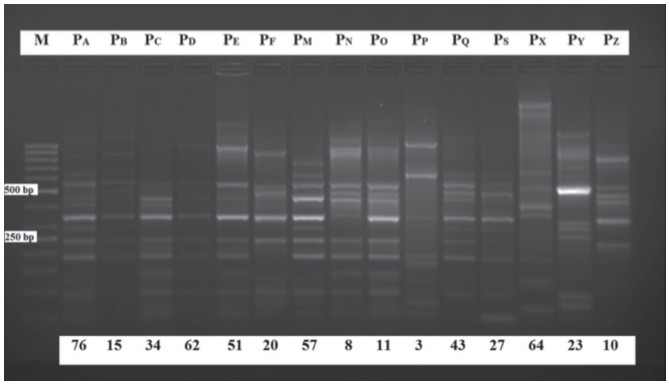

Figure 1. Genotypes of A. *baumannii* isolates to 15 different band patterns obtained by arbitrarily primed-polymerase chain reaction with M-13 primers

M: 50 bp DNA marker

Table 2. ***In vitro*** **effects of antibiotic combinations against 15** ***A. baumannii*** **isolates according to the checkerboard method**

Antibiotic combinations	Synergy n (%)	Additive n (%)	Ineffective n (%)	Antagonism n (%)
CL-MR	15 (100)	0	0	0
CL-RF	15 (100)	0	0	0
CL-CIP	9 (60)	1 (6)	4 (27)	1 (6)
CL-MXF	9 (60)	3 (20)	3 (20)	0
CL-AK	7 (47)	8 (53)	0	0
TGC-MR	7 (46)	6 (40)	2 (14)	0
TGC-RF	8 (53)	2 (14)	5 (33)	0
TGC-CIP	3 (20)	3 (20)	9 (60)	0
TGC-MXF	6 (40)	4 (27)	4 (27)	1 (6)
TGC-AK	4 (27)	6 (40)	4 (27)	1 (6)
TGC-CL	7 (46)	1 (6)	4 (27)	3 (20)

CL: Colistin, MR: Meropenem, RF: Rifampicin, CIP: Ciprofloksasin, MXF: Moxifloxacin, AK: Amikacin, TGC: Tigecycline, n: Number of isolate, synergy FICI ≤0.5, additivitive/indifference 0.5< fractional inhibitory concentration index ≤4 and antagonism fractional inhibitory concentration index >4.0

The most important issue in the *Acinetobacter* infections is the development of resistance against many kinds of antibiotics including primarily preferred carbapenems, CL and sulbactam.[16] Due to the difficulties in the treatment of hospital-acquired infections, broad spectrum antibiotics are commonly chosen. Thus, causative strains become resistant easily. Consequently, new treatment alternatives have been required. TGC, a promising semisynthetic tetracycline, is considered as first choice of the new drugs for the therapy of carbapenem-resistant *A. baumannii* infections. This broad-spectrum antibiotic is shown to be highly effective against MDR *Acinetobacter* spp. isolates.[16-19] However, several studies have currently reported decreased susceptibility to TGC. Certain *in vivo* and *in vitro* researches also highlighted developed resistance to sub-MIC concentrations of TGC. CL is shown as the only effective antibiotic against MDR *A. baumannii* in many countries that have not used TGC yet. In recent years, CL again has become a current issue for the treatment of infections caused by *Acinetobacter* species resistant to all antibiotics except CL. However, there are some major disadvantages of CL when used alone, because of its pharmacokinetic properties, side effects and fast, easy improvement of resistance. Thus, combined use of antibiotics is recommended to prevent the development of resistance and increase the success in the treatment of MDR *A. baumannii* infections since.[7,19,20]

Recent studies in Turkey found that imipenem-netilmicin,[21] RF-ampicillin/sulbactam,[22] CL-TGC,[23] CL-vancomycin[3] in combination showed synergistic interaction, and they were considered as strong choices in the treatment of infections caused by MDR *A. baumannii*.

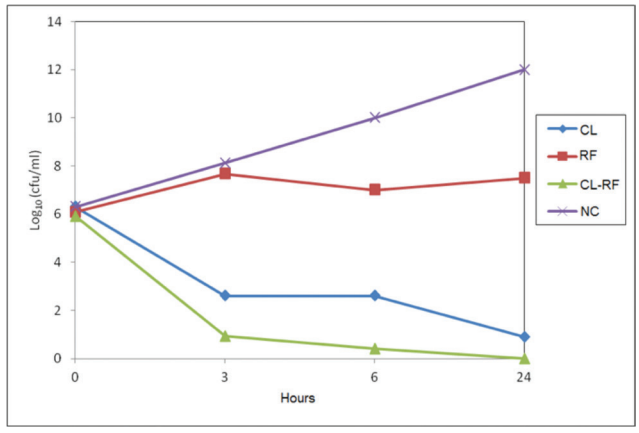

Figure 2. The synergistic effect of CL-RF (0.06-0.25 µg/mL) combination against *A. baumannii* in 3., 6. and 24. hours by time-kill method

CL: Colistin, RF: Rifampicin, NC: Negative growth control without antibiotics

In this study, the interactions of TGC and CL combinations with different antibiotics; AK, CIP, MR, MXF, RF and each other were investigated by CB and TK methods. The best synergistic effect was detected in the CL combinations by CB method, for CL-RF (100%), CL-MR (100%), and in the TGC combinations for TGC-RF (53%). Besides, synergy between TGC and CL was found as 46%.

We also compared the results of CB and TK methods for one of the tested strains. Synergy was determined for the combinations CL with RF and MR by both CB and TK methods. While TGC-RF combination showed synergy by CB, additive interaction was shown by TK method. Percin et al.[3] (2014) investigated CL-vancomycin combinations because of good efficacy by TK as well as CB method, and found consistency of both the results.

Interaction of levofloxacin and CIP alone and their combinations with ceftazidime, cefepime, imipenem, piperacillin/tazobactam AK was investigated in a synergy study conducted in 2005, where 5 *Acinetobacter* spp. were evaluated. The highest synergy was seen in beta-lactam-fluoroquinolone and fluoroquinolone-AK combinations.[24] While in a study conducted in 2011, Tan et al.[8] detected rate of synergy by CB method between polymyxin B-RF and TGC-RF as 19%, those between polymyxin B-TGC was 12%. The same rates obtained by TK methods were 56%, 19% and 44% respectively. When all studied strains were evaluated, the observed synergy rate was 40% by TK method and 17% by the checkerboard method. According to these results, researchers have noted that the best observed *in vitro* synergy rate by TK method was between polymyxin B-RF combination. In a study conducted with 31 MDR and polymyxin B-sensitive *A. baumannii* isolates, Lim et al.[25] reported that the best level of bactericidal activity at 24th hour is in polymyxin B-RF combination (42%), whereas the TGC-RF combination shows very low levels by TK method. In a study conducted by using TK method, initially antagonism was observed in TGC-polymyxin B combination, then variable action was seen where as no interaction was determined when other antibiotics were combined with TGC.[26] In 2013 Lee et al.[4] investigated by TK method the interaction of CL-RF combination for the first time by changing CL concentrations within *in vitro* pharmacodynamic and pharmacokinetic model in aim to achieve clinical concentrations. They observed that this combination prevents the expression of CL resistant subpopulations in CL- susceptible and -resistant MDR *A. baumannii*. In a study conducted in 2012, it was emphasized that the use of inappropriate antibiotic combination increases CL resistance.[27]

Principe et al.[7] for the first time reported that they have noticed the synergy between TGC and CL by TK method in one *A. baumannii* isolate. In two studies conducted by CB method in TGC-CL combination[28] and in TGC-sulbactam, TGC-CL combinations[29] a synergistic effect was observed.

With regard to the assessment of the interaction of the combination used in our study, only one of the 15 strains tested by CB method has also been evaluated by TK method and similar results were obtained with both methods in this strain.

In 2015 a meta-analysis evaluated several studies. It was noted that CL showed *in vitro* synergy and bactericidal activity with many antibiotics against MDR *A. baumannii* strains, especially RF and carbapenem combinations suppressed CL resistance and synergy was observed at over 50% in CL-resistant strains. Synergy rates according to TK method were higher than CB and E-test methods. However, researchers underlined that *in vivo* studies to support *in vitro* studies are insufficient, because some factors like host immune response, bacterial virulence, infection site and antibiotics concentration can alter the effect of the combination of antibiotics. Thus it was declared that there is a need for randomized clinical trials to support the *in vitro* studies.[5,6,30]

CONCLUSION

Today, various methods have been developed for investigation of antibiotic combinations; but there is not a standard approach yet. In this study, both synergy tests showed that CL in combination with RF would be a good option in the treatment of MDR *A. baumannii* infections. Although both methods pose some difficulties such as high work-load, the length of time involved and working with the lowest concentration of antibiotic, they are useful when the reliable results are considered. However, those *in vitro* studies should be supported by *in vivo* studies to determine effective new combinations.

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ORIGINAL ARTICLE

Adenosine A2B Receptors - Mediated Induction of Interleukin-6 in Skeletal Muscle Cells

İskelet Kas Hücrelerinde Adenozin A2B Reseptör Aracılı İnterlökin-6 İndüksiyonu

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ABSTRACT

Objectives: Inflammatory response and cytokine activation are markedly stimulated in skeletal muscle during various conditions. Interleukin-6 (IL-6), a pro-inflammatory cytokine, has pleiotropic effects on skeletal muscle. Adenosine, released by all cell types, binds to a class of G protein-coupled receptors to induce various skeletal muscle effects. The aim of this work was to investigate whether activation of adenosine receptors, particularly adenosine A2B receptors, could stimulate IL-6 gene expression in rat L6 skeletal muscle cells.

Materials and Methods: The rat L6 skeletal muscle cells were cultured in 25 cm^2 flasks. These differentiated cells were treated and then quantitative reverse transcription-polymerase chain reaction (Probe-based) was used to analyze IL-6 gene expression level among different treatment conditions.

Results: Adenosine-5′-*N*-ethyluronamide (NECA), a stable adenosine analogue, concentration- and time-dependently stimulates IL-6 gene expression in skeletal muscle cells. The effect of NECA is inhibited by a selective adenosine A2B receptor antagonist, PSB 603. By using cyclic adenosine monophosphate (cAMP)-arising reagent forskolin, cAMP is found to be involved in the up-regulation of IL-6 induction.

Conclusion: Here, a novel relationship between adenosine and IL-6 up-regulation has been demonstrated for the first time; IL-6 up-regulation induced by NECA is mediated by adenosine A2B receptor activation in skeletal muscle and is dependent on mainly a cAMP pathway. Adenosine A2B receptors are, thus, potentially important pharmacological targets in treating inflammation and related diseases in skeletal muscle tissues.

Key words: Adenosine A2B receptors, skeletal muscle, interleukin 6, cAMP, inflammation

ÖZ

Amaç: İskelet kasında inflamasyon cevabı ve sitokin aktivasyonu, çeşitli şartlarda belirgin şekilde uyarılır. İnterlökin-6 (IL-6), iskelet kasında pleitropik etkilere sahip inflamasyon öncüsü bir sitokindir. Tüm hücre tiplerinden salınan adenozin, iskelet kasında çeşitli etkileri başlatmak üzere G protein eşleşmiş reseptör sınıfına bağlanır. Bu çalışmanın amacı, adenozin reseptörlerinin, özellikle de adenozin A2B reseptörlerinin aktivasyonunun, sıçan L6 iskelet kas hücrelerinde IL-6 gen ifadesini arttırmaya yönelik etkisi olup olmadığını araştırmaktır.

Gereç ve Yöntemler: Sıçan L6 iskelet kas hücreleri 25 cm^2'lik flasklarda kültür edilmiştir. Bu farklılaşmış hücreler, temas ettirilmiş ve ardından farklı temas koşullarında IL-6 gen ifade düzeylerini analiz etmek için nicel ters transkripsiyon-polimeraz zincir reaksiyonu (Prob temelli) yöntemi kullanılmıştır.

Bulgular: Adenozin-5′-*N*-etilüronamide (NECA), dayanıklı adenozin analoğudur, iskelet kas hücrelerinde IL-6 gen ifadesini konsantrasyon ve zamana bağlı arttırır. NECA'nın etkisi, seçici A2B reseptör antagonistleri, PSB 603 tarafından inhibe edilir. Siklik adenozin monofosfattan kaynaklanan belirteç forskolin kullanılarak, cAMP'nin IL-6 indükleyen reseptör artışı ile ilişkili olduğu bulunmuştur.

Sonuç: Bu çalışmada, adenozin ve IL-6 reseptör artışı arasındaki yeni bir ilişki ilk kez gösterilmiştir, şöyle ki NECA tarafından indüklenen IL-6 reseptör artışı, iskelet kasında adenozin A2B reseptör aktivasyonu aracılıklıdır ve ağırlıklı olarak cAMP yolağına bağımlıdır. Bu nedenle, adenozin A2B reseptörleri, iskelet kas dokusunda inflamasyon ve inflamasyon ile ilişkili hastalıkların tedavisinde potansiyel olarak önemli farmakolojik hedeflerdir.

Anahtar kelimeler: Adenozin A2B reseptörleri, iskelet kası, interlökin 6, cAMP, inflamasyon

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INTRODUCTION

Adenosine is a key endogenous signalling molecule produced by all types of cells, and documented as a major local regulator of tissue function. Adenosine can modulate cellular functions via binding to the four members that belong to the cell surface G protein-coupled receptor superfamily (P1 receptors), including adenosine A1, A2A, A2B and A3 receptor subtypes.[1] The adenosine A2A and A2B receptors share a relatively high homology and are coupled to Gs,[2] leading to increased levels of cyclic adenosine monophosphate (cAMP). In addition, the adenosine A2B receptors has been shown to couple to Gq,[3] thereby regulating intracellular calcium levels. In general, the adenosine A2B receptors have a lower affinity for adenosine.[4] Among adenosine receptors, adenosine A2B receptor requires higher concentrations of adenosine in many different cellular types for activation than the adenosine A1, A2, and A3 receptors subtypes.[1] Thus, adenosine A2B receptor can mostly be activated when interstitial adenosine concentrations are increased as a result of tissue hypoxia, injury, inflammation and cell stress,[5] even though adenosine A2B receptors are likely to remain silent under normal physiological conditions.[5]

Within the skeletal muscle tissue, adenosine potentially plays important roles in a large number of physiological processes (such as glucose homeostasis and insulin sensitivity).[6,7,8] Adenosine A2B receptor, in particular, has recently been proposed to act as a potentially functional adenosine receptor in skeletal muscle,[9] however, its pharmacology and biological function(s) remain largely unexplored. Recently, evidence has been accumulated, suggesting that adenosine is a significant modulator of inflammation in response to various stimuli.[10] There is growing evidence that the adenosine system plays an important role in regulating inflammation. Indeed, specific targeting of its components such as the adenosine A2B receptor continues to provide avenues towards the development of potential treatments for at least inflammatory diseases and related disorders, including insulin resistance and type 2 diabetes.

Inflammation is an important contributor to the pathophysiology of diseases related to skeletal muscle dysfunction.[11] Pro-inflammatory cytokines are important contributors to chronic inflammation found in many diseases.[12] One of these inflammatory cytokines is interleukin-6 (IL-6). IL-6, a mediator of inflammation, is a pleiotropic cytokine that has been proposed to be involved in both immune- and nonimmuno-regulation in most cell types and tissues outside the immune system, including skeletal muscle tissue.[13] Indeed, IL-6 is a biologically active cytokine which has a broad range of activities regulating not only inflammatory responses but also in cell proliferation, differentiation, growth and metabolism in skeletal muscle cells.[14,15,16]

Elevated levels of cytokines, such as IL-6 also seems to be the main pro-inflammatory cytokine involved in the pathophysiology of insulin resistance and type 2 diabetes and obesity.[17,18,19,20] Elevated circulating IL-6 levels have been observed in obese individuals and type 2 diabetic patients.[21] Several association studies and numerous clinical studies have suggested that IL-6 plasma concentration are associated with insulin resistance and increased with weight gain.[20] Moreover, some reports provide evidences for high circulating IL-6 levels, as a risk factor for the manifestation of type 2 diabetes.[22] Hence, high circulating IL-6 levels might contribute to the progression of skeletal muscle damage and dysfunction in chronic diseases and might exert pathogenic effects in these diseases. Recent evidence has demonstrated that significant levels of IL-6 is produced in and released from skeletal muscle cells per se in the absence of inflammation and stimulated by complex signalling cascades.[23,24] IL-6 is therefore considered as a myokine and its IL-6 signalling in skeletal muscle tissue has been suggested to be associated with skeletal muscle growth, myogenesis, and regulation of energy metabolism.[13]

Adenosine modulates the functions of many inflammatory cells such as macrophage.[25,26] Moreover, increasing evidence suggests that adenosine signalling plays a role in regulating cytokine network processes. In addition, adenosine increases the release of IL-6 from various cells.[27] In previous studies, it has been reported that skeletal muscle tissue is a source of IL-6 production.[23] As discussed above, increasing evidence indicates that skeletal muscle plays an active role in the inflammatory process by secreting cytokines. However, the effect of adenosine or adenosine analogue on inflammatory cytokine expression by skeletal muscle cells has not been determined. At the same time, adenosine-based approaches are currently being developed for the treatment of various diseases where inflammatory modulation is a key component.[28] Generally, adenosine receptors, in particular adenosine A2B receptors, are increasingly recognized as important orchestrators of inflammation. In fact, adenosine A2B receptor activation enhances the inflammatory responses of mast cells, fibroblasts, epithelial cells and smooth muscle cells.[29] However, the role of this receptor in skeletal muscle cells is yet unexplored.

Extensive *in vitro* and *in vivo* studies have identified potent pro-inflammatory or/and anti-inflammatory functions for all adenosine processes. Recent interest in the endocrine skeletal muscle has potentially revealed the presence of a functional adenosine system in skeletal muscle, however, the effects of adenosine and adenosine receptors modulation on downstream inflammatory signalling, in particular IL-6, still remains unclear. In the current study, an adenosine receptors, in particular adenosine A2B receptors, agonist and antagonist were evaluated for their effects on IL-6 messenger RNA (mRNA) expression level.

MATERIALS AND METHODS

Materials

N-ethylcarboxamidoadenosine (NECA), forskolin, 8-[4-[4-(4-chlorophenzyl)piperazide-1-sulfonyl) phenyl]]-1-propylxanthine (PSB 603) and 2-(4-[2-carboxyethyl]-phenethylamino) adenosine-52 -nethyluronamide (CGS21680) were obtained from Tocris Bioscience, UK; dimethyl sulphoxide reagent was sourced from Santa Cruz, USA; and Trizol and charcoal stripped serum were brought from Applied Biosystem, USA. Maxima Probe quantitative polymerase chain reaction (qPCR) Master Mix (2X) and Thermo Scientific RevertAid First Strand complementary DNA (cDNA) Synthesis were obtained from Thermo Scientific Company, USA. RNeasy Mini Total RNA Purification kits and RNase-Free DNase Set were brought from Qiagen, Germany. Fetal bovine serum (FBS) was supplied by Capricorn Scientific, USA. Horse serum was from Sigma company, Germany. Ham-F 10 was sourced from PAA Company, USA. Dulbecco's modified essential medium (DMEM) was from Caisson, USA.

Cell culture

Rat L6 skeletal muscle cell line and myoblast cell line were originally obtained from the American Type Culture Collection (USA). Cells were maintained as an attached monolayer culture in DMEM with high glucose (4500 mg/L) and L-glutamate supplemented with 10% (v/v) heat-inactivated FBS and 100 µg/mL penicillin-streptomycin. Cells were incubated at 37°C in a 90% humidified atmosphere of 5% CO_2. The cells were passaged upon reaching a state of approximately 60%-70% confluency, and the medium was changed three times per week (Figure 1).

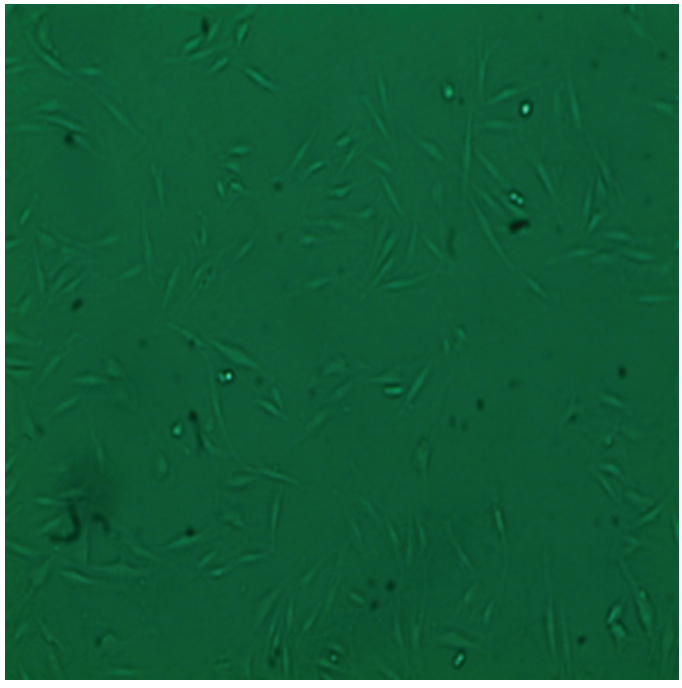

Figure 1. Representative myoblasts derived from passage number 7 myoblasts taken after 1 day seeding into 25 cm^2 (10X)

Confluent cells in 25 cm^2 flasks were cultured for a further 14 days (to allow myotube formation), according to the protocol mentioned in[30] with slight modifications (Figure 2). 70%-90% confluent myotubes (approximately 2 weeks in culture) were serum-starved (incubated in Ham-F 10 medium alone) for 19 hours or 7 days. Then, cells (Figure 2) were treated for 1 hour with vehicle (0.1% dimethyl sulphoxide), NECA 100 nM and 10 µM, PSB 603 100 nM, 1 µM and 10 µM, forskolin 100 nM, NECA and PSB 603 (cells were pre-treated with PSB 603 for 10 min prior to the addition of NECA). Following treatment, cells were washed with ice cold PBS, then lysed with TRIzol (Invitrogen product name) (2 mL per flask).

RNA extraction and cDNA synthesis

Rat L6 skeletal muscle cells (in 25 cm^2 flasks) were scraped in 2 mL of ice cold TRIzol (Applied Biosystems, USA) and RNA was then isolated according to the manufacturer's instructions. Total RNA clean-up and on-column DNAse digestion was performed using RNeasy purification columns (Qiagen, Germany). RNA concentration and purity was determined using a spectrophotometer (JENWAY Genova Nano). For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using RevertAid First Strand cDNA Synthesis (Thermo Scientific, USA) in a total volume of 20 µL for 5 min at 25°C, followed by 1 hour at 42°C, and the reaction was terminated at 70°C for 5 min.

Taqman quantitative real-time polymerase chain reaction

The relative standard curve method based on Taqman quantitative real-time PCR (qRT-PCR) was used to quantify gene expression. Samples were prepared in a total reaction volume of 25 µL [13 µL Maxima Probe qPCR Master Mix 2X reagent, 1.5 µL forward primer (10 µM), 1.5 µL reverse primer (10 mM), 2.5 µL Probe (2 µM), 5 µL water, and 5 µL cDNA]. The qRT-PCR analysis was performed using a RT-PCR system (Applied Biosystems, USA). Gene expression was determined relative to reference gene, TATA. Primers and probes for all genes (Table 1) were designed using Primer Express software (Applied Biosystems, USA) and synthesised by Integrated DNA Technologies, Inc., USA. The standard curve method was used, with a slope between -3.2 and -3.6 and R^2 values of more than 99%, indicating that amplification efficiency was nearly 100%.

Data analysis

Data are expressed as means ± standard error of mean of triplicate or quadruplicate wells generated from at least three independent experimental group. All mRNA data were analysed using one-way ANOVA with a Tukey test. Analysis was performed using GraphPad Prism, version 5.03 (GraphPad Software Inc). The level of statistical significance was set at $p<0.05$.

RESULTS

N-ethylcarboxamidoadenosine stimulates IL-6 mRNA gene expression in skeletal muscle cells

To assess whether stimulation of adenosine A2 receptors could induce IL-6 mRNA gene expression in rat L6 skeletal muscle

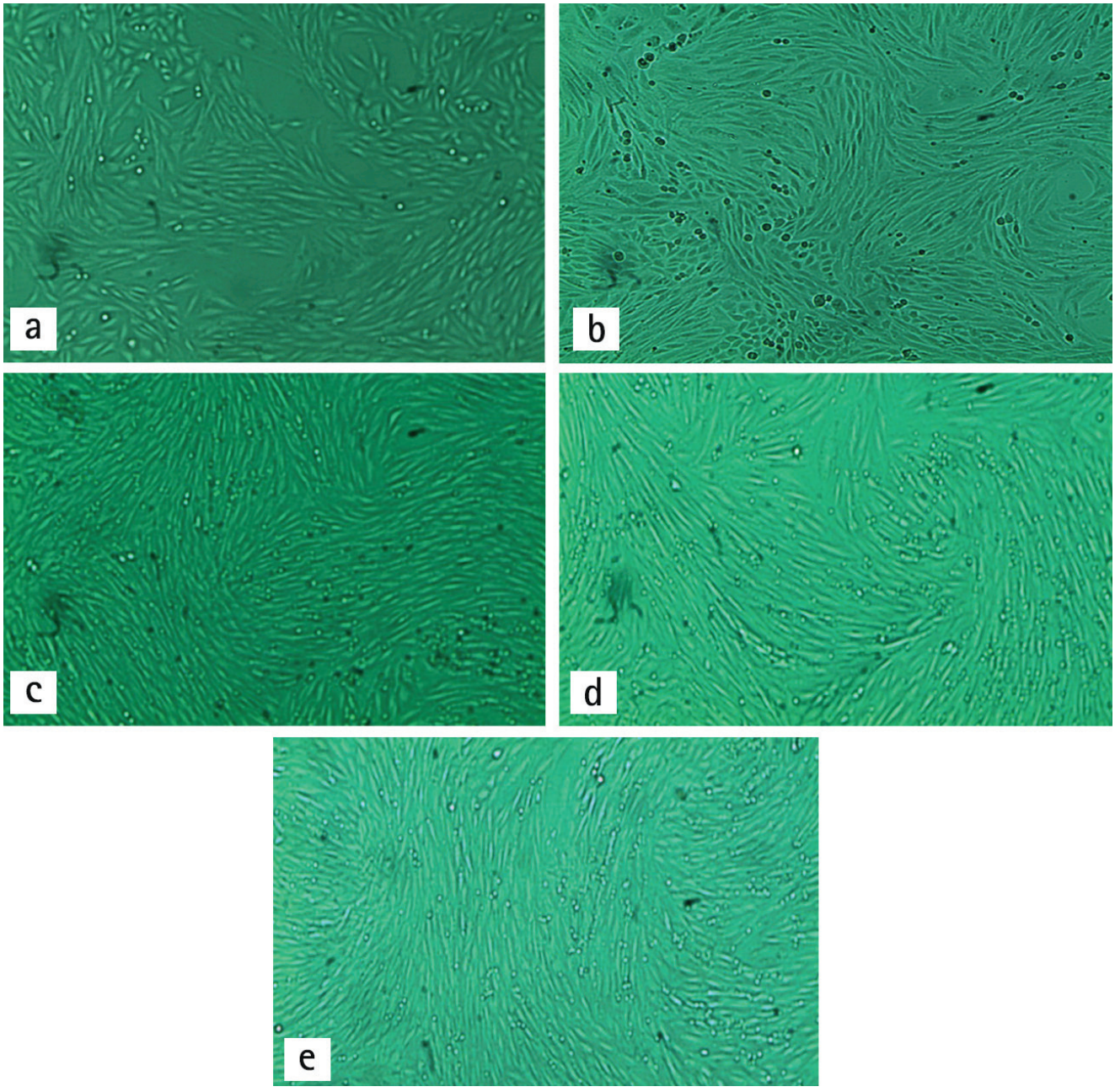

Figure 2. Representative myoblasts/myotubes derived from passage number 7 (a) myoblasts taken at (Ham-F10, 10% fetal bovine serum, 1% P/S) during 3-4 days of tissue culture (10X) (b) myoblasts taken at (Ham-F10, 6% hoarse serum, 1% P/S) during 4-5 days of tissue culture (10X) (c) myoblasts taken at (Ham-F10, 2% hoarse serum, 1% P/S) during 6-7 days of tissue culture (10X) (d) myotubes taken at (Ham-F10, 2% charcoal serum, 1% P/S) during 11-12 days of tissue culture (10X) (e) myotubes taken at (Ham-F10, 1% P/S) after 16 hours starvation (10X)

Table 1. List of gene primer and probe sequences

Gene	Sequences (5'→3')	Amplicon size (bp)
IL-6	Probe: 5'-CTCTCCGCAAGAGACTTCCAGCCAGTT-3' Forward primer: 5'-GCCCTTCAGGAACAGCTATGA-3' Reverse primer: 5'-TGTCAACAACATCAGTCCCAAGA-3'	80
TATA-BOX	Probe 5'-TCCCAAGCGGTTTGCTGCAGTCA-3' Forward primer 5'-TTCGTGCCAGAAATGCTGAA-3' Reverse primer 5'-GTTCGTGGCTCTCTTATTCTCATG-3'	73

IL-6: Interleukin-6

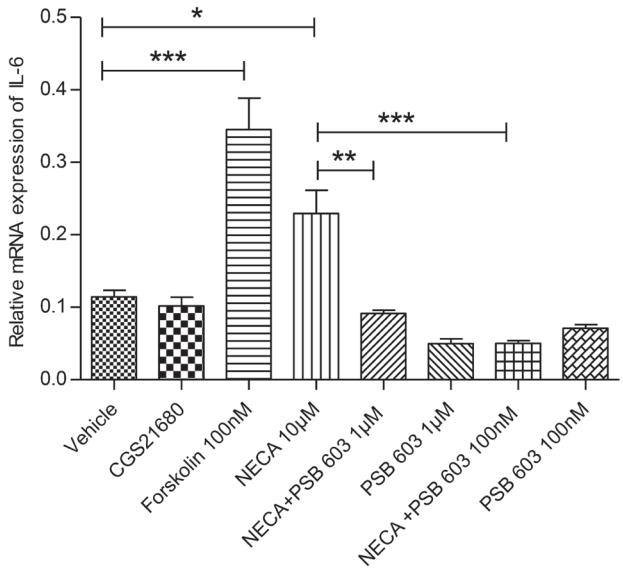

Figure 3. Effects of 5'-*N*-ethylcarboxamidoadenosine (NECA), 2-(4-[2-carboxyethyl]-phenethylamino) adenosine-52-nethyluronamide (CGS21680), and 8-[4-[4-(4-chlorophenzyl) piperazide-1-sulfonyl) phenyl]]-1-propylxanthine (PSB 603) on interleukin 6 (IL-6) messenger RNA (mRNA) gene expression in rat L6 skeletal muscle myotubes using charcoal serum rat L6 skeletal muscle myotubes (70-90% confluent) were serum starved for 7 days and then stimulated for 1 hour IL-6 mRNA levels were measured relative to TATA-BOX using real-time quantitative polymerase chain reaction; stimulation was performed with vehicle (0.1% dimethyl sulphoxide), NECA (10 µM), PSB 603 (100 nM and 1 µM), CGS21680 (100 nM), and forskolin (100 nM), data were represented as means ± standard error of mean of at least three independent experimental groups *denotes $p<0.05$, **denotes $p<0.01$ and ***denotes $p<0.001$, data were analysed using a one-way ANOVA test followed by a Tukey test

cells, the effects of NECA were relatively quantified using a non-selective adenosine receptor agonist on IL-6 mRNA gene expression by qRT-PCR (probe-based). Starved skeletal muscle cells were incubated with NECA (10 µM) for one hour, 3 hours and 24 hours, and mRNA gene expression of IL-6 was subsequently quantified.

Incubation of one week starved L6 skeletal muscle cells with 10 µM of the non-selective adenosine analogue NECA for one hour increases significantly ($p<0.001$) mRNA gene expression of IL-6 (an average around 2.3-fold change compared to vehicle) (Figure 3). Moreover, incubation of 19 hours starved L6 skeletal muscle cells with 10 µM (but, not 100 nM) of the non-selective adenosine analogue NECA for 3 hours (but, not for either one hour or 24 hours) increases significantly ($p<0.05$) mRNA gene expression of IL-6 (an average around 2.2-fold change compared to vehicle) (Figure 4).

Adenosine A2B receptors mediates N-ethylcarboxamidoadenosine-induced IL-6 mRNA gene expression in skeletal muscle cells

To determine which subtype of adenosine A2 receptors mediate the increase IL-6 mRNA gene expression level, the adenosine receptor agonist CGS21680 (subtype A2A selective) was used. The concentration applied for CGS21680 could selectively activate the indicated subtype (Ki=27 nM).[31]

Furthermore, since no selective agonist exists for adenosine A2B receptors, the effect of a selective antagonist to the adenosine A2B receptors (PSB 603) was investigated. PSB 603 exhibits a strong affinity to adenosine A2B receptors and very weak affinity to three other adenosine receptors subtypes. Adenosine A2B receptors display >17000-fold selectivity over other adenosine receptors (Ki values: 0.553, >10000, >10000, and >10000 nM for A2B, A1, A2A, and A3 receptors, respectively).[32]

As shown in Figure 3 and Figure 4a, 4b, NECA (10 µM) increases the mRNA gene expression level of IL-6 significantly. In contrast, the adenosine A2A receptor selective agonist CGS21680 (100 nM) does not cause a significant increase in the mRNA gene expression level of IL-6.

To investigate the effect of a selective adenosine A2B receptor antagonist, PSB 603, rat L6 skeletal muscle cells were incubated with 10 µM NECA in combination with PSB 603 (which was added to cells 10 min prior to adding NECA) in concentrations of 100 nM, 1 µM or 10 µM, which blocks IL-6 mRNA gene expression significantly ($p<0.01$, $p<0.05$ and $p<0.05$, respectively) (Figure 3, 4a, 4b).

It is interesting to note that treatment the 19 hours starved cells for 24 hours with PSB 603 and NECA up-regulates IL-6 mRNA expression level. However, treatment the 19 hours starved cells for 24 hours with either PSB 603 or NECA alone does not modulate the IL-6 mRNA expression level.

Collectively, these results indicate that adenosine A2B receptors are the functionally expressed receptors of adenosine A2 receptors in skeletal muscle, whereas no functional expression of the adenosine A2A receptors was detected using mRNA gene expression levels for IL-6 as a functional readout.

It is worth to mention that the adenosine A2B receptors antagonists/inverse agonists, PSB 603 (at 100 nM, 1 µM and 10 µM), does not mediate a significant change in baseline IL-6 mRNA gene expression levels in skeletal muscle cells (Figure 3, 4). However, even though treatment the 19 hours starved skeletal muscle cells with 100 nM NECA does not up-regulates the IL-6 mRNA expression, incubation that cells with 100 nM NECA in combination with PSB 603 (which was added to cells 10 min prior to adding NECA) decrease baseline IL-6 mRNA gene expression level significantly ($p<0.01$) (Figure 4a, 4b).

Role of cyclic adenosine monophosphate pathway in the mRNA gene expression of IL-6

In previous studies, activation of adenosine A2B receptors in skeletal muscle by NECA increased cAMP accumulation,[33,34] and the NR4A mRNA gene expression.[9]

Experiments were conducted to investigate if the adenylyl cyclase pathway is involved in the activation of IL-6 transcription profile in skeletal muscle cells. In rat skeletal muscle cells, transcripts of adenosine A1, A2, and A3 receptors

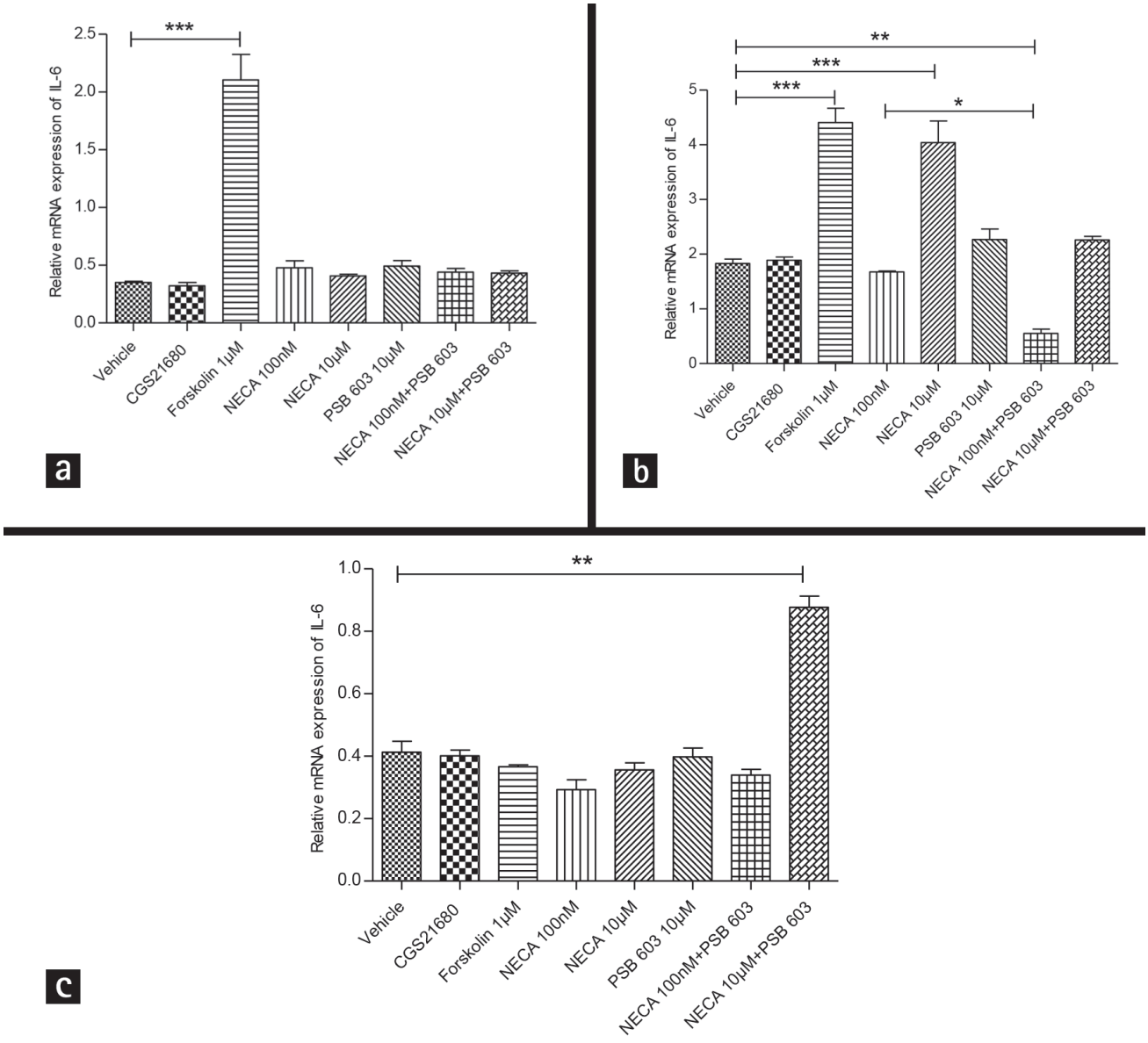

Figure 4. Effects of 5'-*N*-ethylcarboxamidoadenosine (NECA), 2-(4-[2-carboxyethyl]-phenethylamino) adenosine-52-nethyluronamide (CGS21680) and 8-[4-[4-(4-chlorophenzyl) piperazide-1-sulfonyl) phenyl]]-1-propylxanthine (PSB 603) on interleukin 6 (IL-6) messenger RNA (mRNA) gene expression in rat L6 skeletal muscle myotubes using charcoal serum, rat L6 skeletal muscle myotubes were stimulated for the indicated time from 1 hour to 24 hours and interleukin-6 mRNA levels, relative to TATA-BOX, was measured by quantitative real time polymerase chain reaction; stimulation was performed with vehicle (0.1% dimethyl sulphoxide), NECA (100 nM and 10 µM), PSB 603 (10 µM), CGS21680 (100 nM) and forskolin (1 µM) (a) Stimulation was performed up to 1 hour (b) Stimulation was performed for up to 3 hours (c) Stimulation was performed for up to 24 hours, data were represented as means ± standard error of mean of three independent experimental group (n=3; *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001), data were analyzed using one way ANOVA test followed by Tukey test

were detected.[33,34] In addition to gene expression, using cAMP accumulation as a functional readout, it has been confirmed the presence of functional adenosine A2B receptors in skeletal muscle cells,[33,34] whereas the presence of functional adenosine A1, A2A, and A3 receptors were not detected. Furthermore, using NR4A expression as a functional downstream signalling readout, it has been confirmed the presence of functional adenosine A2B receptors in skeletal muscle cells,[9] whereas the presence of functional adenosine A2A receptors were not detected.

Many physiologic roles of adenosine are mediated through cell surface adenosine receptors. In this present study, i provided evidence that the adenosine A2B receptors subtype mediates the effect of the adenosine analogue NECA on IL-6 expression. Our results show the following:

(1) The nonselective agonist NECA increases the expression of IL-6, whereas selective agonist for adenosine A2A receptors CGS21680 had no effect. This agonist is very potent and, at this concentration (100 nM), it fully activates their cognate receptors without significant activation of the adenosine A2B receptors.[31]

For this purpose, the adenylyl cyclase activator forskolin was used in the present study to understand the potential role of the cAMP pathway in NECA induces IL-6 mRNA gene expression in skeletal muscle.

Forskolin (100 nM and 1 μM) increases the expression of IL-6 significantly (an average around 3.5 and 2.4 fold change compared to vehicle, respectively) (Figure 3, 4a, 4b), a result similar to that of NECA. These findings support the idea that the cAMP pathway plays an important role in NECA-induces IL-6 mRNA gene expression. However, treatment the 19 hours starved skeletal muscle cells for 24 hours with forskolin (1 μM) does not induce the expression of IL-6.

DISCUSSION

The novel findings of this present study are that stable adenosine analogue NECA increases the expression of IL-6 by skeletal muscle cells, and that this effect of NECA is mediated by the adenosine A2B receptor subtype. To our knowledge, this is the first paper on the effect of adenosine analogue and its receptor subtype on inflammatory cytokine expression by skeletal muscle cells, and it may represent a novel mechanism for the role of adenosine analogue in skeletal muscle cytokine network. Several reports demonstrated the presence of adenosine receptors in skeletal muscle cells from different species.[35] This is the rationale for determining the effect of this agonist at a concentration of 100 nM.

(2) The effects of NECA on cytokine expression are dependently blocked by selective antagonist of the adenosine A2B receptors subtype, PSB 603. Collectively, these findings provide strong evidence for the role of the adenosine A2B receptors in up-regulating the expression of IL-6 caused by NECA.

These results are in agreement with those obtained in various cell types of different origins, including intestinal[36] and airway epithelial cells,[37] macrophages,[38,39] pulmonary fibroblasts,[40] bronchial smooth muscle cells,[41] astrocytoma cells,[42] astroglioma cells,[43] astrocytes[44] and cardiomyocytes,[45] osteoblasts,[46] and pituitary folliculostellate cells,[47] that all show NECA-induced IL-6 release was via the adenosine A2B receptors. Moreover, numerous *in vivo* studies have also demonstrated that adenosine A2B receptors activation can stimulate the release of IL-6, an important pro-inflammatory cytokine.[36,48] Accordingly, adenosine A2B receptors have been suggested to mediate the pro-inflammatory actions of adenosine. Yet, those results in this current study and above studies contradict a gene-knockout study in which it was reported that adenosine A2B receptors knockout mice show evidence of increased inflammation at baseline, in that levels of cytokines such as IL-6 were elevated in naive adenosine A2B receptors knockout mice.[39,49]

In this present study, cAMP elevation in skeletal muscle cells induces IL-6 expression in a similar to that effect of NECA is expected, because it is well documented that the Gs protein-coupled adenosine A2B receptor increases the formation of cAMP. In fact, several studies have supported the role of cAMP pathway. Indeed, some researchers have also shown that cAMP elevation induced IL-6 release in various cells.[50,51,52] In addition, recently, it has been demonstrated that NECA increased cAMP concentration in rat skeletal muscle cells.[33,34] These data suggest that adenosine A2B receptors mediate IL-6 expression through mainly a cAMP-dependent mechanism in rat skeletal muscle cells, although future studies are recommended to validate and investigate the exact signaling mechanism.

It is interesting to note that PSB 603 might act as a positive allosteric modulator for adenosine A2B receptors in case of treatment the 19 hours starved skeletal muscle cells with PSB 603 and NECA (10 μM but not 100 nM) (cells were pre-treated with PSB 603 for 10 min prior to the addition of NECA) for 24 hours (but not 1 or 3 hours). In this situation, it seems that cAMP is not involved in the (NECA 10 μM and PSB 603) induces IL-6 expression as forskolin does not induce that. However, whether or not Gq-signalling or other downstream targets is involved in the regulation of (NECA 10 μM and PSB 603) induces IL-6 expression in skeletal muscle cells needs to be investigated further. In the literature, the adenosine A2B receptors driven production of these pro-inflammatory molecules has been attributed to both Gs and Gq pathways.[36,53]

It is reported in the current study that the NECA effect in Figure 3 is made on skeletal muscle cells that are starved for one week, and this is not physiologically relevant. However, the idea behind experimenting such a condition is that starvation might change the rate of RNA synthesis for IL-6 and/or the expression level of adenosine receptors subtypes.[54,55]

It should be highlighted that the 1 hour and 24 hours NECA stimulations did not work, but the 3 hours did on 19 hour-starved cells (Figure 4). The reason behind that that adenosine A2B receptors, as a G-protein coupled receptor, might need enough time to couple to G-protein and, then induce downstream signaling pathway to target IL-6 mRNA gene expression. While the cells concentrations of NECA do not explain the disparate effects of 3 hours and 24 hours treatment, duration of exposure may be a pivotal factor. The demonstration of adenosine A2B receptors expression level/density in skeletal muscle cells might fulfill a necessary condition for the specific action of a adenosine A2B receptors activator to alter expression of adenosine A2B receptor-responsive genes.

It is difficult to determine what a 2.5-fold increase in IL-6 transcripts at a single time point translates into IL-6 production. However, it is important to suggest that the relationship between the protein synthesis and RNA content in skeletal muscle cells might not be in a definite linear correlation.[56] One may argue that the changes in expression of IL-6 involved in inflammation observed in the present study may be transient and not reflected by changes in protein expression and therefore have little

significance for inflammation developing in response to stable adenosine analogue NECA. The main reason total protein measurements were not included in the present study is the short duration of the treatment (*i.e.* 1 hour, 3 hours and up to 24 hours), which in line with my main aim was employed to identify the transcriptional events that precede the induction of inflammation under the activation of adenosine A2B receptors. It has shown previously[57] that most of the inflammatory transcripts studied (such as IL-6) in skeletal muscle cells are not translated into protein within that time frame. Therefore my future studies using longer durations of NECA (after 24 hours) will be recommended to investigate the translational changes in response to treatment. The use of primary skeletal muscle cells in future studies may also provide a better opportunity to investigate both transcriptional and translational changes over the time course of prolonged durations of NECA (up to four days).

In this regard, it is clear that adenosine receptors, in particular adenosine A2B receptors, are important molecular targets for adenosine-based therapeutics for inflammation. Approaches utilizing adenosine receptor-based therapeutics will be dependent on further investigation of signalling mechanism for adenosine A2B receptors in skeletal muscle, timing and duration of treatment, and monitoring of beneficial and adverse effects.

CONCLUSION

I showed that the activation of adenosine A2B receptors increases the expression of IL-6 in time- and concentration-dependent manners in rat skeletal muscle cells. These findings provide a novel mechanism whereby adenosine analogue acts as a pro-inflammatory mediator in the skeletal muscle tissue. Furthermore, these findings suggest that the adenosine A2B receptor antagonist at acute early states might have a potential therapeutic utility for the treatment of inflammatory-related skeletal muscle dysfunction.

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ORIGINAL ARTICLE

Real-Time Cell Analysis of the Cytotoxicity of *Origanum acutidens* Essential Oil on HT-29 and HeLa Cell Lines

Origanum acutidens Uçucu Yağının HT-29 ve HeLa Hücre Hatları Üzerine Sitotoksisitesinin Gerçek Zamanlı Hücre Analizi

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ABSTRACT

Objectives: The aim of this study was to investigate the cytotoxic and radical scavenging effects of the essential oil from *Origanum acutidens* (Hand-Mazz.) letswaart and to determine its chemical composition.

Materials and Methods: The essential oil was obtained by hydrodistillation and analyzed by gas chromatography/mass spectrometry (GC-MS). The cytotoxic effect of the essential oil on the growth of human colorectal adenocarcinoma (HT-29) and human cervical adenocarcinoma (HeLa) cell lines was investigated by xCELLigence Real Time Cell Analyzer instrument. Moreover, radical scavenging effect of the oil was determined by using DPPH method.

Results: The main constituents of the oil were found to be carvacrol (61.69%), *p*-cymene (17.32%), γ-terpinene (4.05%), and borneol (3.96%). The essential oil of *O. acutidens* exhibited significant cytotoxic effect against HeLa and HT-29 cell lines at the tested concentrations. The essential oil had moderate DPPH radical scavenging activity compared to butylated hydroxytoluene (BHT).

Conclusion: The data in the present study clearly demonstrated inhibitory effect of the oil on two human cancer cell lines. According to these results and other reported studies, this observed high effect may be attributed to the presence of the carvacrol component in the oil.

Key words*: Origanum acutidens*, carvacrol, antiproliferative, HeLa, radical scavenging

ÖZ

Amaç: Bu çalışmanın amacı, *Origanum acutidens* (Hand-Mazz.) letswaart uçucu yağının sitotoksik ve radikal giderim etkisinin araştırılması ve kimyasal kompozisyonunun belirlenmesidir.

Gereç ve Yöntemler: Uçucu yağ, hidrodistilasyon yöntemi ile elde edilmiş ve gaz kromatografisi/kütle spektrometresi (GC/MS) ile analiz edilmiştir. Uçucu yağın, insan servikal adenokarsinom (HeLa) ve insan kolorektal adenokarsinom (HT-29) hücre hatları üzerine sitotoksik etkisi xCELLigence Gerçek Zamanlı Hücre Analizör cihazı ile araştırılmıştır. Ayrıca, uçucu yağın radikal giderim aktivitesi DPPH yöntemi kullanılarak belirlenmiştir.

Bulgular: Uçucu yağın ana bileşenleri karvakrol (61.69%), *p*-simen (17.32%), γ-terpinen (4.05%) ve borneol (3.96%) olarak bulunmuştur. *O. acutidens* uçucu yağı HeLa ve HT-29 hücre hatlarına karşı test edilen konsantrasyonlarda önemli sitotoksik etki göstermiştir. Uçucu yağ, BHT ile karşılaştırıldığında orta derecede DPPH radikal süpürücü etkiye sahiptir.

Sonuç: Bu çalışmada sunulan sonuçlar, *O. acutidens* uçucu yağının iki insan kanser hücre hattı üzerine olan inhibitor etkisini açıkca göstermektedir. Bu sonuçlara ve rapor edilen diğer çalışmalara gore, gözlenen bu yüksek etki yağda karvakrol bileşiğinin bulunmasına bağlanabilir.

Anahtar kelimeler: *Origanum acutidens,* karvakrol, antiproliferatif, HeLa, radikal giderimi

INTRODUCTION

Cancer has emerged as one of the most alarming diseases in the last few decades throughout the world. The steep rise in the number of cancer cases may be attributed to the changes in food habits, use of tobacco and alcohol, chronic infections, exposure to harmful radiations and chemicals, or more widely due to change in lifestyle and environmental pollution.[1] No extremely effective drug to treat most cancers is available in the market. There is a general call for new drugs that are highly effective, possess low toxicity and have minor environmental impact. Novel natural products offer opportunities for innovation in drug discover.[2] Questions concerning the safety

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of synthetic agents have increased the interest in the use of natural compounds and have encouraged more detailed studies of plant resources, which are a rich source of bionutrients or bioactive phytochemicals.[3]

Essential oils from aromatic plants have been reported to possess anticancer properties as well as antioxidant activity. Most of essential oils have been first characterized for the treatment of inflammatory and oxidative diseases. It appeared that these essential oils could also have anticancer effects as there is a relationship between the production of reactive oxygen species to the origin of oxidation and inflammation that can lead to cancer.[4,5]

Origanum L. (Lamiaceae) is represented by 30 taxa in Turkey, 15 of them are endemic and 5 taxa are hybrid.[6-10] *Origanum* species have traditionally been used as spices and herbal tea. They are traditionally used as a sedative, diuretic, degasifier, sweater and antiseptic, and also in the treatment of gastrointestinal diseases and constipation. This genus is rich in essential oils and bitter substances.[11] Oregano essential oil has been found to be amongst the most effective antioxidant natural agents.[12] The antioxidant effect of these essential oils is attributed to their major components, carvacrol and thymol, and it is the result of various possible mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen quenching capacity.[13]

Origanum acutidens (Hand.-Mazz.) Ietswaart is an endemic, herbaceous, and perennial plant growing mainly in East Anatolia.[8] Essential oil of this plant possesses a variety of biological activities, including antioxidant activity.[14] The aim of this study was, i) to investigate the essential oil composition of *O. acutidens* by gas chromatography/mass spectrometry (GC-MS), ii) to evaluate the cytotoxic effect of the oil on the growth of human cervical carcinoma cell line (HeLa) and HT-29 cell lines by xCELLigence method, and iii) to determine its radical scavenging effect by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

MATERIALS AND METHODS

Chemicals and reagents

DPPH, butylated hydroxyanisole (BHA), 2,6-di-tert-butyl-4-methylphenol (BHT), dimethyl sulfoxide (DMSO), formic acid, Trypsin-ethylenediaminetetraacetic acid, fetal bovine serum (FBS), penicillin/streptomycin and Dulbecco's modified Eagle's medium (DMEM)-high glucose were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany).

Plant materials

Origanum acuditens materials were collected from Bingöl city to Aşağıköy village, 8-9. km, in *Quercus* L. woods (Bingöl, Turkey) in June 2011. The identification of plant materials was confirmed by taxonomist in the Department of Biology, Bingöl University, Turkey. A voucher specimen (*L. Behçet* 8046) was deposited at the Herbarium of the Biology Department, Bingöl University, Turkey.

Essential oil isolation procedure

Two kilograms of dried aerial parts of the plant were shredded and combined with liquid nitrogen in a clean metal container. Then, the plant materials were processed for approximately 3 h in a Clevenger hydro-distillation apparatus and the essential oil was obtained. Anhydrous sodium sulfate was added to eliminate residual water in the oil. Finally, filtration was performed using blue band filter paper. The essential oil was stored in amber glass bottles at 4˚C.

Gas chromatography/mass spectrometry analysis

The essential oil of *O. acutidens* were analyzed with an Agilent Technology 7890A GC system coupled to a 5975C inert MSD with Triple-Axis Detector (Agilent Technologies) on a capillary column [Agilent Technologies HP5-MS (30 m x 0.25 mm I.D. x 0.25 µm film thickness)]. GC temperature program was as follow: From 60 to 150˚C at a rate of 3˚C/min and holding there for 10 min, from 150 to 200˚C at a rate of 5˚C/min and holding there for 3 min, from 200 to 250˚C at a rate of 15˚C/min. Inlet temperature was 250˚C. Spectra were obtained for the range of 50-550 m/z. The GC temperature program was run with helium as carrier gas, at a flow rate of 1 mL/min and injections in split mode (1:50). The mass-spectrometer interface temperature was set to 250˚C. The temperature of the ion source was 230˚C, electron energy 70 eV and quadruples temperature 150˚C. The injection volume was 1 µL.

xCELLigence real time cell analyzer assay

The cytotoxic effect of the essential oil against the HT-29 and HeLa cell lines was determined by using the xCELLigence real-time cell analyzer-single plate (RTCA) instrument (Roche Applied Science, Basel, Switzerland) according to method of Koldas et al.[15] DMEM with 10% FBS and 2% penicillin-streptomycin was used as the cell culture medium during the assessments. First, 50 µL of medium was added to each well of E-plate 96 and the plate was left in the hood for 15 min and then in the incubator for 15 min to let both the E-plate's golden electrode well bottoms and the medium reach a thermal equilibrium. Then, the E-plate was inserted into the RTCA station in the incubator and a background measurement was performed. After ejection of the Eplate from the station, 100 µL HT-29/HeLa cell suspensions were added to the wells to obtain a $2.5x10^4$ cell/well concentration in each well except for three of the wells. The cell concentration (cells/well) was analysed by using fully automated Cedex Hires Analyzer system (Roche Diagnostic Ltd, Rotkreuz, Switzerland) based on the manual Trypan Blue Exclusion Method. Three wells were left without cells to check if there would be an increase in cell index (CI) originating from the medium. 100 µL of medium was added to these wells instead of the cell suspension. After leaving the E-plate in the hood for 30 min, it was inserted to the RTCA station and the second step measurement was initiated for 80 min. During this period, the cells adapted to the bottom of the wells and entered into a growth and division phase. After this step, the E-plate was ejected from the station and solutions of the essential oil that was prepared with DMSO (final concentration of DMSO

was less than 1% in each of the wells) and medium were added to the wells to obtain final concentrations of 250, 100, 50, and 10 µg/mL. Then final volume of the wells was completed to 200 µL by adding medium. After this step, the E-plate was inserted into the station and the main measurement period of 48 hours was initiated.

2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

The free radical scavenging activity of the essential oil was determined by the method of, Blois[16] with some modifications.[17] The solution of DPPH in methanol (0.004%) was prepared fresh daily and 1 mL of this solution in methanol was mixed with 1 mL of sample solution of varying concentrations. Each mixture was kept in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a blank on a ultraviolet (UV) visible light spectrophotometer (Rayleigh, UV-2601). BHT and BHA were used as positive controls. The activity was calculated using the following formula: Scavenging %= $[(A_{control}-A_{sample})/A_{control}]x100$.

Statistical analysis

All experiments were done in triplicate. The results were expressed as means ± standard deviations. Statistical analyses were performed using the SPSS 11.5 (SPSS, Chicago, IL). Differences among means were done by ANOVA, and averages were compared using the Tukey test. The level of statistical significance was taken at $p<0.01$.

RESULTS AND DISCUSSION

Essential oil composition

Hydro-distillated essential oil of *O. acutidens* was analyzed by GC/MS. Twenty eight components were identified representing 98.99% of the oil (Table 1).

According to the GC-MS analysis results, carvacrol was the most abundant component of the oil (61.69%). Other main components of the oil were found to be *p*-cymene (17.32%), γ-terpinene (4.05%), and borneol (3.96%). As seen in Table 1, our findings are in agreement with previously published studies determined carvacrol (64.58-72%) and *p*-cymene (7.5-13.99%) were the major components of the oil.[14,18,19]

Cytotoxic effect by using real time cell analyzer

The cytotoxic effect of the essential oil was examined on HT-29 cell line at the concentrations of 10, 50 and 100 µg/mL by using real time cell analyzer xCELLigence method. The system measures impedance differences in order to derive CI values at time points whose intervals can be set by the operator. These impedance differences and thus the CI values depend on the cell activity at the bottom of the wells.[20] CI is a dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status. If it is decreasing, it shows us that the cancer cells are dying. Our data showed that the essential oil displayed significant cytotoxic effect against HT-29 cell line (Figure 1). The essential oil was most active at the concentration of 50 and 100 µg/mL.

Cytotoxic effect of the essential oil was also examined on HeLa cell line at the concentrations of 50, 100 and 250 µg/mL. The essential oil exhibited excellent cytotoxic effect against HeLa cell line at the tested concentrations [inhibitory concentration $(IC)_{50}<10$ µg/mL]. The Figure 2 belonging to the CI changing according to the cell numbers against to time was obtained.

Begnini et al.[21] reported that *Origanum vulgare* essential oil was composed mostly of 4-terpineol and induced a high cytotoxic effect in HT-29. Sivropoulou et al.[22] investigated cytotoxicity of three *Origanum* essential oils and the oils exhibited high levels of cytotoxicity against four permanent animal cell lines including HeLa. Hussain et al.[23] reported the inhibitory effect of *Origanum*

Table 1. The essential oil composition of *Origanum acutidens*

No	Component	Area %
1	α-Thujene	0.32
2	α-Pinene	0.50
3	Camphene	0.69
4	1-Octen-3-ol	0.26
5	3-Octanone	0.67
6	β-Pinene	0.75
7	α-Phellandrene	0.12
8	α-Terpinene	1.01
9	*p*-Cymene	17.32
10	Eucalyptol	0.70
11	γ-Terpinene	4.05
12	Terpinolene	0.28
13	2-Caren-4-ol	0.23
14	Borneol	3.96
15	4-Terpineol	1.46
16	*p*-Cymen-8-ol	0.23
17	α-Terpineol	0.27
18	Dihydrocarvone	0.30
19	Thymol	0.76
20	Carvacrol	61.69
21	2-Ethyl-5-propylphenol	0.41
22	Caryophyllene	1.52
23	Alloaromadendrene	0.41
24	β-Guaiene	0.33
25	Spathulenol	0.25
26	Caryophyllene oxide	0.50
27	Trienbolone	0.45
28	2-[4-methyl-6-(2,6,6-trimethylcy-clohex-1-enyl)hexa-1,3,5-trienyl] cyclohex-1-en-1-carboxaldehyde	0.56

essential oils (*O. majorana*, *O. vulgare*) on the cell proliferation of two human cancer (MCF-7 and LNCaP) cell lines and the IC_{50} values indicating that the tested *Origanum* essential oils showed prominent cytotoxicity against both the cancer cell lines.

In the present study, carvacrol (61.69%) was found to be the main component of the essential oil. It may be an important component in the cytotoxic effect of this oil. Mehdi et al.[24] demonstrated that carvacrol is an effective anticancer compound with an IC_{50} of 50 mg/L at 48 h inducing growth inhibition in human cervical cells by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assays. In our previous study, we reported the inhibitory effect of the essential oil and the isolated pure or mixture components from *Satureja boissieri* essential oil on HeLa. In that study, the components containing the high concentrations of p-cymene, thymol and carvacrol effectively inhibited the growth of HeLa cells.[25] Akalin and Incesu[26] demonstrated that the carvacrol has cytotoxic effect on H-ras transformed 5RP7 and *N*-ras transformed CO25 cell lines upon time- and concentrations. Therefore, in our present study, the high cytotoxic effect of the essential oil against HeLa and HT-29 cells may be attributed the presence of carvacrol.

Table 2. Radical scavenging activity of the essential oil

	DPPH IC_{50} (mg/mL)
Essential oil	0.387±0.005[a]
BHT	0.022±0.000[b]
BHA	0.003±0.000[b]

DPPH: 2,2-diphenyl-1-picrylhydrazyl, BHT: Buthylated hydroxytoluene, BHA: Butylated hydroxyanisole, IC_{50}: Inhibitory concentration, *Values in the same column with different superscripts are significantly ($p<0.01$) different

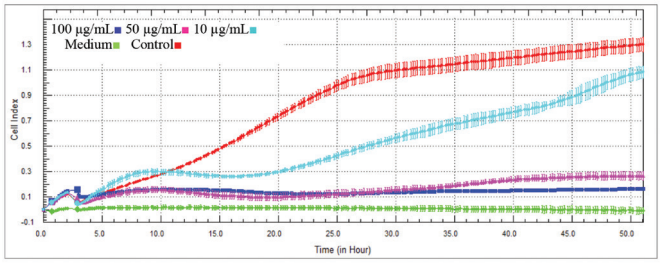

Figure 1. Real-time cell monitoring of the proliferation of HT-29 cells treated with the essential oil for 48 h period

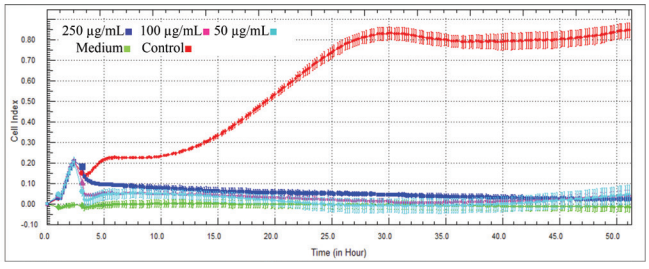

Figure 2. Real-time cell monitoring of the proliferation of HeLa cells treated with the essential oil for 48 h period

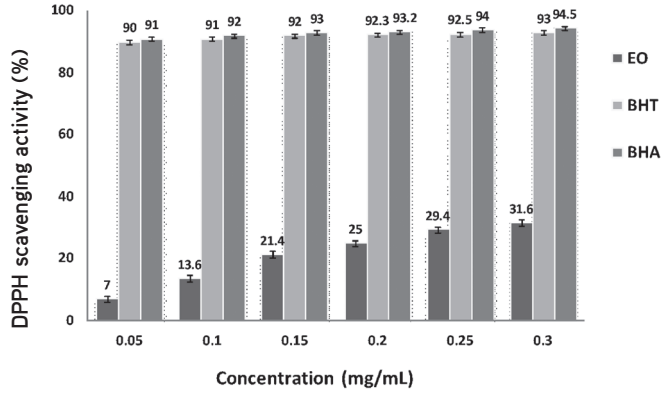

Figure 3. 2,2-diphenyl-1-picrylhydrazyl scavenging activity of the essential oil and synthetic antioxidants at tested concentrations

DPPH: 2,2-diphenyl-1-picrylhydrazyl, EO: The essential oil, BHT: Buthylated hydroxytoluene, BHA: Butylated hydroxyanisole

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of the oil

The free radical scavenging activity of essential oil from *O. acutidens* was measured by DPPH assay. DPPH radical scavenging activity assay is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. This assay measures the ability of a sample to donate hydrogen to DPPH radical. DPPH radical scavenging activities of the essential oil, BHT and BHA are given in Table 2. Lower IC_{50} values indicate higher free radical scavenging activity. The radical scavenging activity of the essential oil increased by increasing the oil concentration (Figure 3). The IC_{50} value of the oil was determined as 0.387±0.005 mg/mL.

The essential oil of *O. acutidens* had moderate radical scavenging activity compared to the synthetic antioxidants. Sokmen et al.[14] was determined the radical scavenging effect of *O. acutidens* essential oil with a IC_{50} value of 133.7±0.5 µg/mL and the oil exhibited lower radical scavenging activity than BHT (19.8±0.5 µg/mL). Hussain et al.[23] reported the radical scavenging activities of *Origanum* oils (*O. majorana* and *O. vulgare*) was less than that of the positive control.

Sharopov et al.[27] investigated the radical scavenging activities of the eighteen different essential oil components and carvacrol showed the one of best radical scavenging activity in the DPPH and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid assays. Lin et al.[28] reported that thymol and carvacrol were the major components attributing the DPPH free radical scavenging activity in the thyme wild essential oil. In the study of Ali et al.[29] carvacrol exhibited 33.9% radical scavenging activity at 5 mM concentration. Therefore, the observed radical scavenging activity of the essential oil is mainly attributed to its main constituent carvacrol.

CONCLUSION

In this study, we have identified the chemical composition of *O. acutidens* essential oil and evaluated the oil's cytotoxic effect on HT-29 and HeLa cell line. Our results clearly show that this essential oil is active against both tumor cell lines tested. These cytotoxic properties could be explained, in part, by the presence of carvacrol which is a main component of the oil. According to the

results, *O. acutidens* essential oil may be suggested as a promising natural agent for alleviating HeLa and HT-29 cell growth.

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ORIGINAL ARTICLE

Encapsulation of Flurbiprofen by Chitosan Using a Spray-Drying Method with *In Vitro* Drug Releasing and Molecular Docking

Püskürtmeli Kurutma Tekniği Kullanarak Flurbiprofenin Kitosan ile Enkapsülasyonu ve *İn Vitro* İlaç Salınımı ve Moleküler Modelleme Çalışmaları

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ABSTRACT

Objectives: This study aimed to prepare chitosan-flurbiprofen micro-nano spheres as environmentally friendly for drug releasing by spray-drying method without any cross-linking agent. It was also aimed to reveal the favorable binding geometries of chitosan and flurbiprofen using molecular modeling.

Materials and Methods: In this study, flurbiprofen was encapsulated with chitosan using spray-drying technique. The used chitosan, flurbiprofen and obtained spheres were characterized via fourier transmission infrared spectrometer (FT-IR), thermogravimetric analysis (TGA), X-ray diffractometer and scanning electron microscopy (SEM). Drug entrapment efficiency was carried out for determination of the drug amount in the micro-nano spheres. *In vitro* release studies of CS-FP spheres were also examined in the simulated biological fluid at pH 7.4. Encapsulation process of flurbiprofen was combined with the docking studies to investigate the possible binding sites of the chitosan.

Results: FT-IR results confirmed that H-bonding system was formed between chitosan and drug. CS-FP spheres with spherical shape were observed by SEM. TGA analysis results showed that thermal stabilities of flurbiprofen and chitosan were decreased after the encapsulation process. The spheres were used for *in vitro* releasing studies in simulated biological fluids. All these analysis results clearly showed that encapsulation was successfully carried out with 73.28% efficiency. Molecular modeling studies showed that CS-FP stable complexes was formed through a hydrogen bonding system between OH group of the drug molecule and chitosan hydroxyl (OH) group with a binding energy of -3.90 kcal/mol. Our computational results supported to spectroscopic results obtained by FTIR.

Conclusion: This study proved that micro-nano spheres can be prepared without using cross-linking agent by spray-drying method. The results of the drug releasing studies showed that release of encapsulated flurbiprofen was completed within 48h. The results of docking analysis can be suggested for the design of new drug carrier systems with chitosan.

Key words: Biodegradable, drug delivery, molecular docking, characterization

ÖZ

Amaç: Bu çalışma, herhangi bir çapraz-bağlama ajanı kullanmadan püskürterek kurutma tekniği ile ilaç salınımı için, kitosan-flurbiprofen mikro ve nano küreleri hazırlamayı amaçlamaktadır. Ayrıca moleküler modelleme kullanarak kitosan ve flurbiprofen arasındaki bağlanma geometrisini açıklamayı amaçlamaktadır.

Gereç ve Yöntemler: Bu çalışmada, püskürterek kurutma tekniği kullanarak flurbiprofenin kitosan ile enkapsülasyonu yapıldı. Kullanılan kitosan, flurbiprofen ve elde edilen kürecikler fourier dönüşümlü kızılötesi spektroskopisi (FT-IR), termogravimetrik analiz (TGA), X-ray difraktometre ve taramalı elektron mikroskopisi (SEM) ile karakterize edildi. Mikro-nano küreciklerdeki ilaç miktarının belirlenmesi için ilaç tutunma verimi çalışıldı. *İn vitro* salınım çalışmaları pH 7.4 te simüle edilmiş biyolojik sıvı içerisinde gerçekleştirildi. Flurbiprofenin enkapsülasyon prosesi, kitosanın muhtemel bağlanma bölgelerini açıklamak için doking çalışmaları ile birleştirildi.

Bulgular: FT-IR sonuçları kitosan ve flurbiprofen arasında H-bağ sisteminin oluştuğunu göstermektedir. Küresel şekilde CS-FP kürecikler SEM ile açıklandı. TGA analizi sonuçları flurbiprofen ve kitosanın termal kararlılıklarının enkapsülasyon sonrası azaldığını göstermektedir. Kürecikler simüle edilmiş biyolojik sıvıda *in vitro* olarak salınım çalışmaları için kullanılmıştır. Tüm bu analizler enkapsülasyonun %73.28 etki ile başarılı bir şekilde gerçekleştirildiğini göstermektedir. Moleküler modelleme çalışmaları bağlanma enerjisi -3.90 kcal/mol olarak kitosan OH grubu ile ilacın hidroksil (OH) grubu arasında H-bağ sisteminin oluşması ile CS-FP kararlı kompleks yapısının oluştuğunu göstermektedir. Bilgisayar hesaplamaları sonuçları FT-IR dan elde ettiğimiz spektroskopik sonuçları desteklemektedir.

Sonuç: Bu çalışma püskürterek kurutma yöntemi ile çapraz-bağ ajanı kullanmadan mikro ve nano küreciklerin hazırlanabileceğini göstermiştir. İlaç salınım çalışması sonuçları, enkapsüle olmuş flurbiprofenin salınımının 48 saat içinde tamamlandığını göstermiştir. Doking analizi sonuçları kitosan ile yeni ilaç taşıyıcı sistemlerin tasarlanması için önerilebilir.

Anahtar kelimeler: Biyobozunur, ilaç salınımı, moleküler modelleme, karakterizasyon

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INTRODUCTION

Although flurbiprofen is one of the non-steroidal anti-inflammatory drugs which is used commonly worldwide for reducing pain and treatment of rheumatoid arthritis and osteoarthritis,[1] it has some side effects on gastrointestinal systems when it is administrated by oral way.[2] To minimize these side effects, biodegradable polymeric micro- and nano spheres have been developed for drug releasing.[3,4] Chitosan is a kind of biopolymer which is with its edible, biodegradable, biocompatible and nontoxic nature[5] and it was used for encapsulation of some compounds such as doxorubicin,[6] curcumin,[7] glycyrrhizin,[8] insulin[9] and cyclosporin A.[10] Chitosan has also been used for delivery of some non-steroidal anti-inflammatory drugs like ibuprofen,[11] naproxen[12] and ketoprofen.[13] In a recently study,[14] genipin cross-linked chitosan microspheres of flurbiprofen was conducted but in that study, the used cross-linked agent (genipin) has an acute toxicity but in the present study it was tried to prepare chitosan-flurbiprofen microspheres for drug releasing without any cross-linked agent.

Several studies of controlled release systems of flurbiprofen have been reported for the preparation of encapsulated drugs using different methods.[15-18] However, these methods are complex and expensive, the adjustment of the particle size is difficult. Compared with the other techniques, spray-drying method is relatively simple, fast and cheap for preparation of encapsulated drugs. One of the main advantages of the spray drying method is adjustable of spherical particles size from submicron to micron by controlling the process parameters.[19]

There are several types of intermolecular interactions between two molecules, such as hydrogen bonds, van der Waals interactions, hydrophobic interactions and electrostatic interactions. The molecular docking study is an effective method predicted these interactions and preferred orientation when bound of ligand to macromolecular target to create a stable complex.[20] In this study, the interaction of flurbiprofen with chitosan will be explained by using molecular docking.

In the present work, we reported the preparation of environmentally friendly chitosan-flurbiprofen micro-nano spheres by using spray-drying method without any cross-linked agent. The morphology and bonding characterization of the spheres were carried out by with scanning electron microscope (SEM) and fourier transform infrared spectroscopy (FTIR) analysis, respectively. The stability of spheres was tested using thermo gravimetric analysis and X-ray diffraction (XRD) analysis. In order to determine of delivering ability of carbon spheres CS-FP spheres, *in vitro* drug release mechanism was examined in the simulated biological fluid at pH 7.4. Encapsulation efficiency was carried out for determination of the drug amount in the spheres. It was also aimed to reveal the favorable binding geometries of chitosan and flurbiprofen using molecular modeling to support of the spectroscopic analysis of spheres.

MATERIALS AND METHOD

Materials

Low molecular weight chitosan (PCode: 1001654970) and flurbiprofen (CAS number: 5104-49-4) were purchased from Sigma Aldrich Co. (USA). Ethanol and acetic acid of analytical grade were obtained from Merck Chemical Co. (Germany).

Preparation of CS-FP micro-nano spheres

In this study, CS-FP spheres were prepared by modification of the method described by Kim et al.[21] CS-FP spheres were prepared using a Buchi B-290 mini spray dryer. 1.0 g of chitosan was added to 25 mL of acetate buffer (pH 5.5-6.0). Flurbiprofen (0.75 g) was added in 25 g of ethanol/distilled water in proportions of 10:15 w/w. Flurbiprofen was dissolved by stirring at 500 rpm for 10 min and the final mixture was heated to 70°C until a clear solution was obtained. The clear solution was mixtured with chitosan solution using a magnetic stirrer at 500 rpm and 70°C for 2 min. Prepared solutions without a cross-linking agent were spray dried using a spray drier at a flow rate of 5 mL/min. The process conditions such as aspirator setting, pump setting, inlet and outlet temperatures were set at 100%, 20%, 100°C and 70°C, respectively. When the solution was evaporated, CS-FP spheres were formed. Dried product was collected in a collecting flask.

Entrapment efficiency

In order to decide the amount of flurbiprofen in drug loaded spheres, drug entrapment efficiency study was carried out by modification of method of Kawadkar and Chauhan.[14] For this purpose, 10 mg of CS-FP spheres was weighted and ingested 5 mL of 3N hydrochloride. It was stirred using magnetic stirrer at 50°C for 1 h and cooled up to the room temperature. The mixture was taken into the extraction flask and 5 mL of chloroform was added to mixture for extraction process. It was strongly shaken to complete extraction of flurbiprofen. After the separating of two phases, organic extract was distinguished from the other phase. Chloroform was completely evaporated at 70°C. The organic extract was dissolved in ethanol and filtered using 125 µm pore size filter membrane. 0.2 mL of clear solution was diluted to 5 mL. The absorbance of filtered clear solution was measured at 247 nm. Entrapment efficiency was calculated using equation [1] given below[22]:

$$\%EE = \frac{\text{Calculated amount of flurbiprofen}}{\text{Total amount of flurbiprofen}} \times 100$$

Characterization of CS-FP micro-nano spheres

Scanning electron microscopy analysis

Surface morphologies of the used chitosan, flurbiprofen and spray-dried CS-FP spheres were investigated using a SEM, FEI, Quanta FEG 250. These materials were made electrically conductive by coating with gold as a thin layer using a Gatan Precision Etching Coating System.

Thermo gravimetric analysis

The EXSTAR S11 TG/DTA 7300 system was used for the thermal characteristics of chitosan, flurbiprofen and CS-FP spheres. In order to obtain TG curves, samples were analyzed at a heating rate of 10°C/min from 25 to 650°C under a nitrogen flow.

Fourier transform infrared spectroscopy

Interactions between the active agent of drug and biopolymer were examined by FTIR spectroscopy (Perkin Elmer mark

FTIR Spectrophotometer, USA). The spectra were recorded for flurbiprofen, chitosan and CS-FP spheres at the scanning range of 4.000-650 cm^{-1}.

X-ray diffraction

A Bruker AXS D8 Advance Model XRD was used for investigation of the effect of encapsulation on crystallinity of drug. XRD peaks of chitosan, flurbiprofen and CS-FP spheres were obtained at 40 kV, 30 mA and the scanning angle range of 5-90°C of 2θ.

In vitro drug release studies

In vitro drug release studies were performed in phosphate buffer saline (PBS) medium at pH 7.4 for a period of 48 h. 10 mg of CS-FP spheres were reconstituted in 25 mL of PBS and moved in dialysis bag. The dialysis bag was placed in 300 mL of the same PBS at 37°C and stirred at 100 rpm using magnetic stirrer. In order to determine the amount of FP released from the dialysis bag at different time intervals (1 min, 5 min, 10 min, 15 min, 20 min, 30 min, 1 h, 2 h, 6 h, 24 h, 48 h), 2 mL of the samples was picked up and then replaced with the 2 mL of fresh PBS buffer. The concentration of drug released to the medium was determined by measuring the absorbance at 247 nm using a ultraviolet spectrophotometer. A calibration curve of FP released to the medium was plotted by concentration against the absorbance. Releasing percentage of FP was calculated from the following equation [2]:

$$\text{Drug release (\%)} = \frac{\text{Released FP}}{\text{Total FP}} \times 100$$

Molecular docking studies

To determine the interactions between chitosan and flurbiprofen, molecular docking analysis were performed using ArgusLab 4.0.1 docking software which is a free molecular modeling package.[23] The chemical structure of chitosan was obtained from the literature.[24] The three dimensional structures were constructed for flurbiprofen and chitosan using Spartan 06 V1.2.0 software.[25] Geometrical optimization of energy minimized structures of the molecules was performed using semiemprical PM3 method in Spartan'06 V1.2.0 software. Whole structures of the lowest energy conformer of chitosan and flurbiprofen were defined as a potential binding site and a ligand, respectively. Argusdock exhaustive search docking engine with grid resolution of 0.40 Å was used for docking runs. Docking precision was set to high precision with a maximum of 150 candidate poses and flexible ligand docking mode was activated for docking runs. A score was used as the scoring function estimated the free binding energy and binding site box size was set to automatically as 15x15x25.

RESULTS AND DISCUSSION

Encapsulation efficiency

The encapsulation efficiency explains that it is the percentage of the amount of the drug that loaded to drug carrier agent. In this study, it was found as 73.28%. Kawadkar and Chauhan[14] found the encapsulation efficiency of flurbiprofen loaded chitosan microparticles between 63.39% and 80.97%. Our result was in accordance with the previous study.[14]

Morphology of CS-FP micro-nano spheres

The surface morphology of encapsulated drugs is one of the most important elements in order to explain the effectiveness of drug delivery systems. Intermolecular interactions between polymer and drug molecule may change the surface morphology. Therefore, SEM analysis was used to investigate the surface morphology and shape of CS-FP spheres. SEM images of the chitosan, pure drug and CS-FP spheres are shown in Figure 1. Surface morphologies of chitosan were revealed in the form of fibrous and porous, while flurbiprofen surface was consisted of flake like appearance. As seen from the Figure 1, surface morphology of CS-FP spheres was spherical in shape with their diameter changed from 700 nm to 13 µm. The appearance of the spheres is fairly smooth throughout the surface. These results indicate that the spheres are separated from the chitosan and pure drug and the flurbiprofen successfully encapsulated into chitosan particles.

Thermal stability

Thermal stability of chitosan, flurbiprofen and CS-FP spheres were determined and the results are presented in Figure 2. For chitosan, two decomposition phases were observed. The first decomposition (7.18%) between 30 and 100°C was due to evaporation of water and the second mass lose (56.32) between 250 and 650°C was because of degradation of polysaccharide structure. The maximum degradation temperature for chitosan was recorded as 298.2°C. For flurbiprofen, very small degradation (0.1%) was recorded between 30 and 100°C and 99.7% mass loss was recorded between 130 and 260°C. This small mass loss in the first step was because of evaporation of little amount of water and the huge degradation in the second step was due to degradation of flurbiprofen. After encapsulation, CS-FP spheres were degraded in three stages. In the first stage, 7.4% mass loss was observed between 30 and 150°C (maximum

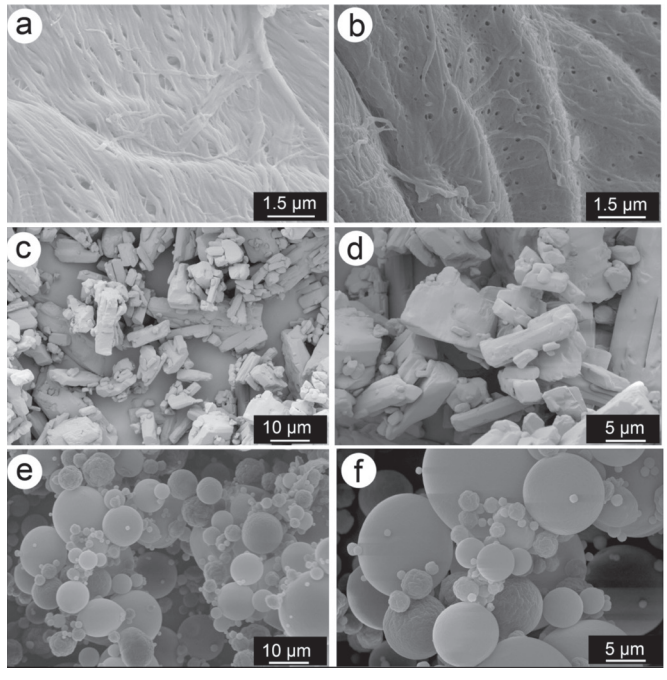

Figure 1. Scanning electron microscope pictures of (a, b) chitosan (c, d) flurbiprofen (e, f) chitosan-flurbiprofen micro-nano spheres

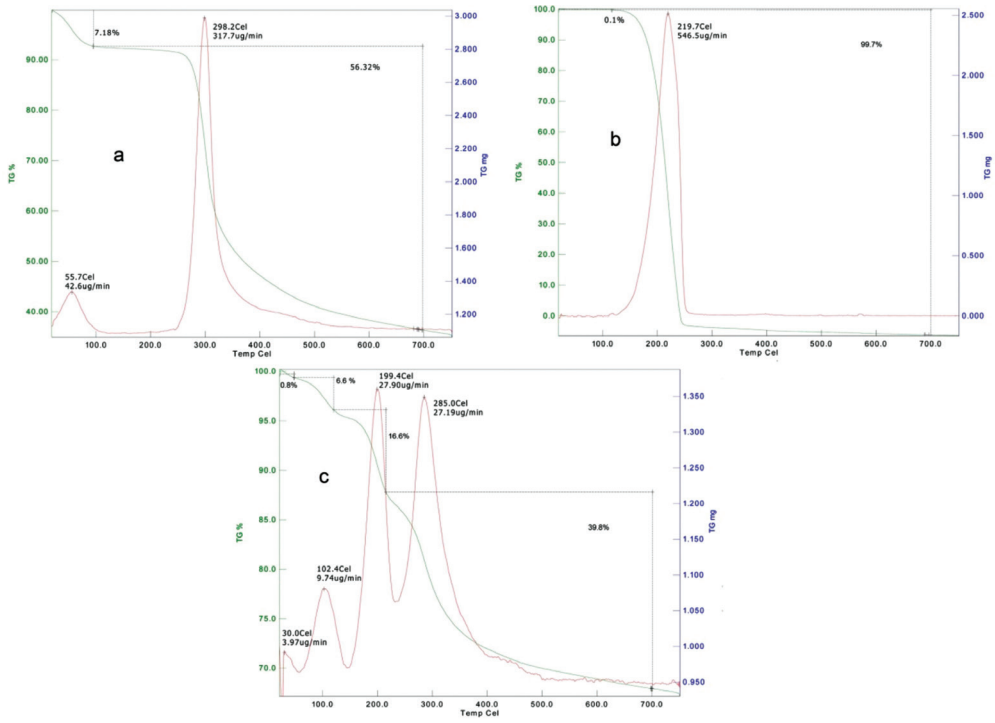

Figure 2. Thermal characteristics of (a) chitosan (b) flurbiprofen and (c) chitosan-flurbiprofen micro-nano spheres

degradation 102.4˚C) and it can be attributed to evaporation of water. In the second stage, 16.6% mass loss was recorded between 150 and 250˚C (maximum degradation 199.4˚C) and it can be attributed to the degradation of flurbiprofen. In the third stage, 39.8% mass loss was observed between 250 and 650˚C (maximum degradation 285˚C) and it can be ascribed to the degradation of chitosan. After encapsulation, thermal stabilities of flurbiprofen and chitosan were decreased. These characteristic degradations for both chitosan and flurbiprofen in CS-FP spheres showed the successful encapsulation.

X-ray diffraction

XRD patterns of chitosan, pure drug and encapsulated sample are given in Figure 3. The broad peak observed at 2θ=19˚ is the characteristic peak for the chitosan. The XRD pattern of flurbiprofen revealed the crystalline structure of drug observed by five sharp peaks at 2θ of 7˚, 11˚, 16˚, 21˚ and 24˚. The XRD pattern of flurbiprofen compared with the XRD pattern of encapsulated sample, CS-FP spheres showed no sharp peaks, whereas a broad peak from 11˚ to 24˚ was observed. Mean of this broad peak is that flurbiprofen was kept in an amorphous state in the chitosan. These results suggest that flurbiprofen successfully encapsulated in the chitosan.

Fourier transform infrared spectroscopy

FTIR spectroscopy of flurbiprofen, chitosan and CS-FP spheres were instructed to explain drug-biopolymer interaction. FTIR spectrums of flurbiprofen, chitosan and CS-FP spheres were compared in Figure 4. As seen from the Figure 4a, the characteristic sharp peaks of flurbiprofen at 1694.7, 1414.7 and 1216.1 cm^{-1} were due to C=O stretching, O-H bending and C-F stretching, respectively. The characteristic band of flurbiprofen due to the hydrogen bonds of the carboxyl group appeared in the range of the 3400-2400 cm^{-1}. Characteristic bands of chitosan

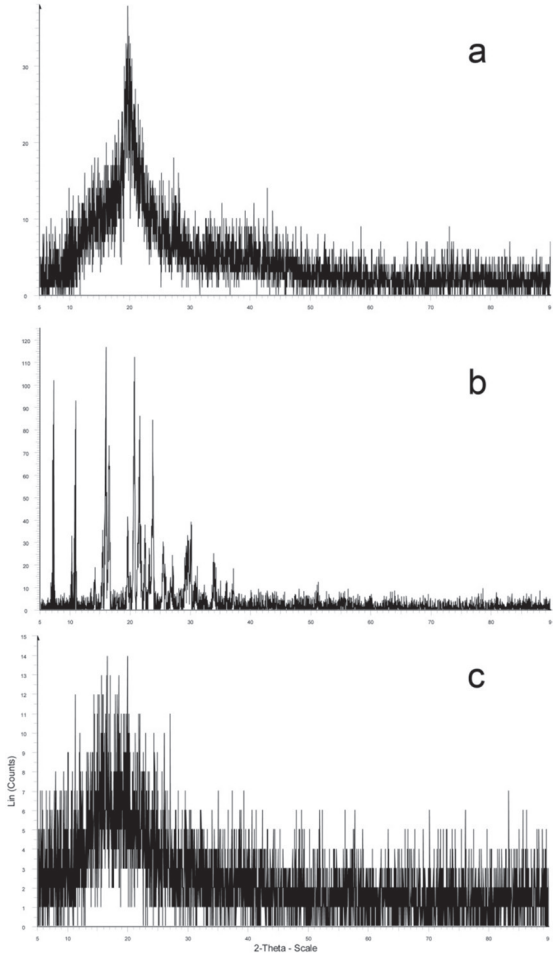

Figure 3. X-ray diffraction pattern of (a) chitosan (b) flurbiprofen and (c) chitosan-flurbiprofen micro-nano spheres

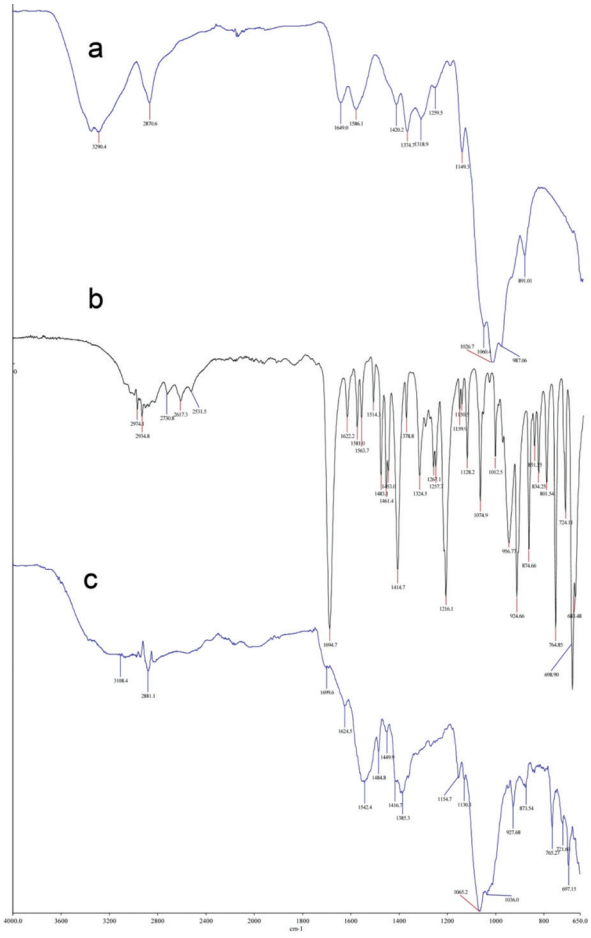

Figure 4. Fourier transform infrared spectroscopy spectrums of (a) chitosan (b) flurbiprofen and (c) chitosan-flurbiprofen micro-nano spheres

were seen from the Figure 4b at 3290.4 cm^{-1}, 1649.0 cm^{-1}, 1586.1 cm^{-1} and 1318.9 cm^{-1} which correspond to OH and NH stretching, amide I (C=O), amide II (NH_2) and amide III (C-N), respectively.[26] Spectrum of CS-FP spheres (Figure 4c) compared with the other spectrums, there are some changes indicating the structural differences of chitosan after the encapsulation process. It is seen that the O-H and N-H stretching bands were shifted to lower wavenumbers at 3108.4 cm^{-1} due to H bonding system. Furthermore, peaks observed at 927.68 cm^{-1}, 765.27 cm^{-1}, 721.6 cm^{-1} and 697.15 cm^{-1} indicate the presence of the substitute aromatic rings of flurbiprofen. These changes greatly showed that flurbiprofen successfully encapsulated into chitosan particles.

In vitro release

In order to determine the releasing behavior of drug from the chitosan matrix, drug loaded spheres were examined in PBS to simulate biological environment. The cumulative percentages of CS-FP spheres released at pH 7.4 were presented in Figure 5. As seen from the Figure 5, flurbiprofen released from spheres was completed within 48h. Release profile of CS-FP spheres was found to be biphasic, with an initial fast releasing for 4h followed by a slower releasing rate. While cumulative percentage of flurbiprofen released from chitosan is about 54.9% for the first 4h, it is 99.37% for 48h. The main

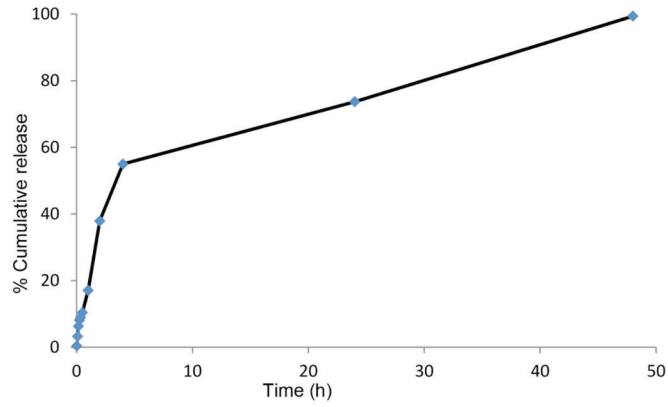

Figure 5. Releasing profile of chitosan-flurbiprofen micro-nano spheres

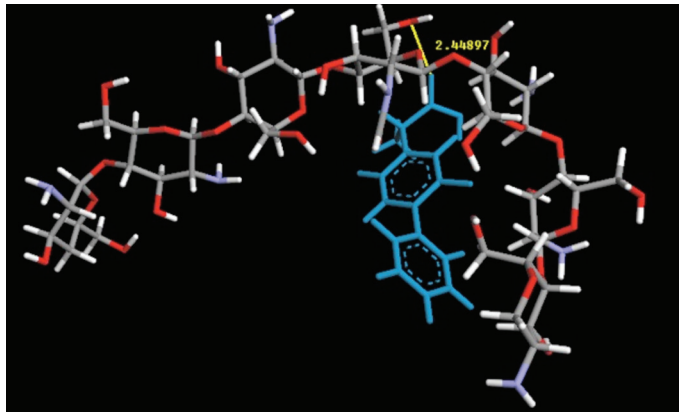

Figure 6. The best docked complexes structures of chitosan-flurbiprofen micro-nano spheres with ΔG binding energy= -3.90 kcal/mol

reason of the fast releasing of the first phase can be explained by adsorption of flurbiprofen on the spheres surface. In the second phase, flurbiprofen encapsulated in the chitosan was passed to the dispersion environment. Dudhani and Kosaraju[27] revealed that the releasing behavior of micro or nanoparticles is generally biphasic. Biphasic patterns of drug-chitosan systems were reported by earlier studies.[11,28,29] Biphasic releasing patterns were observed both in the present study and the study by Kawadkar and Chauhan[14] but the releasing velocity of the present study was recorded about two times faster than that of the study by Kawadkar and Chauhan.[14] While spray-drying method was used for producing the spheres, cross-linked agent was used in Kawadkar and Chauhan[14] study. It can be concluded that this difference in the releasing velocity is because of the spheres producing methods. Consequently, our results proved that releasing behavior of flurbiprofen from CS-FP spheres produced by using spray-drying method was effective in order to improve controlled drug delivery system.

Molecular docking

The experimental results of encapsulation process of flurbiprofen were combined with the docking studies to investigate the possible binding sites of the chitosan. For this purpose, flurbiprofen was docked with the chitosan. The flexible docking results showed that flurbiprofen fitted into the active sites of the chitosan. The binding modes of the best docked CS-FP complexes with the lowest energy are shown in the Figure 6. The binding energy of the docked structures of the CS-FP

stable complexes was calculated as a value of -3.90 kcal/mol. As seen from the Figure 6 flurbiprofen was surrounded by the chitosan and bonded to chitosan through a new hydrogen bond between carboxyl oxygen of drug (O-174) and chitosan O-35 atoms. The distance of H bond is evaluated as 2.449 Å. Our docking results supported to the spectroscopic results obtained by FTIR shown the H-bonding.

CONCLUSION

This study showed that it is also possible to produce CS-FP micro and nano spheres by using spray-drying method without any cross-linked agent. Structural details of the spheres were examined by using SEM, XRD and FTIR analysis. *In vitro* drug releasing and encapsulation efficiency were also studied. Our drug releasing results showed that release of encapsulated flurbiprofen was completed within 48h. Also it was determined that the releasing velocity of the spheres with spray-drying method was much faster than releasing velocity of spheres with genipin cross-linked. Here molecular docking explained the interaction between chitosan and flurbiprofen. The results of our docking studies can be useful for the design of new drug carrier systems with chitosan. The results of molecular docking can be suggested to use in further studies to explain of interactions between chitosan and the others drug active agents.

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Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

A Comparative Study of Different Proportions of Superdisintegrants: Formulation and Evaluation of Orally Disintegrating Tablets of Salbutamol Sulphate

Salbutamol Sülfatın Oral Dağılan Tabletlerinin Formülasyonu ve Değerlendirilmesi: Süper Dağıtıcıların Farklı Oranlarının Karşılaştırmalı Çalışması

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ABSTRACT

Objectives: Superdisintegrants play important role in disintegration of orally disintegrating tablets (ODTs). Action of three different superdisintegrants, viz. croscarmellose sodium, sodium starch glycolate and Indion 414, were studied individually or in their binary combinations for their fast disintegrant action in ODTs of salbutamol sulphate prepared by direct compression.

Materials and Methods: ODTs were prepared in three different superdisintegrant combinations A, B and C. In each combination, five formulations were prepared with superdisintegrants in ratios 10:90, 25:75, 50:50, 75:25, and 90:10. Three ODT formulations were prepared with single superdisintegrant and two ODT batches were prepared from marketed ODT excipient blends, viz. Prosolv-ODT and F-Melt. Prepared ODT formulations were evaluated and compared for weight variation, hardness, friability, wetting time, disintegration and drug release.

Results: All ODTs disintegrated quickly in 32 s or less. ODT formulation F3, containing croscarmellose sodium and sodium starch glycolate, disintegrated very quickly in 19.28±3.11 s. Results of F3 were compared with the batches (F19 and F20) containing marketed coprocessed excipients and found in good agreement for various evaluation parameters. Formulation F20 was hygroscopic, while F3 did not suffer this disadvantage.

Conclusion: Thus, we may conclude that superdisintegrants in combinations may offer additive and/or synergistic disintegration possible due to their different mechanism.

Key words: Wetting, disintegration, dissolution, croscarmellose sodium, sodium starch glycolate, Indion 414

ÖZ

Amaç: Farklı üç süper dağıtıcının, ki bunlar; kroskarmeloz sodyum, sodyum nişasta glikolat ve Indion 414, doğrudan basım ile hazırlanan salbutamol sülfat oral dağılan tabletlerinde (ODT) hızlı dağıtma etkisi için ayrı ayrı veya ikili kombinasyonlarında çalışıldı.

Gereç ve Yöntemler: ODT'ler farklı üç süper dağıtıcı kombinasyonunda; A, B ve C, hazırlandı. Herbir kombinasyonda süperdağıtıcıların 10:90, 25:75, 50:50, 75:25 ve 90:10 oranlarında beş formülasyon hazırlandı. Üç ODT formülasyonu tek süper dağıtıcı ile ve iki ODT serisi piyasadaki ODT eksipiyanları ile ki bunlar, Prosolv-ODT ve F-Melt, hazırlandı. Hazırlanan ODT formülasyonları ağırlık sapması, sertlik, friabilite, ıslanma zamanı, dağılma ve etken madde salımı için değerlendirildi ve karşılaştırıldı.

Bulgular: Tüm ODT'ler 32s veya daha kısa sürede dağıldı. Croscarmellose sodyum, sodyum nişasta glikolat içeren F3 ODT formülasyonu 19.28±3.11 s de çok hızlı dağıldı. F3'ün sonuçları piyasadaki koproses eksipiyanları içeren serilerle (F19 ve F20) karşılaştırıldı ve çeşitli değerlendirme parametreleri için iyi bir uyum olduğu bulundu. F20 formülasyonu higroskopik iken F3 formülasyonunda bu dezavantaj yoktu.

Sonuç: Böylece, kombinasyondaki süper dağıtıcıların farklı mekanizmalarına bağlı olarak olası ilave ve/veya sinerjik dağılmayı sağlayabileceği sonucuna varabiliriz.

Anahtar kelimeler: Islanma, dağılma, çözünme, kroskarmeloz sodyum, sodyum nişasta glikolat, Indion 414

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INTRODUCTION

Novel drug delivery system aims to enhance safety and efficacy of drug molecule by formulating a convenient dosage form for administration and to achieve better patient compliance. One such approach is oral disintegrating tablets (ODT) which are those solid dosage forms when put on tongue, disintegrate or dissolve instantaneously, releasing the drug, within a few seconds without the need of water. ODT is also known as fast melting, fast dispersing, rapid dissolve, rapid melt and/or quick disintegrating tablet. ODT can be administered easily to dysphagic adults, pediatrics, geriatrics, mentally ill, uncooperative and nauseated patients.[1] Disorder of dysphagia is associated with many medical conditions including stroke, Parkinson's disease, AIDS, thyroidectomy, head and neck radiation therapy and other neurological disorders including cerebral palsy. One study showed that 26% out of 1576 patients experienced difficulty in swallowing tablets due to their large size, followed by their surface, shape and taste.[2,3] The centre for drug evaluation and research, US Food and Drug Administration defined ODT as "A solid dosage form containing medicinal substances, which disintegrates rapidly, usually within a matter of seconds, when placed upon the tongue".[4] The disintegration time for ODTs varies from a few second to more than a min depending upon the formulation and size of the tablet. ODT also offer rapid disintegration followed by fast dissolution and absorption, which may produce rapid onset of action. Increase in bioavailability is expected for drugs which are absorbed from the mouth pharynx and oesophagus as the saliva passes down into the stomach. Convenience of administration, accurate dosing when compared to liquids, liquid medication in the form of solid form, no need of water to swallow and good mouthfeel are some other salient features of ODTs.[5]

Salbutamol (albuterol) is a β2 adrenergic agonist, it cause bronchodilation through β2 receptor stimulation via increased cyclic adenosine monophosphatedan formation in bronchial muscle cell which cause relaxation. Its action start within 15-20 min and lasts for 3-4 hour.[6] It is therefore used to abort and terminate attacks of asthma. Muscle tremors is the dose related side effect of this drug. Asthma is common problem to large number of people and asthmatic attack to a patient needs a quick onset of action for instant relief, ODT of salbutamol sulphate will help to achieve a quick action by faster disintegration and/or dissolution than conventional tablet. ODTs of salubutamol sulphate have been reported in several research reports.[7-12] In one study *Plantago ovata* husk and powder was used as disintegrant in preparing Salbutamol sulphate ODTs.[7] *Plantago ovata* husk and powder was superior to croscarmellose sodium (CCS) and sodium starch glycoate in disintegration action. CCS, alginic acid, sodium starch glycolate (SSG), modified guar gum and modified agar were used as disintegrants in preparing ODTs of Salbutamol sulphate.[8] Quantities of PVP K30, sodium starch glycoate and microcrystalline cellulose (MCC) were optimized to achieve faster disintegration and adequate mechanical strengths.[9] Both, intragranular and extragranular incoroporation of crocarmellose XL-10 in formulations were found effective and superior disintegrant action when compared with CCS, starch glycolate and low substituted hydroxyl propyl cellulose (L-HPC) in preparing salbutamol sulphate ODTs by direct compression and wet granulation.[11] In another attempt salbutamol sulphate ODTs were prepared with combination of superdisintegrant (crospovidone) and subliming agents (camphor, menthol, thymol and ammonium bicarbonate) to achieve quick disintegration with optimum mechanical strength.[12] Primojel, L-HPC, Kollidon CL and Primojel were used individually in achieving fast disintegration of ODTs of salbutamol sulphate.[10]

The use of superdisintegrants in ODTs has become popular for various reasons. For tablet containing sparingly water soluble drugs, the start of dissolution is often delayed by poor wettability of tablet or slow liquid penetration into tablet matrix this causes increase in disintegration time and retards the drug release. This can be overcome by addition of superdisintegrants.[13] The mechanism of action of superdisintegrants may be by wicking, swelling, which is found to effect primarily for tablet disintegrants, while other mechanisms, such as deformation recovery, particles repulsion theory, heat of wetting and evolution of a gas etc., may play a role in particulate cases of tablet disintegration.

SSG is the sodium salt of a carboxymethyl ether of starch or of a cross linked carboxymethyl ether of starch. It is effective at a concentration of 2-8%. SSG swells 7-12 folds in less than 30 seconds and it swells in three dimensions and high level serve as sustain release matrix. It can take up more than 20 times its weight in water and the resulting high swelling capacity combined with rapid uptake of water accounts for its high disintegration rate and efficiency.

CCS is the sodium salt of a cross linked, partly *O* (carboxymethylated) cellulose. CCS swells 4-8 folds in less than 10 seconds and the mechanism of disintegrant action is swelling and wicking both. Its swelling is in two dimensions and it is used as a starch free disintegrant for direct compression and granulation. CCS and SSG, both are sodium salts, anionic and their polymer backbones are composed mostly of glucose units. ODT tablets containing CCS and SSG disintegrate almost instantaneously when they will come in contact with even slight amount of saliva or water.[14]

Indion 414 is a weak acid cation exchange resin based on crosslinked polyacrylic acid (Indion 414 brochure).[15] It is supplied as potassium salt for use in pharmaceutical formulations as disintegrant and taste masking agent. A concentration of 0.5-2% is sufficient as disintegrant in conventional tablets. However, in ODTs, amounts higher than this may be added to achieve faster disintegration. Additionally it does not have an adhesive tendency and is insoluble in water and common solvents. Indion 414 provides good mechanical strength to the tablet which facilitate easy transportation, and packing. It swells to about 700% when it comes in contact with water or gastrointestinal fluid causing rapid disintegration without formation of lumps. In one research report, swelling index of 800 was estimated for Indion 414 and 750 for SSG and 700 for CCS in simulated saliva.[15]

The aim of present study is to formulate salbutamol sulphate ODTs using three different superdisintegrants, *viz.* SSG, CCS and

Indion 414, and their binary combinations to find out the effect on various evaluation parameters of prepared ODTs. Marketed ODT blends Prosolv-ODT and F-melt were also used to prepare salbutamol sulphate ODTs and results were compared to ODTs containing various combinations of superdisintegatnts.

MATERIALS AND METHODS

Materials

Salbutamol sulphate was purchased from Yarrow Chem. Products, Mumbai. CCS was received as gift from JRS Pharma, Germany. SSG and MCC were purchased from Signet Chem. Corporation, Mumbai. Indion 414 was received as a gift from Ion Exchange (India) Ltd, Gujarat. Mannitol was *ex gratis* sample from Service Techniques Et Laboratories, Lestrem, France. Magnesium sterate, talc, saccharin sodium was purchased from Loba Chemie Pvt Ltd. Mumbai. F-melt type C and Prosolv ODT were received as *ex gratis* samples from Fuji Chemical Industry Co. Ltd., Tokyo and JRS Pharma, Germany. All other chemicals and reagents used were of analytical grade.

Methods

ODT formulations

ODTs of salbutamol sulphate were prepared by direct compression. CCS, SSG and Indion 414 were used as superdisintegrants. Formulations were prepared in three different combinations A, B and C. In each combination, two superdisintegrants were used and five different formulations were prepared. Total amount of superdisintegrants in the formulation was fixed as 25 mg in the tablet of total weighing 200 mg (12.5% w/w superdisintegrant of total weight of tablet). Proportion of both superdisintegrants were varied in a sequential pattern, 10:90, 25:75, 50:50, 75:25 and 90:10, to achieve five different formulations of one combination. Compositions of various tablet formulations are provided in the Table 1. Batches F16, F17, F18 were prepared with individual superdisintegrant SSG/CCS/Indion 414 respectively at 12.5% w/w. Batch F19 and F20 were prepared with marketed ODT blends, Prosolv-ODT and F-Melt, respectively.

MCC was used as a filler in same amount *i.e.* 50 mg (25% w/w of total tablet weight) in all the tablets. Mannitol was used as filler and diluent of the tablet in same amount *i.e.* 105 mg (52.5% of total tablet weight-200 mg) in all tablet formulations. Talc, 4 mg, was used as glidant and magnesium stearate, 4 mg, was used as lubricant. Saccharin sodium, 8 mg, was used as a sweetening agent. Drug and excipient except talc were mixed geometrically in a mortar pestle and with the help of pestle and spatula for around half an hour then finally talc was added to the powder blend and mixed in a polybag.

Evaluation of powder blends

Powder blends have been evaluated for bulk density, tapped density and angle of respose Compressibility Index and Hauser's ratio were calculated from bulk and tapped density of the powder blends.

Bulk density

Apparent bulk density (g/mL) was determined by pouring bulk powder into a graduated cylinder and measuring the volume and weight. Bulk density (ρ_b) was calculated by the following formula.

$\rho_b = M/V_b$, where, M=mass of the powder and V_b=bulk volume of the powder

Tapped density

Apparent bulk density (g/mL) was determined by pouring bulk powder into a graduated cylinder and measuring the initial volume and weight. Then the cylinder was allowed to fall under its own weight onto a hard surface from a constant height, the tapping was continued until no further change in volume was noted. Tapped density (ρ_t) can be calculated by the following formula.

$\rho_t = M/V_t$, where, M=mass of the powder and V_t=final volume of the tapped powder

Carr's index

It was calculated according to the following equation.

Carr's index (%)=$[(\rho_t - \rho_b) / \rho_t] \times 100$

Hausner's ratio

It was calculated by the following formula.

Hausner Ratio=ρ_b/ρ_t

Angle of repose

The powder was allowed to flow through the funnel fixed on a tripod stand at definite height. The angle of repose is then calculated by measuring the height (h) and radius (r) of the heap of powder, formed. Angle of repose was calculated by the formula $\theta=\tan^{-1}(h/r)$.

Preparation and evaluation of tablets

Bioconvex ODTs were prepared in direct compression of the powder blends in a rotary tablet machine CMD3-16 (Cadmach, Ahmedabad) operated manually with one set of punch only. The compositions of the prepared ODTs are given in Table 1.

Weight variation

Tablets (n=20) were selected at random and average weight was calculated. Percentage deviation from the average was calculated.

Hardness

Hardness of the tablets was determined as force required to fracture the tablet when placed diametrically in a monsanto hardness tester. The tester consists of a barrel containing a compressible spring held between two plungers. The lower plunger is placed in contact with the tablet and a zero reading was observed. The upper plunger was forced against a spring by turning a thread bolt until the tablet fractures. As the spring was compressed, a pointer rides along a gauge in the barrel to indicate the force, which was a measure of hardness.

Table 1. Composition of various salbutamol sulphate ODTs

Ingredient	Amount (mg)									
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Salbutamol sulphate	4	4	4	4	4	4	4	4	4	4
SSG	2.50	6.25	12.50	18.75	22.5	2.50	6.25	12.5	18.75	22.50
CCS	22.5	18.75	12.50	6.25	2.50	-	-	-	-	-
Indion 414	-	-	-	-	-	22.5	18.75	12.5	6.25	2.50
MCC	50	50	50	50	50	50	50	50	50	50
Mannitol	105	105	105	105	105	105	105	105	105	105
Talc	4	4	4	4	4	4	4	4	4	4
Magnesium stearate	4	4	4	4	4	4	4	4	4	4
Saccharin sodium	8	8	8	8	8	8	8	8	8	8
Prosolv ODT	-	-	-	-	-	-	-	-	-	-
F-Melt	-	-	-	-	-	-	-	-	-	-
Ingredient	**Amount (mg)**									
	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20
Salbutamol sulphate	4	4	4	4	4	4	4	4	4	4
SSG	-	-	-	-	-	25	-	-	-	-
CCS	2.50	6.25	12.5	18.75	22.5	-	25	-	-	-
Indion 414	22.5	18.75	12.5	6.25	2.50	-	-	25		
MCC	50	50	50	50	50	50	50	50	-	-
Mannitol	105	105	105	105	105	105	105	105	-	-
Talc	4	4	4	4	4	4	4	4	-	-
Magnesium stearate	4	4	4	4	4	4	4	4	-	-
Saccharin sodium	8	8	8	8	8	8	8	8	-	-
Prosolv ODT	-	-	-	-	-	-	-	-	196	-
F-Melt	-	-	-	-	-	-	-	-	-	196

ODTs: Oral disintegrating tablets, SSG: Sodium starch glycolate, CCS: Croscarmellose sodium, MCC: Microcrystalline cellulose

Wetting time

The method reported by Bi et al.[16], was followed to measure tablet wetting time. A piece of tissue paper (12 cm x 10.75 cm) folded twice was placed in a small petridish (Internal diameter=6.5 cm) containing 6 mL of Sorenson's buffer pH 6.8 having small volume of dye orange red. A tablet was put on the paper, and the time for complete wetting was measured. Test was conducted on three tablets and an average and standard deviation was calculated.

Percent friability

It is measured of mechanical strength of tablets. Roche friabilator (FB II, Electrolab, Mumbai) was used to determine the friability by following procedure. A preweighed tablet was placed in the fribaiator. Fribaiator consists of a plastic-chamber that revolves at 25 rpm, dropping those tablets at a distance of 6 inches with each revolution. The tablets were rotated in the friabalator for at least 4 min. At the end of test tablets were dusted and reweighed, the loss in the weight of tablet is the measure of friability and is expressed in percentage as:

Friability (%)=(Loss in weight / Initial weight) x 100

In vitro disintegration time

The test was carried out on 6 tablets using the apparatus (DIA 32, Ketan Dial, Mumbai) specified in I.P. 1996, distilled water at 37°C±2°C was used as a disintegration media and the time taken for complete disintegration of the tablet with no palpable mass remaining in the apparatus was measured. One-way analysis of variance (ANOVA) followed by Tukey multi-comparison test was used at a confidence to investigate differences among the studied groups of samples.[17,18]

In vitro drug release studies

In vitro drug release studies were carried out by using USP XXIII Dissolution Apparatus II (Disso 2000, LabIndia, Mumbai) (Paddle type) at 50 rpm. The drug release profile was studied in 500 mL of pH 6.8 phosphate buffer by maintaining at 37±0.5°C. Aliquots (5 mL) of dissolution medium were withdrawn at specific time intervals, filtered and the amount of drug released was determined spectrophotometrically (2101, Systronics, Ahmedabad). Three trials for each batch were performed and average percentage drug release with standard deviation was calculated and recorded.

RESULTS

Precompression characteristics of different batches of powder blends

For each designed formulation, blend of drug and excipients was prepared and evaluated for micromeritic properties as shown in Table 2. Angle of repose was 8.8±0.637 to 16.7±0.921, bulk density ranged 0.55±0.031 to 0.82±0.072, tapped density was in range 0.62±0.072 to 0.98±0.12. Carr's Compressibility Index for different formulations ranged between 5.88±0.39 to 21.33±0.92, Hausner's ratio ranged from 1.066±0.009 to 1.27±0.08.

Evaluation of oral disintegrating tablets

Hardness of the tablets was in the range 3.8±0.22 to 4.7±0.11 kg, weight variation and friability test results were found within acceptable limits (Table 3).

Disintegration time

All the batches showed good disintegration time less than a min which qualifies them as ODTs. ODTs of batch F3 showed the lowest disintegration time of 19.28±3.11 s. Equal amounts of both disintegrants (SSG and CCS) in this batch gave best results and tablets disintegrated in shortest time. One-way ANOVA followed by Tukey multi-comparison test ($p<0.05$) showed significant difference in disintegration time of F3 formulation when compared to F1, F2, F4, F5, F16 and F17 formulations. When we used SSG or CCS as a single superdisntegrant in formulation F16 and F17 tablets disintegrated relatively slower, disintegration time was 28.37±2.26 s and 33.57±2.29 s respectively. In ODTs prepared with SSG+CCS, combination of disintegrants is proved as beneficial in reducing the disintegration time.

When SSG and Indion 414 were used as disintegrants in combination (Batch F6-F10), disintegration time increased when one of the disintegrant is at lowest concentration and the other at highest concentration (Table 3). One-way ANOVA followed by Tukey multi-comparison test ($p<0.05$) showed significant difference in disintegration time of F6 formulation when compared to F7, F8, F9, F10, F17 and F19 formulations. When disintegration time of batches F6-10 was compared to Batch F17 and F19, it was observed that combination was beneficial in reducing the disintegration time of tablets.

In combination of CCS and Indion 414 (Batch F11-F15), disintegration time reduces with increasing the amount of CCS and corresponding decrease in the amount of Indion 414, which indicate a more prominent role of Indion 414 as disintegrant. One-way ANOVA followed by Tukey multi-comparison test ($p<0.05$) showed significant difference in disintegration time of F11 formulation when compared to F12, F13, F14, F15, F17 and F18 formulations. All the tablets from batches from F10-F15 disintegrated in more than 30s time. When disintegration time of batches F11-15 were compared to Batch F17 and F18, it was observed that this combination was found not beneficial in reducing the disintegration time of tablets because disintegration time were longer than the disintegration time for batches F17 and F18. So, this combination of superdisintegrant failed to get any significant increase in disintegration time.

On the basis of disintegration time for batches F1-15, combination SSG+CCS in formulation F3 was best among other combinations studied on the basis on disintegration test.

Friability

Friability for batches F1-F19 was found within the acceptable limits. Batch F20 prepared with F-melt was greater than 1%

Table 2. Precompression characteristics of different powder blends

Batch	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Angle of repose (°)	Carr's index	Hausner ratio
F1	0.55±0.03	0.62±0.07	11.32±0.12	11.29±0.51	1.13±0.05
F2	0.66±0.08	0.71±0.02	9.68±0.32	7.04±0.44	1.08±0.01
F3	0.67±0.04	0.72±0.03	10.20±0.11	6.94±0.39	1.07±0.00
F4	0.65±0.05	0.73±0.02	12.80±0.43	10.95±0.76	1.12±0.01
F5	0.64±0.04	0.72±0.04	8.80±0.64	11.11±0.37	1.16±0.06
F6	0.59±0.03	0.75±0.04	13.20±0.76	21.33±0.92	1.27±0.08
F7	0.62±0.07	0.74±0.02	14.60±0.57	16.21±0.83	1.19±0.01
F8	0.69±0.07	0.72±0.09	11.10±0.32	4.16±0.17	1.04±0.05
F9	0.66±0.04	0.73±0.07	12.20±0.43	9.68±0.21	1.11±0.06
F10	0.68±0.05	0.74±0.06	16.70±0.92	8.10±0.47	1.08±0.02
F11	0.66±0.06	0.73±0.07	15.80±0.76	9.58±0.66	1.10±0.01
F12	0.63±0.01	0.74±0.06	14.10±0.44	14.86±0.36	1.17±0.01
F13	0.69±0.03	0.75±0.05	9.92±0.66	8.01±0.27	1.08±0.03
F14	0.64±0.03	0.69±0.02	13.24±0.19	7.24±0.31	1.08±0.01
F15	0.67±0.08	0.72±0.07	11.90±0.22	6.94±0.83	1.09±0.01
F16	0.63±0.02	0.67±0.04	12.70±0.12	5.97±0.36	1.07±0.01
F17	0.59±0.01	0.66±0.03	11.30±0.42	10.61±0.59	1.12±0.03
F18	0.64±0.03	0.68±0.05	11.90±0.24	5.88±0.39	1.07±0.02
F19	0.61±0.04	0.69±0.04	12.30±0.29	11.59±0.18	1.13±0.05
F20	0.82±0.07	0.98±0.1	14.40±0.17	16.32±0.17	1.20±0.10

All values are mean ± standard deviation of three determinations

which might be due to lower compressibility of the co-processed excipient. Friability of 0.92% was observed in batch F3, which gave maximum disintegration time. A minimum friability of 0.39% was achieved in batch F9.

Drug release studies

Drug release profiles of different ODTs are provided as Figure 1, 2, 3, 4, 5. Fastest drug release of 102.2±2.11% in 20 min was achieved for salbutamol sulphate ODTs of Batch F3, among batches F1-15. Batch F3 utilized disintegrants SSG and CCS, which might have showed additive/synergistic action. ODTs of Batch F20, prepared with marketed ODT excipient blend F-melt, release 98.50±2.4% drug in 15 min. Batch F3 was the best formulation among prepared ODT blends which gave drug release which may be related to its best shortest disintegration time. Minimum drug release of 91.47±2.59% in 30 min was achieved for ODTs of Batch F18, which contained a single disintegrant Indion 414. While, batch F16 and F17, which contained individual superdisintegrant SSG and CCS respectively, showed relatively faster drug release of 94.57±1.39% in 25 min and 93.19±2.12% in 30 min.

DISCUSSION

Angle of repose was less than 25° for all powder blends which indicated excellent flow properties. On the other hand, powder blends for F1-F5, F7-F19 formulations scored either good or excellent on scale of flowability based on Hauser's ratio and compressibility index as per USP.[19] Powder blends for F7 and F20 formulations were fair to flow. However, powder blend for

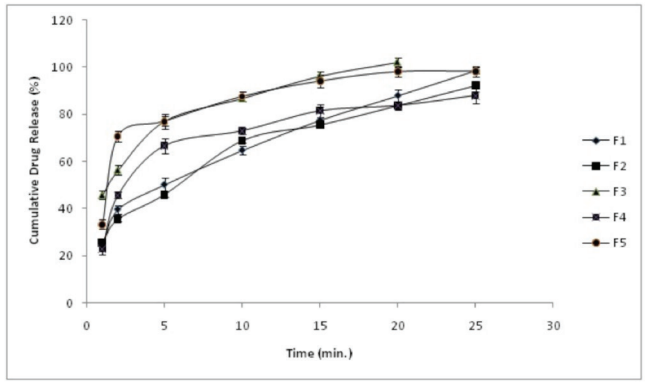

Figure 1. Drug release profile of salbutamol sulphate oral disintegrating tablets for batches F1-F5 (n=3, mean ± standard deviation is reported)

Table 3. Characterization of prepared salbutamol sulphate ODTs

Batch	Weight variation (%) (n=20)	Diameter (mm) (n=3)	Thickness (mm) (n=3)	Hardness (kg/cm²) (n=3)	Wetting time (s) (n=3)	Friability (%) (n=20)	Disintegration (s) (n=6)
F1	1.38	8.20±0.00	3.33±0.05	4.40±0.06	64.28±3.06	0.80	31.92±1.67
F2	2.24	8.19±0.15	3.33±0.07	4.10±0.12	58.33±6.11	0.64	25.65±2.28
F3	3.36	8.20±0.00	3.30±0.00	3.90±0.67	52.32±4.12	0.92	19.28±3.11
F4	4.82	8.20±0.00	3.40±0.11	3.90±0.33	65.40±6.20	0.76	24.37±2.98
F5	4.22	8.19±0.15	3.30±0.00	4.10±0.42	58.82±5.60	0.48	26.92±3.62
F6	2.91	8.20±0.00	3.30±0.00	4.10±0.42	58.13±3.26	0.77	27.67±1.89
F7	3.29	8.20±0.00	3.31±0.06	3.90±0.55	63.66±4.89	0.81	28.82±2.88
F8	5.12	8.20±0.00	3.10±0.17	4.00±0.37	66.24±5.12	0.96	32.39±3.28
F9	6.90	8.20±0.00	3.20±0.10	3.80±0.22	59.29±6.38	0.39	29.61±2.93
F10	3.37	8.19±0.21	3.30±0.05	4.20±0.79	43.28±3.19	0.56	22.60±1.62
F11	4.17	8.20±0.00	3.36±0.04	4.70±0.11	57.33±5.26	0.67	32.38±1.77
F12	1.12	8.20±0.00	3.30±0.00	3.90±0.82	63.28±6.29	0.84	33.44±2.29
F13	3.72	8.20±0.00	3.20±0.00	4.20±0.29	67.58±3.72	0.58	34.71±1.49
F14	4.49	8.20±0.00	3.46±0.20	4.10±0.31	65.72±4.11	0.69	34.82±2.81
F15	3.12	8.20±0.00	3.34±0.07	4.20±0.67	61.49±3.81	0.88	35.10±3.71
F16	2.29	8.23±0.04	3.40±0.07	4.10±0.17	62.42±2.98	0.52	28.37±2.26
F17	3.46	8.19±0.15	3.34±0.06	3.90±0.21	64.57±2.19	0.87	33.57±2.29
F18	3.49	8.19±0.06	3.30±0.00	4.20±0.31	66.67±4.01	0.79	31.56±3.12
F19	4.47	8.23±0.06	3.33±0.06	3.90± 0.10	55.66±3.21	0.71	32.13±2.11
F20	6.68	8.26±0.06	3.23±0.12	3.90±0.06	44.33±2.51	1.01	15.50±2.42

ODTs: Oral disintegrating tablets, All values are mean ± standard deviations, Number of tablets used for testing has been reproted in each coloum heading

formulation F6 was passable All powder blends were flowable and posed no difficulty in tabletting by direct compression. Hardness and friability of all the batches were found within the acceptable range. Tablets from all the batches disintegrated quickly in less than 40s and thus qualifies the criterion for ODTs.

In the combination A of superdisintegrants (batch F1-F5), SSG and CCS were used as disintegrants. As we increased the amount of SSG in combination and moved towards the batches F2, F3, F4, and F5, at the point where both superdisintegrants were in equal amount in formulation *i.e.* batch F3, it took lowest time (19.28±3.11 s) to disintegrate and showed fastest drug release (102.2±2.11% in 20 min) among these five batches (F1-F5). Total concentration of 12.5% disintegrant was used in preparing ODTs. F4 contained 9.375% and 11.25% of SSG, which is very much higher than its optimum concentration for disintegrant effect. At 8% or higher concentration it swells to a gel which increases disintegration time of ODTs.[20] Hence, it is anticipated that the gelling effect due to high concentration of SSG acts adversely to reduces the disintegration time of ODTs of batches F4 and F5. In one similar study, disintegrant blend of CCS and SSG in 1:1 ratio was used to prepare ODTs of certirizine hydrochloride (HCl)[21] and Ondansetron HCl.[22] Quick water uptake and good swelling ability of CCS and SSG is ascribed to the carboxylic moieties in their structure which upon hydration results in gelation.[23] Particle size and amorphous content in the disintegrant may also affect easy water accessibility.

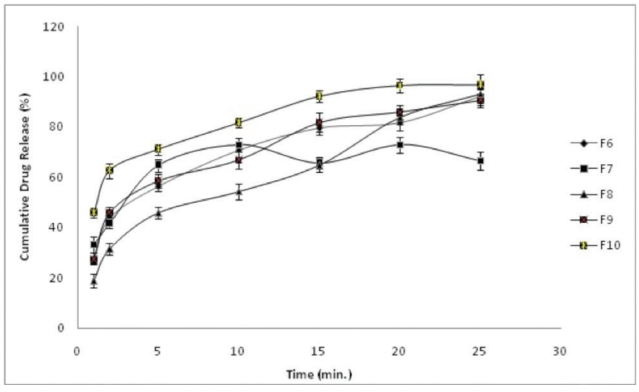

Figure 2. Drug release profile of salbutamol sulphate oral disintegrating tablets for batches F6-F10 (n=3, mean ± standard deviation is reported)

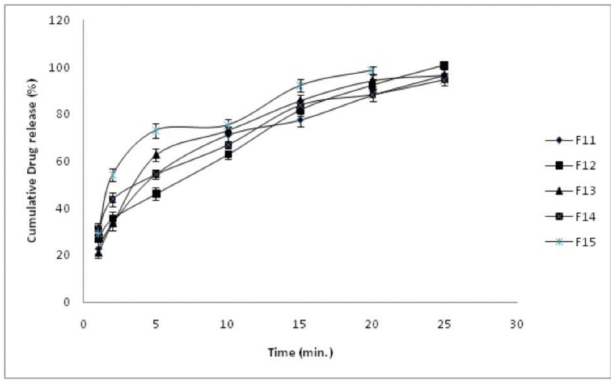

Figure 3. Drug release profile of salbutamol sulphate oral disintegrating tablets for batches F10-F15 (n=3, mean ± standard deviation is reported)

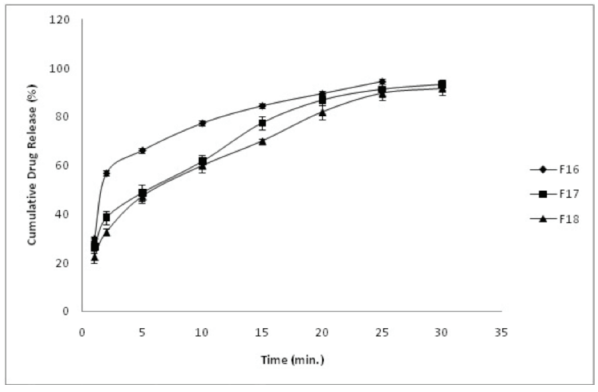

Figure 4. Drug release profile of salbutamol sulphate oral disintegrating tablets for batches F16-F18 (n=3, mean ± standard deviation is reported)

In the combination B (batch F6-F10), SSG and Indion 414 were used as disintegants. Among these batches (F6-F10), the best formulation F10 with 90% of SSG and 10% of Indion 414, disintegrated in 22.6±1.62 s and 96.73±4.12% drug release was achieved in 25 min. As the concentration of Indion 414 increased with simultaneous decrease in SSG, the formulations took more time to disintegrate probably due to insoluble nature of Indion 414 particles.[24]

In the combination C (batch F11-F15), CCS and Indion 414 were used, formulation F12 was the best formulation in combination C which showed a complete disintegration in 33.44±2.29 s and 100.7±1.77% in 25 min. An optimum swelling action contributed by Indion 414 and wicking action of CCS was anticipated when these are combined in 3:1 ratio.

ODT batches F16, F17, and F18 were prepared with single disintegrant SSG, CCS, and Indion 414, respectively. As a single superdisintegrant SSG gave best results of disintegration time 28.37±2.26 s and 94.57±1.39% drug release in 25 min. Furthermore, drug release at initial time points, especially at 2 min, was significantly higher in case of F16 formulation containing SSG as disintegrant. However, no significant difference in disintegration time was observed for CCS and Indion 414.

Among all ODT batches (F1-F18), F3 was the best formulation with fastest disintegration time of 19.28±3.11 s and acceptable friability. Combination of disintegrants in ODT batch F3 was

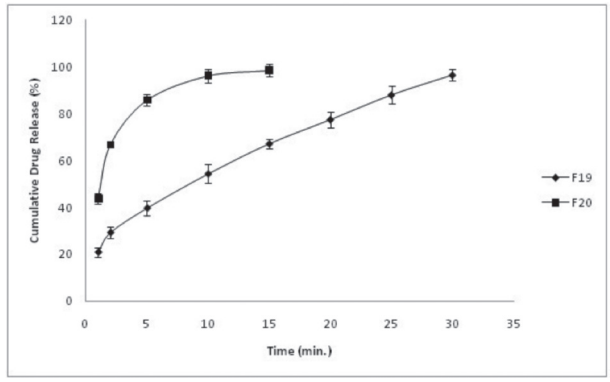

Figure 5. Drug release profile of salbutamol sulphate oral disintegrating tablets for batches F19-F20 (n=3, mean ± standard deviation is reported)

more effective than formulation F19 prepared with marketed blend, Prosolv ODT, which disintegrated in 32.13±2.11 s. Highest cumulative drug release (%) and faster disintegration of salbutamol sulphate ODTs in batch F3 was probably due to the additive/synergistic mechanisms of disintegrant action, *i.e.* high swelling index of SSG (7-12 folds) in three dimensions, along with swelling and wicking action of CCS. Increasing the amount of any one of the superdisintegrant and reducing the other, we observed an increase in disintegration time of tablet.

ODTs of batch F19, prepared by with marketed blend Prosolv-ODT, disintegrated completely in 32.13±2.11 s and 96.55±2.33% drug release was achieved in 30 min. Friability was 0.71% and wetting time was 55.66±3.21 s. Weight variation of 4.71% and hardness of 3.9±0.1 kg was achieved. Prosolv ODT is the unique combination of soluble and insoluble ingredients manufactured using JRS Pharma's (http://www.jrspharma.com/) co-processing technology. Prosolv contains MCC, colloidal silicon dioxide, mannitol, fructose, and crospovidone.[25,26] Prosolv® ODT is an ODT excipient matrix which allows a rapid formulation development and quality tablet manufacture.

ODT batch F20 prepared with F-melt marketed blend gave fastest disintegration of 15.5±2.42 s, and dissolution of 98.50±2.49% after 15 min and a fastest wetting time 44.33±2.52 s. However, the friability was greater than 1%. Furthermore, weigh variation was maximum (6.68%) and hardness was towards lower side 3.9±0.058 kg. Low hardness and friabile ODTs prepared with F-melt swelled and disintegrated quickly resulting in faster dissolution of the drug. F-melt was difficult to handle due to its hygroscopic nature. F-melt (http://www.f-melt.com/) is a spray-dried excipient used in ODTs that contain five different pharmaceutical ingredients consisting of carbohydrates (mannitol, xylitol and microcrystalline cellulose), disintegrant (crospovidone), and inorganic excipients (magnesium aluminosilicate, dibasic calcium phosphate anhydrous).[25] ODTs can be easily manufactured through direct compression only by adding F-melt to the active ingredient and lubricant. F-melt type C is recommended for faster disintegration needs in both pharmaceutical and nutraceutical formulations.[27] F-melt contains mannitol, xylitol, MCC, crospovidone, dibasic calcium phosphate anhydrous and magnesium aluminosilicate.[25] F-melt was hygroscopic at humidity conditions higher than 75% relative humidity. We also observed that F-melt is not suitable esthetically at higher humidity conditions. At accelerated stability conditions for 6 months, benzocaine ODTs diameter was increased by 2 mm due to swelling of tablet by moisture uptake.[25] F-melt contains crospovidone which can swells upon contact with water or moisture. Carr's Compressiblity Index for F20 was 16.32±0.172 and 1.195±0.098 which was probably due to hygroscopic nature of F-melt.

CONCLUSION

Salutamol sulphate ODTs of batch F3 were best ODTs prepared with combination of superdisintegrants. The present work revealed that binary combination of disintegrants is more effective than individual use disintegrants. Among all combinations of disintegrants, SSG and CCS (1:1 ratio) gave best results for both disintegration and drug release. ODTs of batch F3 disintegrate a little slower than ODT prepared with F-Melt (batch F20) but faster than ODTs containing Prosolv ODT (batch 19). F-Melt is a hygroscopic blend and need special handling, storage, and processing care. However, composition of F3 does not pose any problem of hygroscopicity. This aspect of F3 makes it a suitable alternative to a marketed blend like F-Melt or Prosolv ODT.

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Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Synthesis, Antibacterial and Lipoxygenase Inhibition Studies of *N*-(Alkyl/aralkyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides

N-(Alkil/aralkil)-*N*-(2,3-dihidro-1,4-benzodioksin-6-il)-4-metilbenzensülfonamitlerin Sentezi ile Antibakteriyel ve Lipoksijenaz İnhibitör Özellikleri

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ABSTRACT

Objectives: The present research work was aimed to synthesize some new sulfonamides bearing 1,4-benzodioxin ring, which might have suitable antibacterial potential and can be used as possible therapeutic agents for inflammatory ailments.

Materials and Methods: The synthesis was accomplished by the reaction of 2,3-dihydro-1,4-benzodioxin-6-amine (**1**) with 4-methylbenzenesulfonyl chloride (**2**) using 10% aqueous Na_2CO_3 to afford *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**3**). Further the parent molecule 3 was reacted with different alkyl/aralkyl halides (**4a-e**) to achieve *N*-(alkyl/aralkyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides (**5a-e**), using polar aprotic solvent; *N*,*N*-dimethylformamide (DMF) and catalytic amount of lithium hydride as base. The characterization of synthesized compounds was conducted by contemporary spectral techniques e.g., IR, 1H-NMR and EI-MS. Then these molecules were subjected to screening against various bacterial strains and their inhibitory potential against Lipoxygenase was also ascertained.

Results: The screening results against various Gram-positive and Gram-negative bacterial strains revealed that *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**3**), *N*-(2-bromoethyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5a**) and *N*-(2-phenethyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5b**) showed good inhibitory activity as compared to standard Ciprofloxacin. Moreover, *N*-(3-phenylpropyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5c**) and *N*-(4-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfon-amide (**5e**) displayed decent inhibition against lipoxygenase enzyme relative to standard Baicalein.

Conclusion: On the basis of results obtained it can be concluded that the synthesized sulfonamides may provide an overall indispensable basis to introduce new drug candidates for the cure of inflammatory and other associated diseases.

Key words: 2,3-dihydro-1,4-benzodioxin-6-amine, 1H-NMR, antibacterial potential, lipoxygenase

ÖZ

Amaç: Mevcut araştırma çalışmaları, uygun antibakteriyel potansiyele sahip olabilen ve inflamatuar hastalıklar için olası terapötik maddeler olarak kullanılabilen, 1,4-benzodioksin halkası taşıyan bazı yeni sülfonamidleri sentezlemek için hazırlanmıştır.

Gereç ve Yöntemler: Sentez, 10% sulu Na_2CO_3 kullanılarak 2,3-dihidro-1,4-benzodioksin-6-amin (**1**) ile 4-metilbenzensülfonil klorit (**2**) 3-dihidro-l,4-benzodioksin-6-il)-4-metilbenzensülfonamid (**3**). Ayrıca, ana molekül 3, *N*-(alkil/aralkil)-*N*-(2,3-dihidro-l,4-benzodioksin-6-il)-4-hidroksi-4-karboksilik asit elde etmek için farklı alkil/aralkil halojenürler (**4a-e**) Metilbenzensülfonamidler (**5a-e**), polar aprotik çözücü kullanarak; *N*,*N*-dimetilformamit (DMF) ve baz olarak katalitik miktarda lityum hidrid. Sentezlenen bileşiklerin karakterizasyonu çağdaş spektrum teknikleri örneğin IR, 1H-NMR ve EI-MS ile gerçekleştirildi. Daha sonra bu moleküller çeşitli bakteri soylarına karşı taramaya tabi tutuldu ve Lipoksigenaz'a karşı önleyici potansiyelleri de tespit edildi.

Bulgular: Çeşitli Gram-pozitif ve Gram-negatif bakteri suşlarına karşı tarama sonuçları, *N*-(2,3-dihidro-1,4-benzodioksin-6-il)-4-metilbenzensülfonamit (**3**), N-Bromoetil)-*N*-(2,3-dihidro-1,4-benzodioksin-6-il)-4-metilbenzensülfonamid (**5a**) ve *N*-(2-fenetil)-*N*-(2,3-dihidro-1,4-benzodioksin-6-il)-4-

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metilbenzensülfonamit (**5b**) standart Ciprofloxacine kıyasla iyi inhibitör aktivite gösterdi. Ayrıca *N*-(3-fenilpropil)-N-(2,3-dihidro-1,4-benzodioksin-6-il)-4-metilbenzensülfonamit (**5c**) ve *N*-(4-klorobenzil)-*N*-3-dihidro-l,4-benzodioksin-6-il)-4-metilbenzensülfon-amid (**5e**), standart Baikaleine göre lipoksijenaz enzimine karşı iyi inhibisyon sergiledi.

Sonuç: Elde edilen sonuçlara dayanarak, sentezlenen sülfonamidlerin inflamatuvar ve diğer ilişkili hastalıkların tedavisi için yeni ilaç adayları oluşturmak için vazgeçilmez bir temel oluşturabileceği sonucuna varılabilir.

Anahtar kelimeler: 2,3-dihidro-1,4-benzodioksin-6-amin, 1H-NMR, antibakteriyel potansiyel, lipoksijenaz

INTRODUCTION

Sulfonamides or sulfa drugs bearing SO_2NH- group derived from sulfanilamide, a class of compounds which are being utilized as synthetic antibiotics. In the history of medicines it was amongst the first antibiotic drug which has been used in 1930's.[1] Sulfa drugs do not possess any odor and they are mostly white in color or have slightly colored solids, soluble in water and exhibits more than two polymorphic forms.[2,3,4] Sulfonamides are capable of inhibiting bacterial growth, they also contest against p-aminobenzoic acid for dihydropteroatesynthetase enzyme, which is necessary for the biogenesis of folic acid (required for the growth of cell) by bacteria.[5,6] Sulfonamides possess antimicrobial activity against Gram-positive and Gram-negative bacteria and act as carbonic anhydrase inhibitors.[7,8,9,10] In combination with Trimethoprim, sulfonamides are used for the treatment of urinary tract infections and prevent parasitic and malarial infections.[11] In addition to antiviral agents sulfonamides are also used as antitumor agents, diuretics, anti-leprotic, tuberculostatics and oral hypoglycemic drugs.[12,13,14] Sulfasalazine (Figure 1); an antibiotic is used to manage the long-term inflammation of bowel diseases.[15] Aliphatic sulfonamide derivatives act as antifungal agents.[16]

Dioxane rings containing compounds can introduce variety of new substituents into common skeleton and provide new synthetic routes for generation of various organic compounds. These compounds have two special characteristics: (i) under thermal or photochemical conditions they are readily available for alkyletenes (ii) if C-C double bond is present in the dioxane ring then it will act as an enol form of masked acylacetic acids (unit cells in organic synthesis). Some medically important compounds whether synthetic or not, encompass benzodioxane moiety. Compounds encompassing benzodioxane ring system exhibits different biological activities such as, anti-microbial, antioxidant[17], anti-hepatotoxic and anti-inflammatory.[18,19]

The incredible pharmacological importance of sulfonamide stimulated us to carry out synthesis and bioactivity studies of *N*-alkyl/aralkyl-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides. So, in search of new and potent therapeutic agents, we have synthesized a series of sulfonamides bearing 1,4-benzodioxin ring system. The structures of synthesized compounds were characterized by fourier transform infrared spectroscopy (FTIR), ^{1}H-NMR and EI-MS techniques. Our effort endured fruitful as some of the molecules depicted good inhibitory potential against the some bacterial strains and lipoxygenase enzyme.

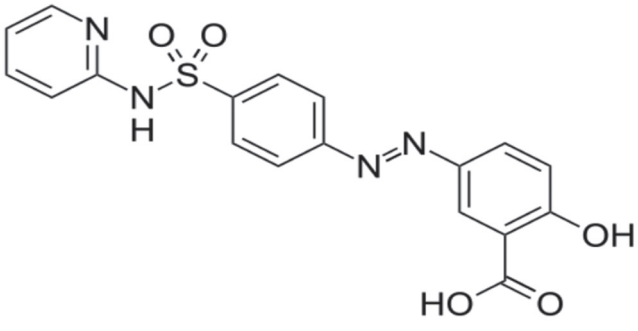


Figure 1. Structure of Sulfasalazine

EXPERIMENTAL

Measurements

Required chemicals/solvents were of analytical grade and procured from authorized dealers of Sigma Aldrich/Fluka. Thin Layer Chromatography (TLC) coated with silica gel G-25-UV_{254} was used to monitor reactions on every step in various percentages of *n*-hexane and ethyl acetate as mobile phase. Open capillary tubes were used in Gallen-Kamp melting point apparatus to record the melting points. The spectra of FTIR were recorded on a Jasco-320-A spectrophotometer in KBr disc and the wave number was in cm^{-1}. ^{1}H-NMR spectra were recorded by Bruker spectrometer in $CDCl_3$ operating at 400 MHz at 25˚C. The chemicals shifts (δ) were taken in ppm and coupling constants (*J*) were recorded in Hertz (Hz). Mass spectra (EI-MS) were measured on Finnigan MAT-312 instrument having the data system.

Synthesis

N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide ***(3)***

2,3-Dihydro-1,4-benzodioxin-6-amine (1.22 mL; 0.01 mol; **1**) and 4-methylbenzenesulfonyl chloride (0.90 g; 0.01 mol; **2**) were taken in a round bottom flask having 30 mL of distilled water. The pH of the suspension was adjusted and maintained at 9.0-10.0 by adding aqueous solution of 10% Na_2CO_3 at room temperature. The reaction solution was stirred for 2-3 hours and progress of the reaction was inspected the by TLC till single spot. The product was obtained by the slow addition of concentrated HCl at pH 2.0-3.0 as brown coloured precipitates which were collected by filtration, washed with distilled water and air-dried to afford pure *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**3**). The synthesis of compound **3** and its derivative **5a** was coherent with the reported method.[20]

*N-(Alkyl/aralkyl)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides (**5a-e**)*

N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (0.1 g; **3**) solubilised in 10 mL of *N*,*N*-

dimethyl formamide (DMF) followed by the addition of lithium hydride (LiH) (0.004 g; LiH) in the mixture which was stirred for 2-3 hours at room temperature. After stirring, various alkyl/aralkyl halides (**4a-e**) were added slowly to the mixture and were further stirred for 2-3 hours. The progress of reaction was monitored via TLC till single spot. After reaction completion the reaction mixture was quenched with cold distilled water to get precipitates of *N*-(alkyl/aralkyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides (**5a-e**) which were collected by the filtration or solvent extraction (using $CHCl_3$) depending upon the nature of the derived compound.

*N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**3**)*

Greyish brown powder, Yield: 82%, m.p: 150˚C; Molecular formula: $C_{15}H_{15}NO_4S$; Molecular mass: 305 gmol^{-1}; HR-MS: $[M]^+$ 305.3509 (calculated for $C_{15}H_{15}NO_4S$; 305.3506). IR (KBr, cm^1) ν_{max}: 3500 (N-H), 3080 (Ar C-H), 1650 (Ar C=C), 1390 (SO_2-), 1175 (C-O-C); ^{1}H-NMR ($CDCl_3$, 400MHz, δ in ppm): 9.87 (s, 1H, NH), 7.58 (d, *J*=8.4 Hz, 2H, H-2' & H-6'), 7.32 (d, *J*=8.0 Hz, 2H, H-3' & H-5'), 6.68 (d, *J*=8.4 Hz, 1H, H-8), 6.65 (d, *J*=2.4 Hz, 1H, H-5), 6.50 (dd, *J*=2.4, 8.8 Hz 1H, H-7), 4.14 (s, 4H, CH_2-2 & CH_2-3), 2.33 (s, 3H, CH_3-7'); EI-MS (m/z): 305 [M$^+$; $C_{15}H_{15}NO_4S$], 241 $[C_{15}H_{15}NO_2]^+$, 218 $[C_{10}H_9NO_2S]^+$, 170 $[C_8H_7O_2]^+$, 155 $[C_7H_7SO_2]^+$, 91 $[C_7H_7]^+$.

*N-(2-Bromoethyl)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5a**)*

Tea pink powder; Yield: 97%; m.p: 142˚C; Molecular formula: $C_{17}H_{18}BrNO_4S$; Molecular mass: 412 gmol^{-1}; HR-MS: $[M]^+$ 412.2997 (calculated for $C_{17}H_{18}BrNO_4S$; 412.2992). IR (KBr, cm^{-1}) ν_{max}: 3017 (Ar C-H), 1677 (Ar C=C), 1390 (SO_2-), 1150 (C-O-C); ^{1}H-NMR ($CDCl_3$, 400 MHz, δ in ppm): 7.59 (d, *J*=8.4 Hz, 2H, H-2' & H-6'), 7.37 (d, *J*=8.0 Hz, 2H, H-3' & H-5'), 6.64 (d, *J*=8.4 Hz, 1H, H-8), 6.63 (d, *J*=2.4 Hz, 1H, H-5), 6.53 (dd, *J*=2.4, 8.8 Hz, 1H, H-7), 4.16 (s, 4H, CH_2-2 & CH_2-3), 3.83 (t, *J*=7.2 Hz, 2H, CH_2-1''), 3.36 (t, *J*=7.2 Hz, 2H, CH_2-2''), 2.34 (s, 3H, CH_3-7'); EI-MS (*m/z*): 412 [M$^+$; $C_{17}H_{18}BrNO_4S$], 321 $[C_{10}H_{11}BrNO_4S]^+$, 318 $[C_{16}H_{16}NO_4S]^+$, 277 $[C_9H_{11}BrNO_2S]^+$, 257 $[C_{10}H_{11}BrNO_2]^+$, 240 $[C_{10}H_9NSO_4]^+$, 218 $[C_{10}H_9NSO_2]^+$, 170 $[C_8H_7O_2]^+$, 155 $[C_7H_7SO_2]^+$, 149 $[C_8H_7NO_2]^+$, 107 $[C_2H_4Br]^+$, 91 $[C_7H_7]^+$.

*N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-N-(2-phenethyl)-4-methylbenzenesulfonamide (**5b**)*

Greyish brown powder; Yield: 85%; m.p: 110˚C; Molecular formula: $C_{23}H_{23}NO_4S$; Molecular mass: 409 gmol^{-1}; HR-MS: $[M]^+$ 409.4988 (calculated for $C_{23}H_{23}NO_4S$; 409.4994). IR (KBr, cm^{-1}) ν_{max}: 3015 (Ar C-H), 1689 (Ar C=C), 1395 (SO_2-), 1155 (C-O-C); ^{1}H-NMR ($CDCl_3$, 400MHz, δ in ppm): 7.53 (d, *J*=8.4 Hz, 2H, H-2' & H-6'), 7.34 (d, *J*=8.0 Hz, 2H, H-3' & H-5'), 7.28-7.12 (m, 5H, H-2''' to H-6'''), 6.62 (d, *J*=8.4 Hz, 1H, H-8), 6.60 (d, *J*=2.4 Hz, 1H, H-5), 6.51 (dd, *J*=2.4, 8.8 Hz, 1H, H-7), 4.85 (t, *J*=7.6 Hz, 2H, CH_2-8''), 4.15 (s, 4H, CH_2-2 & CH_2-3), 2.37 (s, 3H, CH_3-7'), 2.02 (t, *J*=7.6 Hz, 2H, CH_2-7''); EI-MS (*m/z*): 409 $[M]^+$, 345 $[C_{23}H_{23}NO_2]^+$, 332 $[C_{17}H_{18}NO_4S]^+$, 318 $[C_{16}H_{16}NO_4S]^+$, 254 $[C_{16}H_{16}NO_2]^+$, 241 $[C_{15}H_{15}NO_2]^+$, 218 $[C_{10}H_9NSO_4]^+$, 197 $[C_9H_{11}NO_2S]^+$, 155 $[C_7H_7SO_2]^+$, 105 $[C_8H_9]^+$, 91 $[C_7H_7]^+$.

*N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-N-(3-phenylpropyl)-4-methylbenzenesulfonamide (**5c**)*

Light brown powder; Yield: 93%; m.p: 130˚C; Molecular formula: $C_{24}H_{25}NO_4S$; Molecular mass: 423 gmol^{-1}; HR-MS: $[M]^+$ 423.5260 (calculated for $C_{24}H_{25}NO_4S$; 423.5267). IR (KBr, cm^{-1}) ν_{max}: 3027 (Ar C-H), 1679 (Ar C=C), 1157 (C-O-C), 1398 (SO_2-), 681; ^{1}H-NMR ($CDCl_3$, 400MHz, δ in ppm): 7.57 (d, *J*=8.4 Hz, 2H, H-2' & H-6'), 7.35 (d, *J*=8.0 Hz, 2H, H-3' & H-5'), 7.27-7.07 (m, 5H, H-2''' to H-6'''), 6.67 (d, *J*=8.4 Hz, 1H, H-8), 6.62 (d, *J*=2.4 Hz, 1H, H-5), 6.57 (dd, *J*=2.4, 8.8 Hz, 1H, H-7), 4.13 (s, 4H, CH_2-2 & CH_2-3), 2.61 (t, *J*=6.0 Hz, 2H, CH_2-9''), 2.30 (s, 3H, CH_3-7'), 1.80 (t, *J*=8.0 Hz, 2H, CH_2-7''), 0.93-0.89 (m, 2H, CH_2-8''); EI-MS (m/z): 423 [M$^+$; $C_{24}H_{25}NO_4S$], 381 $[C_{21}H_{19}NO_4S]^+$, 268 $[C_{17}H_{18}NO_2S]^+$, 240 $[C_{10}H_{10}NO_4S]^+$, 208 $[C_{10}H_{10}NO_2S]^+$, 197 $[C_9H_{11}NO_2S]^+$, 155 $[C_7H_7SO_2]^+$, 105 $[C_8H_9]^+$, 119 $[C_9H_{11}]^+$, 91 $[C_7H_7]^+$.

*N-(2-Chlorobenzyl)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5d**)*

Grey powder; Yield: 96%, m.p: 127˚C; Molecular formula: $C_{22}H_{20}ClNO_4S$; Molecular mass: 429 gmol^1; HR-MS: $[M]^+$ 429.9169 (calculated for $C_{22}H_{20}ClNO_4S$; 429.9176). IR (KBr, cm^1) ν_{max}: 3019 (Ar C-H), 1673 (Ar C=C), 1382 (SO_2-), 1095 (C-O-C), 765 (C-Cl); ^{1}H-NMR ($CDCl_3$, 400MHz, δ in ppm): 7.56 (d, *J*=8.4 Hz, 2H, H-2' & H-6'), 7.36 (dd, *J*=2.6, 8.2 Hz, 1H, H-3''), 7.30 (d, *J*=8.0 Hz, 2H, H-3' & H-5'), 7.19 (dd, *J*=2.4, 8.6 Hz, 1H, H-6''), 7.15-7.10 (m, 2H, H-4'' & H-5''), 6.66 (d, *J*=8.4 Hz, 1H, H-8), 6.64 (d, *J*=2.4 Hz, 1H, H-5), 6.55 (dd, *J*=2.4, 8.8 Hz, 1H, H-7), 4.86 (s, 2H, CH_2-7''), 4.12 (s, 4H, CH_2-2 & CH_2-3), 2.35 (s, 3H, CH_3-7'); EI-MS (*m/z*): 431 [M+2; $C_{22}H_{20}ClNO_4S]^+$, 429 [M$^+$; $C_{22}H_{20}ClNO_4S$], 365 $[C_{22}H_{20}NO_2Cl]^+$, 274 $[C_{15}H_{13}ClNO_2]^+$, 240 $[C_{10}H_{10}NO_4S]^+$, 208 $[C_{10}H_{10}NO_2S]^+$, 155 $[C_7H_7SO_2]^+$, 125 $[C_7H_6Cl]^+$, 111 $[C_6H_4Cl]^+$, 91 $[C_7H_7]^+$.

*N-(4-Chlorobenzyl)-N-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5e**)*

Brown powder; Yield: 90%; m.p: 132˚C; Molecular formula: $C_{22}H_{20}ClNO_4S$; Molecular mass: 429 gmol^{-1}; HR-MS: $[M]^+$ 429.9171 (calculated for $C_{22}H_{20}ClNO_4S$; 429.9176). IR (KBr, cm^{-1}) ν_{max}: 3039 (Ar C-H), 1679 (Ar C=C), 1149 (C-O-C), 1379 (SO_2-), 769 (C-Cl); ^{1}H-NMR ($CDCl_3$, 400 MHz, δ in ppm): 7.54 (d, *J*=8.4 Hz, 2H, H-2' & H-6'), 7.31 (d, *J*=8.0 Hz, 2H, H-3' & H-5'), 7.13 (d, *J*=8.4 Hz, 2H, H-3'' & H-5''), 7.05 (d, *J*=8.4 Hz, 2H, H-2'' & H-6''), 6.67 (d, *J*=8.4 Hz, 1H, H-8), 6.61 (d, *J*=2.4 Hz, 1H, H-5), 6.52 (dd, *J*=2.4, 8.8 Hz, 1H, H-7), 4.77 (s, 2H, CH_2-7''), 4.10 (s, 4H, CH_2-2 & CH_2-3), 2.32 (s, 3H, CH_3-7'); EI-MS (m/z): 431 [M+2; $C_{22}H_{20}ClNO_4S]^+$, 429 [M$^+$; $C_{22}H_{20}ClNO_4S$], 365 $[C_{22}H_{20}NO_2Cl]^+$, 274 $[C_{15}H_{13}ClNO_2]^+$, 240 $[C_{10}H_{10}NO_4S]^+$, 208 $[C_{10}H_{10}NO_2S]^+$, 155 $[C_7H_7SO_2]^+$, 125 $[C_7H_6Cl]^+$, 111 $[C_6H_4Cl]^+$, 91 $[C_7H_7]^+$.

Antibacterial assay

The antibacterial activity was evaluated by using the referenced method but with minor modifications.[21-23] The antibacterial activity was carried out in sterile 96-wells microplates under aseptic circumstances. This technique is based on the principle that as the microbial growth increases in a log phase of growth, the number of microbial cells multiply exponentially which in turn increases absorbance of broth medium. Micro organisms used in this study included; three Gram-negative bacteria i.e. *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*

and two Gram-positive bacteria namely *Bacillus subtilis* and *Staphylococcus aureus*. All the stains were obtained from the local hospital. They are clinically cultured samples/clinical pathogens and were tested and verified by the experts. The tested strains were nourished on stock agar culture medium. The samples being analyzed were diluted in suitable solvents and 20 µL of each sample was pipetted into every well. Fresh bacterial culture maintained overnight was suitably diluted with fresh nutrient broth and was 180 µL quantity of this bacterial culture was poured into every well. The starting absorbance of the culture was strictly maintained at 540 nm between 0.12-0.19. The total volume kept in each well was 200 µL. These microplates covered with lids were incubated for 16-24 hours at 37°C. Before and after incubation, the absorbance was measured at 540 nm using microplate reader, and index of bacterial growth was noted by the difference in absorbance before and after incubation. The formula for calculating the percentage inhibition is:

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Results are mean of three sets of test samples (n=3, ±standard error of mean). Standard used was ciprofloxacin. Suitable dilutions ranging from 5-30 µg/well were used to measure the minimum inhibitory concentration (MIC). EZ-Fitz Perrella Scientific Inc. Amherst USA software was used to calculate the results.

Lipoxygenase assay

Lipoxygenase activity was assayed according to the method reported[24,25,26] with slight modifications. A total volume of 200 µL lipoxygenase assay mixture having 150 µL sodium phosphate buffer (100 mM, pH 8.0), 10 µL test compound and 15 µL purified lipoxygenase enzyme. The contents were mixed and pre read at 234 nm and pre-incubated for 10 min at 25°C. The reaction was initiated by addition of 25 µL substrate solution. The change in absorbance was observed after 6 min at 234 nm. All reactions were performed in triplicates. The positive and negative controls were included in the assay. Baicalein (0.5 mM well^{-1}) was used as a positive control.

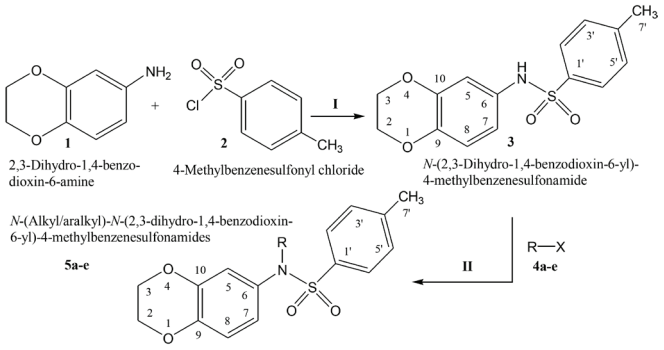
IC_{50} values (concentration at which there is 50% enzyme inhibition) of compounds was calculated using EZ-Fit Enzyme kinetics software (Perella Scientific Inc. Amherst, USA).

Statistical analysis

All the measurements were done in triplicate and statistical analysis was performed by Microsoft Excel 2010. Results are presented as mean ± standard error of mean.

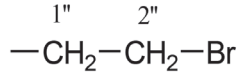
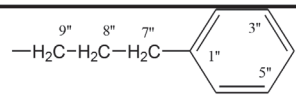
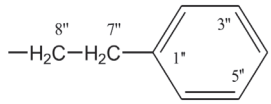
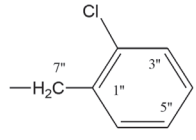
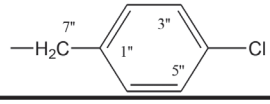
RESULTS AND DISCUSSION

N-(Alkyl/aralkyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfon-amides (**5a-e**) were synthesized following pathway sketched in Scheme 1 and Table 1. All conditions suitable for reactions and detailed procedures have been discussed in experimental section. The projected structures of newly synthesized molecules were confirmed via IR, ^{1}H-NMR and EI-MS techniques. In search of potent anti-bacterial and lipoxygenase inhibitors, these synthesized molecules were screened against various Gram-positive and Gram-negative bacterial strains (Table 2) and lipoxygenase enzyme (Table 3).



Scheme 1. Outline for the synthesis of *N*-(Alkyl/aralkyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides (5a-e), Reagents & Conditions: (I) Aq. 10% Na_2CO_3 soln./pH 9-10/stirring at reverse transcription for 3 hrs, (II) *N, N*-dimethylformamide/LiH/stirring at reverse transcription for 2-3 hrs

Table 1. Different alkyl/aralkyl halides (4a-e) utilized in the synthesis of *N*-(alkyl/aralkyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamides (5a-e)

Compound	-R	Compound	-R
4a, 5a		**4c, 5c**	
4b, 5b		**4d, 5d**	
		4e, 5e	

Chemistry

N-2,3-Dihydro-1,4-benzodioxine-6-amine (**1**) was reacted with 4-methylbenzenesulfonyl chloride (**2**) in the presence of 10% Na_2CO_3 under dynamic pH control at 9-10 under stirring for 2-3 hours at room temperature to achieve *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**3**). Further, alkyl/aralkylation of the parent compound 3 was done utilizing different alkyl/aralkyl halides (**4a-e**) in DMF as a polar aprotic solvent and LiH as the base to yield the new target compounds (**5a-e**). Compound **3** and **5a** were synthesized by method reported in literature and the spectral data was also found to be in concordance with the literature data.[20] The molecule **5e** was obtained as brown powder having melting point 132°C. The molecular formula $C_{22}H_{20}ClNO_4S$ was deduced through its EI-MS, having molecular ion peak at *m/z* 429 $[M]^+$ and by counting the number of protons via integration curves in its ^{1}H-NMR spectrum. The mass spectrum of this molecule has been shown in Figure 2 while its suggested mass fragmentation has been sketched in Figure 3. The IR spectrum showed absorption bands at ν 3039, 1679, 1149, 1379 and 709 cm^{-1} for the bond stretching of C-H, Ar C-H, Ar C=C, C-O-C, SO_2 and C-Cl respectively. In ^{1}H-NMR spectrum, two discrete A_2B_2 type spin systems were observed in the aromatic region. The ortho-coupled doublets resonating at δ 7.54 (2H, H-2′ & H-6′ and δ 7.31 (2H, H-3′ & H-5′) along with a methyl signal at δ 2.32 (H-7′) corroborated the presence of 4-methylbenzenesulfonyl moiety in the molecules. Similarly, the other ortho-coupled doublets at δ 7.13 (2H, H-3″ & H-5″) and δ 7.05 (2H, H-2″ & H-6″) along with a benzylic methylene signal at 4.77 (s, 2H, CH_2-7″) were helpful to ascertain the substitution of 4-chlorobenzyl moiety on nitrogen atom of the targeted sulfonamide. The 6-substituted 1,4-benzodioxane nucleus in the molecule was clearly demonstrated by its three typical signals in aromatic region at δ 6.67 (d, *J*=8.4 Hz, 1H, H-8), 6.61 (d, *J*=2.4 Hz, 1H, H-5) and 6.52 (d, *J*=2.4, 8.8 Hz, 1H, H-7) along with a broad singlet in aliphatic region at δ 4.10 (4H, CH_2-2 & CH_2-3). On the basis of above collected evidences, the projected structure of **5e** was confirmed as *N*-(4-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide. Similarly, the structural analysis of other synthesized molecules (**5a-e**) was affected in an analogous manner e.g. appearance of an A_2B_2 spin system

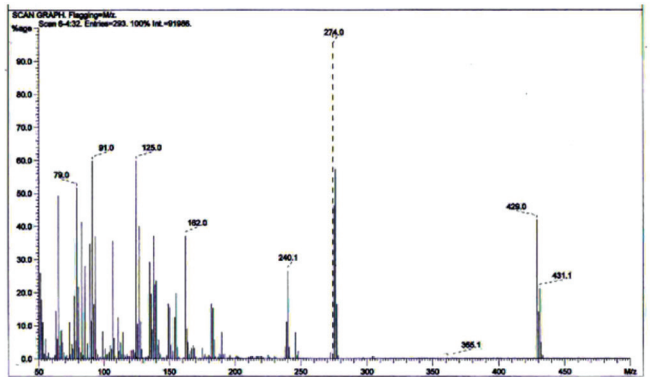


Figure 2. EI-MS spectrum of *N*-(4-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5e**)

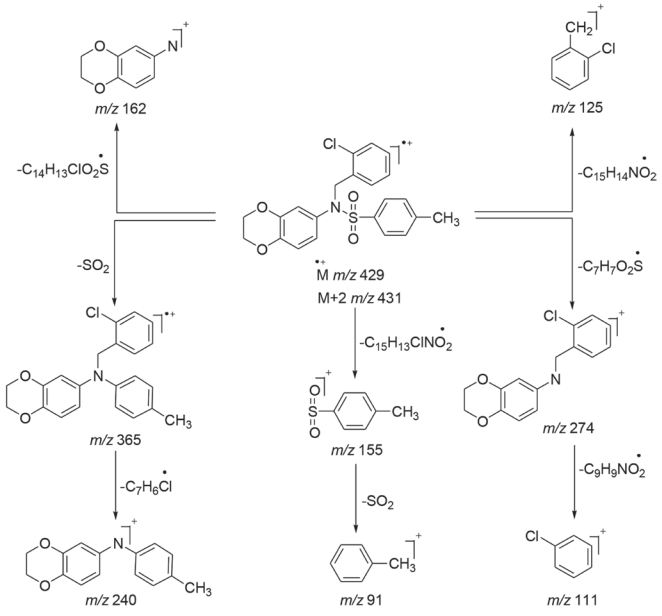


Figure 3. Suggested mass fragmentation pattern of *N*-(4-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (**5e**)

Table 2. Antibacterial activity (% age inhibition and minimum inhibitory concentration) of synthesized 3 and 5a-e

Codes	Antibacterial activity					
	S. typhi		*E. coli*		*B. subtilis*	
	Inhibition %	MIC (μg mL^{-1})	Inhibition %	MIC (μg mL^{-1})	Inhibition %	MIC (μg mL^{-1})
3	45.57±0.25	-	79.57±0.53	09.22±0.70	80.29±0.50	08.41±0.98
5a	62.45±0.81	13.00±0.89	69.14±0.63	09.66±0.33	70.86±0.64	11.46±0.90
5b	57.98±0.56	15.72±0.54	77.14±0.65	10.11±0.04	35.14±1.00	-
5c	48.00±0.24	-	34.86±0.63	-	44.00±0.91	-
5d	60.38±0.81	13.51±0.56	46.03±0.68	-	47.81±0.49	-
5e	56.09±0.48	16.12±0.13	24.00±0.75	-	34.29±0.54	-
Ciprofloxacin	**91.05±0.68**	**7.83±0.78**	**92.32±0.42**	**8.01±0.12**	**92.02±053**	**7.22±0.67**

MIC: Minimum inhibitory concentration

Table 3. Enzyme inhibition activity (% age inhibition and IC_{50}) of synthesized 3 and 5a-e

Codes	Lipoxygenase assay		
	Conc. (mM)	Inhibition %	IC_{50} (mM)
3	0.5	86.41±0.58	255.38±0.61
5a	0.5	59.41±0.91	405.39±0.39
5b	0.5	89.93±0.63	168.31±0.47
5c	0.5	98.71±0.42	085.79±0.48
5d	0.5	60.93±0.41	314.91±0.25
5e	0.5	84.71±0.64	089.32±0.34
Baicalein	**0.5**	**93.79±1.27**	**22.41±1.30**

for 4-methylbenzenesulfonyl moiety was observed in all the synthesized derivatives. In **5a**, the appearance of two triplets at δ 3.83 and 3.36, respectively, marked the amalgamation of bromoethyl moiety at *N*-atom of the parent sulfonamide. In **5b**, the insertion of phenethyl group was confirmed by appearance of a multiplet at δ 7.28-7.12 for phenyl group and two triplets at δ 4.85 and δ 2.02, for two adjacent methylene groups in the molecule. The phenylpropyl group in **5c** was characterized by a five-proton multiplet in aromatic region and three methylene signals in aliphatic region. In this case, the central methylene appeared as a multiplet resonating at δ 0.93-0.89. Similarly, the presence of typical signals of 2-chlorobenzyl moiety in 5d, pointed out to the successful thesis of targeted molecule.

Pharmacological screening

Antibacterial activity

Synthesized derivatives were screened for their antibacterial activity against three Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*) and two Gram-positive bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*). The results of screening are tabulated in Table 2. The synthesized compounds showed moderate antibacterial potential as compared to the standard ciprofloxacin. It was revealed that none of the synthesized compounds showed any activity against *Staphylococcus aureus* (+) and *Pseudomonas aeruginosa* (-).

Against *S. typhi* *N*-(2-bromoethyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5a) and *N*-(2-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5d) showed comparatively better inhibition having IC_{50} value of 13.00±0.89 μg mL^{-1} and 13.51±0.56 μg mL^{-1} respectively, relative to the reference standard; ciprofloxacin (7.83±0.78 μg mL^{-1}). Parent sulfonamide 3 and *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-*N*-(3-phenylpropyl)-4-methylbenzenesulfonamide (5c) was inactive against *S. typhi*. Results against *E. coli* revealed that compounds 3, *N*-(2-bromoethyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5a) and *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-*N*-(2-phenethyl)-4-methylbenzenesulfonamide (5b) showed inhibitory potential having IC_{50} values of 9.22±0.70 μg mL^{-1} and 9.66±0.33 μg mL^{-1}, respectively, as compared to standard ciprofloxacin (MIC; 8.01±0.12 μg mL^{-1}). Screening results against *B. subtilis* revealed that Only 3, and *N*-(2-bromoethyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5a) showed inhibitory potential with IC_{50} values of 8.41±0.98 μg mL^{-1} and 11.46±0.90 μg mL^{-1}, respectively, relative to ciprofloxacin (MIC; 7.22±0.67 μg mL^{-1}). Rest of the compounds did not show any activity against the bacterial strains.

Lipoxygenase activity

All the synthesized compounds were screened against lipoxygenase enzyme. Amongst the screened compounds, *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-*N*-(3-phenylpropyl)-4-methylbenzenesulfonamide (5c) and *N*-(4-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5e) were identified as possible inhibitors of liquid oxygen having IC_{50} value of 85.79±0.48 mM and 89.32±0.34 mM respectively, relative to the Baicalein, a reference standard (22.41±1.3 mM). Rest of the compounds showed very low inhibitory potential. The results depicted by the screening are elaborated in Table 3.

CONCLUSION

All the synthesized molecules were achieved in excellent yields by following a simple method. The projected structures of synthesized compounds were well supported by the spectral characterization data by IR, ^{1}H-NMR and EI-MS. Antibacterial potential of the parent compound 3, and its derivatives 5a-e, revealed that none of the compounds were active against *S. aureus* and *P. aeruginosa*. Moreover, *N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-*N*-(3-phenylpropyl)-4-methylbenzenesulfonamide (5c) did not show any inhibitory potential against any bacterial strain. Overall, *N*-(2-bromoethyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5a) was the only compound which showed maximum inhibition against *S. typhi*, *E. coli* and *B. subtilis*. However, against lipoxygenase enzyme, all compounds showed weaker inhibitory potential except *N*-(3-phenylpropyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5c) and *N*-(4-chlorobenzyl)-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-4-methylbenzenesulfonamide (5e) which displayed decent inhibition against lipoxygenase. On the basis of aforesaid results, the synthesized sulfonamides may provide an overall indispensable basis to introduce new drug candidates for the cure of inflammatory and other associated diseases.

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Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

In Situ Hydrogel Formulation for Intra-Articular Application of Diclofenac Sodium-Loaded Polymeric Nanoparticles

Diklofenak Sodyum Yüklü Polimerik Nanopartiküllerin İntra-Artiküler Uygulaması İçin *İn Situ* Hidrojel Formülasyonu

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ABSTRACT

Objectives: The world's population is getting older and the number of people suffering from arthritis is a major problem according to World Health Organization's data. In this respect, the need for more efficient treatment for arthritis becomes an urgent issue. In this research, nanoparticle bearing in situ gelling hydrogel formulation was developed for prolonged local delivery of diclofenac sodium (DS).

Materials and Methods: Emulsion-solvent evaporation technique was used for the preparation of nanoparticles. Particle size, encapsulation efficiency, morphology, and drug release profile of DS loaded biodegradable nanoparticles as well as gel viscosity and gelation time of *in situ* gelling hydrogel formulations were optimized to increase the time interval between each dose application for enhanced patience compliance.

Results: The spherical nanoparticles with a mean particle diameter of 168 nm was obtained and confirmed by both transmission electron microscope and atomic force microscope. Different types of surfactants were tested in the first emulsification step of nanoparticle production process and Arlacel®-C significantly increased the encapsulation efficiency to 89.7%. Thirty days prolonged *in vitro* release of DS was achieved by using the combined formulation of polymeric nanoparticles and *in situ* hydrogel prepared by using poloxomer 407 and chitosan.

Conclusion: Local administration of DS with this novel delivery system could be considered of having potential to minimize side effects associated with decreased amount of drug in dosage form compared to conventional oral dose.

Key words: Diclofenac sodium, drug delivery, PLGA, Poly(ε-caprolactone), thermosensitive hydrogel, poloxamers

ÖZ

Amaç: Dünya Sağlık Örgütü'nün verilerine göre dünya çapında insanların yaş ortalaması ve bağlantılı olarak artrit hastalığından mağdur olan insan sayısı da artmaktadır. Bu açıdan bakıldığında, artrit tedavisi için daha etkili tedavilerin gerekliliği önemli hale gelmiştir. Bu araştırmada, diklofenak sodyumun (DS) uzatılmış lokal taşınımı için nanopartikül içeren in situ hidrojel formülasyonları geliştirilmiştir.

Gereç ve Yöntemler: Nanopartiküllerin hazırlanmasında emülsiyon-çözücü buharlaştırma yöntemi kullanılmıştır. DS yüklü nanopartiküllerin partikül büyüklüğü, enkapsülasyon etkinliği, morfolojisi ve ilaç salım profilleri ile *in situ* hidrojelin jel viskozitesi ve jelleşme süresi, hasta uyuncunu artırmak için her bir dozun uygulanması arasındaki zamanı artırmak amacıyla optimize edilmiştir.

Bulgular: Ortalama partikül büyüklüğü 168 nm olan küresel nanopartiküller elde edilmiş ve geçirimli elektron mikroskobu ve atomik kuvvet mikroskobu ile desteklenmiştir. Nanopartikül üretim sürecinin ilk emülsifikasyon adımında farklı sürfaktanlar denenmiş ve Arlacel®-C diklofenak sodyumun enkapsülasyon etkinliğini %89.7'ye yükseltmiştir. DS'nin 30 güne uzatılmış *in vitro* salımı, nanopartiküllerin ve poloksamer 407/kitozan ile hazırlanmış *in situ* hidrojelin kombine kullanılmasıyla başarılmıştır.

Sonuç: DS'nin bu yenilikçi taşıyıcı sistem ile lokal uygulanmasının, konvansiyonel oral dozaj formuna göre azaltılmış miktarda etkin madde içermesinden dolayı yan etkileri minimize etme potansiyeline sahip olduğu düşünülmüştür.

Anahtar kelimeler: Diklofenak sodyum, ilaç taşınımı, PLGA, Poly(ε-kaprolakton), ısıya duyarlı hidrojel, poloksamerler

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INTRODUCTION

Biodegradable nanoparticulate drug delivery systems are becoming more common each day because of their advantages such as enhanced drug stability, targeted delivery to any desired cell or tissue, and modified release. Poly(lactic-co-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) are two major examples of polyester based polymer family and usually preferred to formulate nanoparticle based drug delivery systems due to their biodegradation to non-toxic and biodegradable monomers. By the approval of United States Food and Drug Administration,[1,2] they have started to used in market and brought immense potential as a drug delivery carrier.

The pathological situation called arthritis affects most of the middle aged population who are over 45 years old. The most common ways of arthritis treatment include the application of oral non steroidal anti-inflammatory drugs, intra-articular medications and surgical interventions. The arthritis patients usually have difficulties during the application of intra-articular medications which are the most preferred treatment among physicians. Utilization of micro- and nano-particles provides enhanced retention time of the medication at joint area, maintains the drug concentration at a desired level via controlled release mechanism and eliminated the potential side effects of the drug by low dose treatment. Elron-Gross et al.[3] proved that intra-articular application of diclofenac sodium (DS) loaded microparticle containing hyaluronan based hydrogel formulation was significantly increased the retention time and therapeutic effect remained stable with lower drug amount compared to conventional oral dose of the drug. The gelation could be achieved with Poloxamer 407 and Poloxamer 188 polymers in thermosensitive hydrogel formulations due to their unique characteristics.[4]

Several polymeric microparticle and nanoparticle systems have been attempted with the goal of increasing the retention time of therapeutic agents within the joint cavity. However, nanoparticles rapidly escape from the joint cavity and micro-sized particulates are prone to phagocytosis by macrophages in synovial linings.[5] Recently, *in situ* forming hydrogels was introduced as an alternative tool to increase the retention time in joints.

In situ forming hydrogels are usually applied as solutions or suspensions and undergo gelation process in the application site due to physicochemical changes such as pH, temperature and ions. This system allows administration of accurate doses, increment of residence time and bioavailability at desired area.[6] Thermosensitive *in situ* hydrogel systems have a liquid character below body temperature (~37°C) and after administration to the body, liquid state turns into a gel system. Poloxamers are widely used to prepare thermosensitive *in situ* hydrogel systems. Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene [poly(propylene oxide)] flanked by two hydrophilic chains of polyoxyethylene [poly(ethylene oxide)]. Due to the dehydration of polymer blocks with temperature, non-chemically cross-linked hydrogel is formed. The gel formation is a result of micellar enlargements and can not be set apart easily from each other, which accounts for the rigidity and high viscosity of poloxamer containing gels.[7] Poloxamer-based hydrogels are biocompatible and can be administered into the body in a minimally invasive manner. Thus, these hydrogels are excellent candidates for long term therapy.[8]

DS, the sodium salt of o-(2,6-dichlorophenylamino)-phenyl acetic acid is a well known nonsteroidal anti-inflammatory drug. Because of having anti-inflammatory, analgesic and antipyretic pharmacological effects, it is widely used in the treatment of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis which leads a quite uncomfortable life especially among elder population. DS is absorbed quickly and almost completely with oral administration and also its terminal plasma half life is as short as 1-2 hours.[9] These physicochemical properties reveal some problems in the treatment procedure such as the requirement of repeated per oral administrations of DS. However, frequently repeated per oral administrations of DS may cause adverse effects at gastrointestinal system.[10] Therefore, new drug delivery strategies are needed for local delivery of DS.

The main purpose of this research is to develop *in situ* gelling hydrogel formulations containing DS loaded nanoparticles. DS loaded nanoparticles were prepared by using PLGA and PCL as polymer and characterized in terms of particle size, morphology, encapsulation efficiency and *in vitro* drug release. Then, *in situ* gelling hydrogel formulations containing DS loaded nanoparticles were prepared with Poloxamer 407 and chitosan. *In vitro* nanoparticle properties such as particle size, zeta potential, particle morphology, drug encapsulation efficiency, drug release profiles and gelation temperature, pH, viscosity features of *in situ* gelling hydrogel formulations were evaluated and optimized for a better arthritis treatment.

EXPERIMENTAL

Materials

DS was kindly provided by Novartis® (Turkey). Poloxamer 407, Poloxamer 188, PLGA (lactide: glycolide 50:50, Mw 24.000-38,000), Poly (ε-caprolactone) (PCL, Mw 70.000-90.000), Chitosan (medium molecular weight, 75-85% deacetylated), Polyvinyl alcohol (PVA) (PVA, Mw 30.000-70.000), Arlacel®-C (sorbitan sesquioleate), and dichloromethane (DCM) (DCM, analytical grade) were purchased from Sigma-Aldrich (Germany). Tween 80 was purchased from Merck (Germany). All other chemicals used were of analytical grade.

Methods

Preparation of nanoparticles

Modified water/oil/water ($W_1/O/W_2$) emulsion-solvent evaporation technique was used for the preparation of nanoparticles.[11,12] Briefly, 100 mg of polymer/polymer mixtures (1:1) of PCL and PLGA were dissolved in 2 mL of DCM to form O phase of the multiple emulsion. DS (75 mg) was separately dissolved in 1 mL of pH 5.8 or pH 7.4 aqueous buffer solution

to form W_1 phase. The primary emulsion (W_1/O) was obtained by directly pouring O phase on W_1 phase and sonication of this mixture for 1 min at 100% amplitude. W_2 phase was formed via solubilizing different stabilizers (1% w/v) in 10 mL of various buffer solutions (pH 5.8 or pH 7.4 phosphate buffers). Finally, primer emulsion was poured quickly into the W_2 phase then sonicated for 2 min at 100% amplitude to generate W_1/O/W_2 emulsion. After formation of the multiple emulsion, DCM was evaporated via magnetic stirring overnight. The final multiple emulsion was centrifuged (40.000 x g) at 10°C for 30 min then washed with 10 mL fresh W_2 phase three times to get rid of surface attached DS molecules and nanoparticles were collected after 24 hours of lyophilisation.

Tween 80 which has an important role to form primary emulsion was added to W_1 phase (25% v/v of 1 mL W_1 phase) in the preparation method of F1-F5. On the other hand, Arlacel®-C was added to O phase (10% v/v of 2 mL O phase) during the formulation of F6 instead of adding Tween 80 to W_1 phase.

Particle size, polydispersity index and zeta potential

A sample of 10 µL of nanoparticles was diluted with ultrapure water to 2 mL. The size distribution and zeta potential were evaluated at 25°C via differential light scattering method using Malvern Zetasizer® Nano ZS (Malvern Instruments, UK).

Encapsulation efficiency

Encapsulation efficiencies of the nanoparticle formulations were determined via indirect method. Briefly, each supernatant fraction collected during washing step at nanoparticle production procedure, stored in a flask and 1 mL supernatant was removed from this flask to determine the amount of non-encapsulated DS via ultraviolet (UV) spectroscopy (Shimadzu DV-1601) at 268 nm. After non-encapsulated DS amount was found, this amount was subtracted from the whole DS amount used at nanoparticle production procedure and encapsulated amount of DS was determined. Encapsulation efficiency was calculated using the Equation 1.

Encapsulation Efficiency (%) = amount of drug in the nanoparticles (mg) / initial amount of drug (mg) x 100
(Equation 1)

Preparation and characterization of in situ hydrogel system

Thermosensitive poloxamer gels were prepared according to the cold method.[13] Chitosan (0.25% w/v) and Poloxamer 407 (18% w/w) polymers were used in the preparation of thermosensitive hydrogel formulation. The gelation temperature was determined based on an observation of the immobilized magnetic bar due to the gelation.[14] Two grams of the prepared solution was transferred to a 5 mL transparent vial containing a magnetic stirring bar, the vial was heated with a constant stirring rate and the temperature at which the rotation of the bar stopped was taken as gelation temperature. The pH of formulation was measured with SenTix 82 pH electrode.[15] Viscosity determinations of prepared formulations were carried out on a Brookfield RVTDV-II viscometer[16] using spindle T-E. Angular velocity increased gradually from 0.5 to 100 rpm. The average of three readings was used to calculate viscosity.

In vitro release

In vitro release studies from DS loaded nanoparticles, DS loaded nanoparticle bearing hydrogel and non-encapsulated DS bearing hydrogel were performed according to previously reported methods with minor modification.[17,18,19] Briefly, 5 mg DS containing different nanoparticle formulations were suspended in 1 mL of pH 7.4 phosphate buffer then placed in dialysis bag. For hydrogel formulations, 5 mg of non-encapsulated DS containing hydrogel or 14 mg of F6 coded nanoparticle bearing (contains 5 mg DS) 1 mL hydrogel were put in dialysis bag directly without any dilution. The dialysis bag (Sigma, 12000-14000 Da MWCO) was suspended in pH 7.4 phosphate buffer (50 mL) maintained at 37±0.5°C. The dispersion was rotated at 50 rpm in an incubated shaker. The 1 mL sample were taken at each specified time interval then replaced with the same amount of fresh release medium and drug concentrations were determined by UV spectrophotometer after a certain dilution of withdrawn volume. Finally, cumulative drug release percent was calculated and each release test was repeated three times.

Differential scanning calorimetry

The physical state of DS entrapped in the nanoparticles was characterized by differential scanning calorimetry (DSC). A sample of 5-10 mg of DS, PLGA, PCL and lyophilized nanoparticle formulation was placed in a standard aluminum pan with a lid. The heating rate was set to 5°C/min between 30-300°C in a differential scanning calorimeter (Shimadzu DSC-60, Japan).

Atomic force microscopy and transmission electron microscopy

The morphology of the nanoparticles was investigated by atomic force microscopy (AFM) and transmission electron microscopy (TEM). AFM images were taken in tapping mode using Nanomagnetics' instrument.[20] Samples were prepared by redispersion of nanoparticles in ultrapure water and placing a droplet onto a mica surface adhered to nickel disc, followed by drying under nitrogen flow. TEM images were taken using FEI Tecnai G2 at 300 kV.[21] During the preparation of TEM samples, after a certain dilution, nanoparticle solutions (10 µL) were placed on copper grids than left for drying at 25°C and viewed.

Gas chromatography analysis for residual solvent detection

Gas chromatography (GC) method was performed to detect the amount of residual DCM which was used during nanoparticle production procedure. Chromatographic test was carried out by using Agilent 6,890N Network GC System equipped with flame-ionization detector and Supelco SP-2380 (60.0 mm x 0.25 mm x 0.25 mm) column. Before GC application, residual DCM was extracted from nanoparticles by using n-hexane via partitioning without dissolving the polymer. A particular amount of DS loaded nanoparticle was treated with n-hexane in a tube for 3 hours with vortexing. Afterwards, nanoparticles were separated by centrifuging and supernatant was injected to the column of GC device. Pure n-hexane and DCM were also injected to define each solvent's peak by itself.[22]

Statistical analysis

All data were expressed as mean ± standard deviation. Student's t test was used to compare differences between

groups. P values less than 0.05 were considered statistically significant. Each experiments and analyses were carried out as 3 replicates.

RESULTS AND DISCUSSION

Particle size, polydispersity and zeta potential

Nanoparticle formulations were successfully formulated with modified w/o/w emulsion-solvent evaporation technique. Nanoparticle characteristics such as particle size, polydispersity index and zeta potential were evaluated. Formulation variables are included in the Table 1. In each step only one formulation parameter has been modified compared to previous nanoparticle formulation, to observe how the modification effected on particles' characteristics in terms of average particle size, zeta potential and encapsulation efficiency.

Particle size of nanoparticles plays an important role for the penetration through physiological barriers, interaction with cell membranes and the therapeutic effect. Smaller particles have various advantages such as bearing a larger surface area which makes the release of encapsulated active substance easier by surface erosion & diffusion and providing penetration and crossing through physiological barriers.[23] Nanoparticulate systems appear more suitable for delivery to inflamed synovial tissue than microparticles due to their ability to penetrate the synovium as reported by Horisawa et al.[24] A colloidal suspension of the fluoresceinamine bound nanoparticles, with a mean diameter of 265 nm, was phagocytosed in the synovium by the macrophages infiltrated through the synovial tissues. In contrast, an aqueous suspension of the fluoresceinamine bound microparticles, with a mean diameter of 26.5 μm, was not phagocytosed in the macrophages and localize inflammatory responses were almost undetected. In this research, various DS loaded nanoparticle batches having a diameter range between 168.1±3.01 to 266.1±6.3 nm with narrow size distribution, were produced (Table 2). The particle size values of F4 and F5 coded nanoparticle formulations showed that the effect of different stabilizers on average particle size may be one of the most significant factor depending on the formulation. After obtaining smaller nanoparticles through the usage of PVA in F5, F6 coded nanoparticle formulation was also formulated with PVA as stabilizer.

Besides the cellular interaction and barrier penetration issues, the average particle size of nanoparticles has another impact which is injectability of the nanoparticle bearing hydrogel. The intra-articular injections are usually applied via a syringe equipped with the 18G[25] or 27G[26] needle. These needles have a diameter of 0.84 mm-0.21 mm respectively and that is why the average particle size has to be small enough not to clog the needles. Since Singh et al.[26] applied nanoengineered particles with average particle size of 303±13 nm and 500±22 nm intra-articularly via 27G needle without any clogging problem, the hydrogel formulation bearing F6 coded nanoparticle with 190.8±3.1 nm average particle size may be applied via syringe equipped with 18G or 27G needle in the present study.

Zeta potential which is an indicator for micro/nano particles' stability, is the estimate of the surface charge that particles gain in the dispersed state. When the zeta potential value of nanoparticles is between ±30 mV, the colloidal systems show no aggregation and they form stable dispersions that depends on the repulsion forces between particles.[27,28] From this point of view, nanoparticles' stability confirmed via zeta potential analysis. The zeta potential value of F6 coded formulation

Table 1. Polymeric nanoparticle formulations

Code	Polymer	PLGA:PCL ratio	Surfactant	Stabilizer	pH (outer phase)
F1	PLGA	1:0	Tween80	Poloxamer 188	7.4
F2	PCL	0:1	Tween80	Poloxamer 188	7.4
F3	PLGA+PCL	1:1	Tween80	Poloxamer 188	7.4
F4	PLGA+PCL	1:1	Tween80	Poloxamer 188	5.8
F5	PLGA+PCL	1:1	Tween80	PVA	5.8
F6	PLGA+PCL	1:1	Arlacel®-C	PVA	5.8

PLGA: Poly(lactic-co-glycolic acid), PCL: Poly(ε-caprolactone), PVA: Polyvinyl alcohol

Table 2. Particle size, polydispersity index, zeta potential and encapsulation efficiency of formulations

Code	Particle size (nm)	Polidispersity index (PI)	Zeta potential (mV)	Encapsulation efficiency (%)
F1	169.2±2.5	0.354±0.001	-55.9±1.3	31.5±0.5
F2	199.5±4.9	0.208±0.006	-46.1±1.3	37.7±0.1
F3	168.1±3.01	0.177±0.006	-44.9±0.5	18.4±1.2
F4	266.1±6.3	0.367±0.011	-39.3±0.4	24.4±1.1
F5	195.7±3.5	0.265±0.010	-23.0±2.8	56.1±0.4
F6	190.8±3.1	0.145±0.008	-29.6±2.7	89.7±0.9

was found as -29.6±2.7 mV which is in the theoretical ±30 mV stability range and no aggregation was observed after three months storage at 25˚C and 60% relative humidity.

Particle size, polydispersity index and zeta potential of F6 nanoparticle formulation was measured after three months storage at 25˚C, 60% relative humidity. Particle size/size distribution (182.0±1.511 nm and 0.231±0.009, respectively) and zeta potential (-28.8±0.69) of F6 formulation was found similar.

Encapsulation efficiency

Encapsulation efficiency of the active substance is a crucial parameter for particularly expensive active substances in drug delivery systems.[23] Two different methods were evaluated for determination of encapsulation efficiency. The encapsulation efficiency results of F1 coded formulation with indirect method and direct method are 31.5±0.5 and 33.1±0.9%, respectively. This result shows that there is no significant difference for determination of encapsulation efficiency between direct and indirect method. We have concluded that the critical experimental step which effects the results of encapsulation efficiency, obtained by different methods is the washing step that removes the drug molecules attached to nanoparticles' surface. That is why all the encapsulation efficiency experiments were carried out with indirect method which is simple, quick and accurate with the application of enough washing steps although the direct method seems more accurate for drug encapsulation analysis.

The pH value of external aqueous phase may have a significant effect on encapsulation process if drug substances have a pH dependent solubility. Since DS has pH dependent solubility such as 0.14 mg DS is soluble in 1 mL of pH 5.8 phosphate buffer and 5.15 mg DS soluble in 1 mL pH 7.4 phosphate buffer at 23±2˚C,[29] a pH value which DS is less soluble in the external phase was selected in order to increase encapsulation efficiency.[22] Lowering the external pH from 7.4 to 5.8 (F4) resulted with an increase on encapsulated DS by 32% and from this point on all other nanoparticle formulations (F5 and F6) were prepared with pH 5.8 external phase which allows less DS escape to outer aqueous phase depending on less solubility.

The stability of the first emulsion (W_1/O) plays an important role in encapsulation efficiency in the $W_1/O/W_2$ emulsion-solvent evaporation technique. Non-ionic surfactants are mostly added the inner aqueous phase.[30] In this research, Arlacel®-C which is usually utilized for hydrophobic drug encapsulation, was used in first emulsification step as a surfactant and significantly increased ($p<0.05$) encapsulation efficiency of DS.

Encapsulation efficiency value also varies depending on some parameters including the solubility of the active substance and the type of selected solvent and polymer.[31] DS is referred as sparingly soluble in water 30th United States Pharmacopoeia 30 and this solubility characteristic facilitates encapsulation of DS to the polymeric structure. The highest encapsulation efficiency was obtained with F6 formulation (89.7%±0.9) which is one of the most critical finding of this research. As indicated by Lai et al.[1] (2014) PVA was found the best stabilizer for the second emulsification step for PLGA nanoparticles and increased encapsulation of DS in F5 and F6 formulations.

Gelation temperature, pH and viscosity of in situ hydrogel

A gelation temperature suitable for *in situ* gel formulations would be 30-36˚C according to Kim et al.[32] *In situ* hydrogel formulation containing DS loaded nanoparticles was found in this range (35.0±2.6˚C) and the pH of this formulation was adjusted to 6.9±0.08 with 100 µL pH 7.4 phosphate buffer which is feasible for intra-articular injection.[33] Viscosity values at 25˚C and 37˚C were compared and a significant increase in viscosity depending on the temperature was observed as a result of gelation ($p<0.05$) (Figure 1).

In vitro release

Effect of polymer/polymer combination (PLGA and/or PCL) on the drug release profile was evaluated at Figure 2. The release profile of F2 formulation indicates that PCL nanoparticles have a slower release profile than PLGA nanoparticles due to PCL has more hydrophobic character and higher molecular weight as well. F3 coded formulation prepared with mixture of PLGA and PCL (1:1) released a greater amount of the drug than F1 and F2 coded formulations containing PLGA or PCL alone during the same period (Figure 2). Utilization of the polymer blends in particle production process may lead particles with different surface porosity characteristics. Cao and Shoichet[34] clearly demonstrated that particles prepared with PLGA/PCL blend had considerable surface porosity compared to particles were composed of PCL or PLGA alone. The increase of the porosity on nanoparticles' surface which may be obtained by using the combination of polymers in F3 coded formulation, might lead to increased drug release. Since one of the aims of this research is providing a side effect free medication with application of the drug with as low dose as possible, all other nanoparticle formulations (F4, F5 and F6) were prepared with the combination of PLGA and PCL.

In Figure 3, effect of stabilizers and surfactants on the drug release profile was evaluated. The surfactant of inner phase has been shown to have an important effect on the drug release. Arlacel®-C including F6 coded formulation was sustained the release of the drug more then other formulations. *In vitro* release of DS was prolonged over 24 hours with F6 formulation (Figure 3). Then, F6 nanoparticles were transferred into *in situ* hydrogel

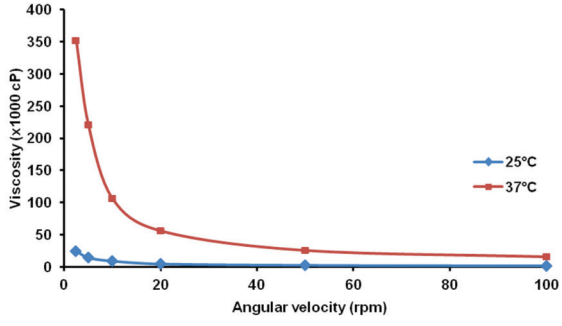

Figure 1. Viscosity versus angular velocity graphs at 25˚C and 37˚C

formulation and *in vitro* release was repeated (1 mL of hydrogel formulation contains 14 mg of nanoparticle that is loaded with 5 mg DS). 98% DS was released from F6 loaded *in situ* hydrogel in a controlled manner during 30 days (Figure 4). Similarly, nanoparticle-based topical ophthalmic gel formulation was prepared by Yang et al.[35] and hydrocortisone was released from nanoparticle-gel system over 30 days. The prolonged release of the DS may be attributed to the poloxamer's property to control the release of the encapsulated drug from hydrogel. Utilization of chitosan together with poloxamer in the hydrogel formulation would be also beneficial in terms of providing bioadhesive effect at application are for stronger attachment of gelling system to articular surface.[36]

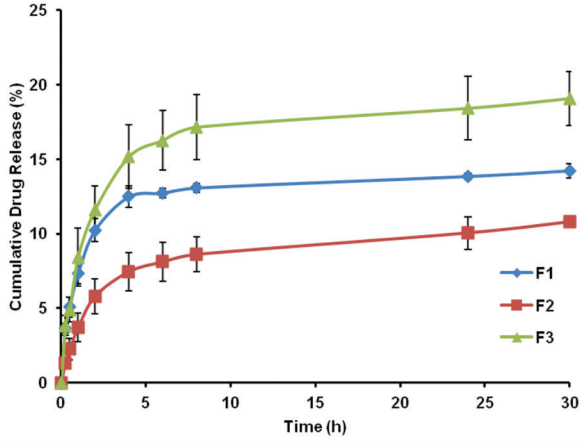

Figure 2. *In vitro* release profiles of F1, F2 and F3 formulations

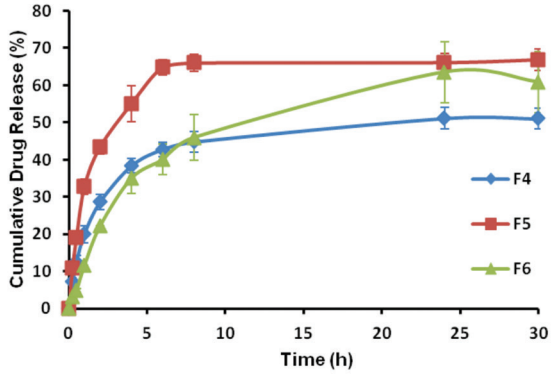

Figure 3. *In vitro* release profiles of F4, F5 and F6 formulations

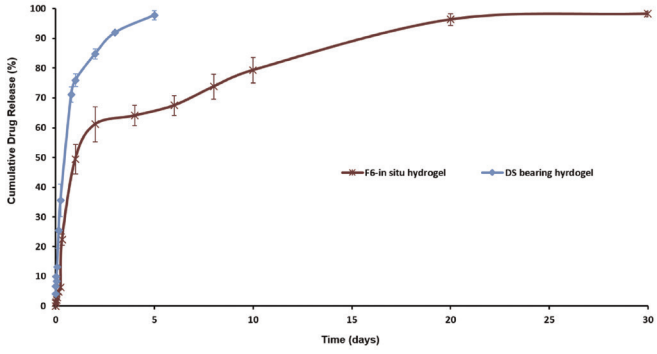

Figure 4. *In vitro* release profile of diclofenac sodium bearing hydrogel formulation and F6 loaded in situ hydrogel formulation

The DS loaded nanoparticles containing hydrogel's release profile was also compared with non-encapsulated DS containing hydrogel to reveal the fact if the 30 days release profile is an attribute of the utilization of nanoparticles or just an advantage of hydrogel. Figure 4 shows that non-encapsulated DS containing hydrogel provide only a 5 days release. The possible reason of obtaining a 30 days release profile with *in situ* gel containing nanoparticles may be the slow hydration of nanocarrier's surface while formulated in the hydrogel. When the low amount of DS released from the carrier there is another barrier which is polymeric hydrogel network, for being released to the medium. Hence, there are two different barriers to pass for DS for being released and these barriers prolong the release of DS.

The parameters and conditions of *in vitro* release test method which we applied, has to be controlled and standardized to obtain a valid performance indicator of formulation. A typical release test includes standardized conditions such as, hydration of dialysis membranes with release medium during 12 hours before the test, putting the same volume of either nanoparticles (suspended in 1 mL of release medium) or hydrogel formulations (1 mL), being sure that there is no air bubbles in dialysis bags, replacing the withdrawn release medium with the fresh medium and maintaining the flow of the release medium on the dialysis membrane's surface to provide gradient between the release medium and inside the membrane. The volume of the release medium has to be large enough compared to continuous phase (the volume that was put inside the membrane) to provide a faster diffusion rate compared to release rate of the drug from the nanocarrier for getting rid of obtaining a release profile depending on partition. But there is one parameter that may affect the results and may be considered as a source of error which is the continuous phase volume's change depending on the osmosis.[37] Since we have used a quite small volume (1 mL) compared to release medium (50 mL) as continuous phase, the possible change at 1 mL volume was not a significant source of error.

The average of three repetitions for each formulation was plotted against time and error bars were presented on graphs. The error bars show the experimental range that may depend on small variations of dilution or withdrawn volume.

Although intra-articular injection is a relatively painful and expensive administration, increased dose application interval to 1 month might decrease the total cost of the treatment and also provides better patience compliance. Apart from this fact, many studies have reported severe side effects could be eliminated by utilization of local delivery. According to the data of Zhang et al.[38] 80% of drug was released from locally administered polymeric micelle formulations in 24 h. *In vivo* pharmacodynamic test based on both acute and adjuvant arthritis model indicated that sustained therapeutic efficacy could be achieved through the local injection of drug-loaded nanoparticles. Most importantly, local delivery of non-steroidal anti inflammatory drug could eliminate side effects such as severe gastric ulceration, which was associated with oral administration.[38]

Differential scanning calorimetry

DSC analyses were performed to reveal the physical state of

DS-nanoparticles. DSC curves of DS, PLGA, PCL and lyophilized nanoparticle formulation were shown in Figure 5. The glass transition temperatures (Tg) of PLGA, PCL and nanoparticles were found 49°C, 56°C and 53.72°C, respectively. Results were in agreement with the reported Tg values of PLGA and PCL in the literature.[39,40] The absence of the characteristic melting point (285.36°C) peaks of DS in nanoparticle formulation indicated that DS was in the amorphous state. Amorphous state suggests better drug dispersion and increased drug-matrix interactions, leading to the conclusion that reduced crystallinity is favored when slow-release kinetic is required.[41]

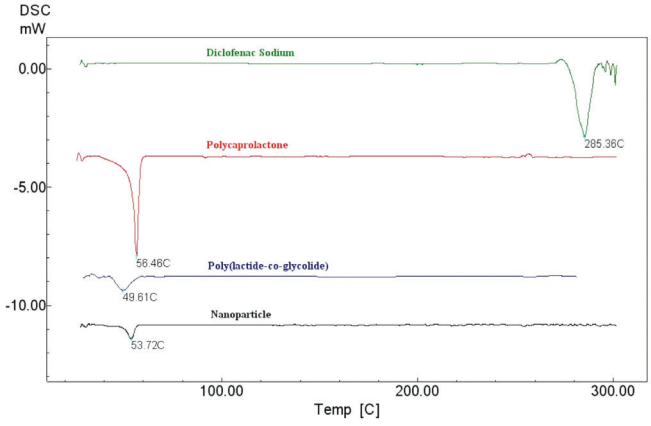

Figure 5. Differential scanning calorimetry thermograms of diclofenac sodium, poly(lactic-co-glycolic acid), poly(ε-caprolactone) and nanoparticles

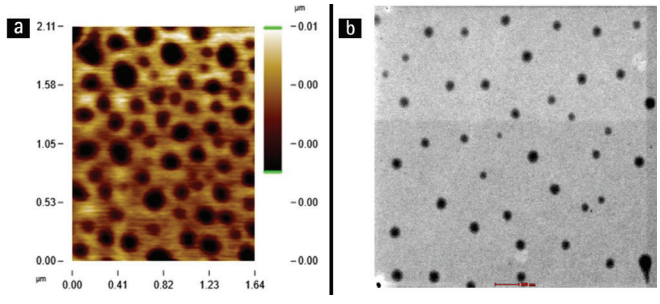

Figure 6. Atomic force microscopy image (a) and transmission electron microscopy photograph (b) of nanoparticles

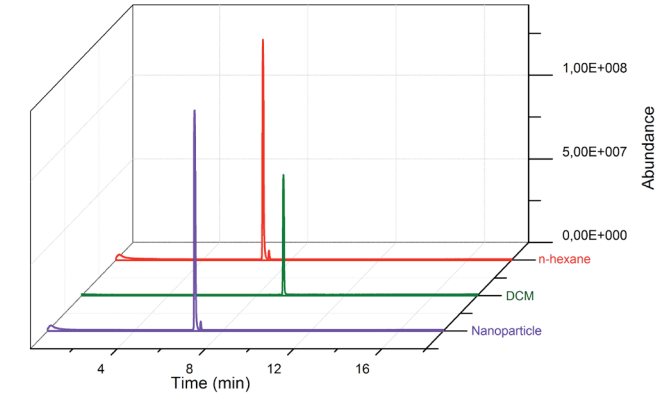

Figure 7. Gas chromatography chromatograms of pure n-hexane, pure dichloromethane and diclofenac sodium loaded nanoparticles

DCM: Dichloromethane

Morphology studies

The shape of the particles injected into the joint is important for triggering an immune response. Irregularly shaped particles have been demonstrated to promote tissue inflammation in comparison with round-shaped drug delivery systems. Round-shaped particles are to be preferred for intra-articular drug delivery.[42] AFM and TEM analyses were performed for visualization of the morphology of nanoparticles. Nanoparticles were shown to have a spherical shape and there was no aggregation (Figure 6). The particle size measured by AFM and TEM confirmed the particle size results obtained by photon correlation spectroscopy.

Residual dichloromethane determination by gas chromatography analysis

During the process of nanoparticle preparation, DCM was used to dissolve polymers before mixing with an aqueous phase. Since DCM has severe toxic effects on body, the amount of residual DCM has to be controlled and determined in the developed formulations. GC chromatograms of pure n-hexane, DCM and F6 coded nanoparticle formulation are presented in Figure 7. The retention times of pure n-hexane and DCM were at 6.74 and 9.21 min, respectively. When the GC chromatogram of the nanoparticle formulation was examined only one peak was observed at 6.74 min, which suggests the presence of only n-hexane. There was no peak signal obtained that proved the existence of DCM in the nanoparticle formulation.

The GC analyses results showed that magnetic stirring and freeze drying steps during nanoparticle production process are effective enough to get rid of residual DCM.

CONCLUSION

Optimization of formulation variables used for preparation of DS loaded nanoparticles such as combination of biodegradable polymers, adding surfactant into the inner phase, adjusting the pH and stabilizer of the outer aqueous phase, were achieved and high encapsulation efficiency of DS was obtained. One of the most challenging intra-articular drug formulation issue[43] which is quick drug release profiles of formulations during the residence time at joints, was overcome via utilizing the combination of a *in situ* hydrogel and polymeric nanoparticles. The projected *in situ* gelling system comprising DS-nanoparticles has ease of application compared to microparticle based hydrogels because of having relatively small particle size and provides a controlled release of the drug over 30 days.

Detailed results and *in vitro* tests suggest that monthly intra-articular application of DS loaded nanoparticle bearing *in situ* hydrogel system could be a promising tool for the treatment of arthritis after further investigations in the future. This system holds the potential of being applicable to other drugs as a reference delivery platform in terms of enhancing patience compliance with less dose interval and also eliminating systemic side effects.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Cutaneous Wound Healing after Topical Application of *Pistacia atlantica* Gel Formulation in Rats

Pistacia atlantica Jel Formülasyonunun Haricen Uygulamasının Sıçanlarda Oluşturulan Deri Yaraları Üzerindeki İyileştirici Etkisi

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ABSTRACT

Objectives: This study has been undertaken to investigate the antioxidant activity of the gel formulation from *Pistacia atlantica* oil extraction on enzymatic antioxidants in experimental wound created in rats.

Materials and Methods: Under anesthesia, a square-shaped skin defect (2x2 cm) was created aseptically by surgical incision. Then, animals were randomly allocated to four groups (I- untreated controls, II- topically treated with base gel, III- topically treated with 5% gel, IV- topically treated with 10% gel).

Results: The results suggest that topical application of *Pistacia atlantica* oil gels improved reepithelialization with continuous stratum basalis and a mature granulation tissue and adnexa (hair follicles and sweat gland) compared with control and base gel groups. Treatment with 10% oil gel significantly enhanced the tensile strength, ultimate stress, yield strength and stiffness in this group compared with the control and base gel groups at 21 days post injury. The collagen fibers showed a more organized pattern and the tissue alignment was greater as compared to the control and base gel-treated groups at the same stage.

Conclusion: The results suggest that topical application of *Pistacia atlantica* oil gel improved the morphological, biochemical and biomechanical properties of experimentally-induced wound defects in rats.

Key words: *Pistacia atlantica*, wound healing, histopathology, biomechanic, skin

ÖZ

Amaç: Bu çalışma, sıçanlarda oluşturulan deneysel yara modelinde *Pistacia atlantica* yağı ile hazırlanmış jel formülasyonunun, enzimatik antioksidanlar üzerindeki etkisini araştırmak amacıyla yapılmıştır.

Gereç ve Yöntemler: Genel anestezi altına alınan hayvanlarda, aseptik koşullarda, insizyon yapmak suretiyle kare şeklinde yaralar (2x2 cm) oluşturulmuştur. Daha sonra, hayvanlar rasgele dört gruba ayrılmıştır (I- tedavi edilmemiş kontrol grubu, II- haricen uygulanan baz jel grubu, III- haricen %5 jel uygulanan grup, IV- haricen %10 jel uygulanan grup).

Bulgular: Sonuçlar, *Pistacia atlantica* yağı ile hazırlanmış jel formülasyonunun haricen uygulanmasının, kontrol ve baz jel gruplarına kıyasla, bütünlüğü bozulmamış stratum bazalisi, olgun granülasyon dokusunu, kıl folikülleri ve ter bezleri ile reepitelizasyonu arttırdığını göstermektedir. Yara oluşumundan 21 gün sonra, %10 yağlı jel ile tedavi edilen hayvanların dokularındaki gerilme kuvveti, kopma gerilimi ve bükülme direnci, kontrol ve baz jel grubu ile karşılaştırıldığında önemli ölçüde artmıştır. Aynı evredeki kontrol ve baz jel ile muamele edilen gruplara kıyasla, kollajen liflerin daha organize bir düzenlenme ve dokunun daha düzgün bir iyileşme gösterdiği belirlenmiştir.

Sonuç: *Pistacia atlantica* yağ jelinin haricen uygulanmasının, sıçanlarda oluşturulan deneysel yaralar üzerinde morfolojik, biyokimyasal ve biyomekanik yönden düzeltici rol oynadığı ortaya koyulmuştur.

Anahtar kelimeler: *Pistacia atlantica*, yara iyileşmesi, histopatoloji, biomekanik, deri

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INTRODUCTION

Plants have immense potential for the management and treatment of wounds. A large number of plants are used by tribal and folklore in many countries for the treatment of wounds. Several plants have been experimentally used as traditional medicines to treat skin disorders and wound injuries.[1]

Pistacia atlantica (*P. atlantica*) trees grow in large populations in the western, central and eastern parts of Iran. The fruits of *P. atlantica*, which have been used traditionally in treatment of peptic ulcers[2] are called "Bene" in Iran and have been utilized as food.[3] Bene hull oil (BHO) comprises of the whole fruit and yields up to 30% oil.[3,4,5] *P. atlantica* oil contains saturated fatty acids, mono unsaturated fatty acids and the polyunsaturated fatty acids (PUFAs).[3]

The BHO contains tocopherols and tocotrienols as the main constituents in unsaponifiable matter.[6] The oil displays antioxidant activity which is important for human health.[6]

BHO contains tocopherols and phenolic constituents,[7] both of which are very important natural antioxidants by providing an effective protection against oxidative stress.[8,9]

The PUFAs, besides their structural function, they can modulate cell-cell interactions and intracellular signaling.[10] Linoleic acid is an essential fatty acid (EFA) of 18 carbons, cannot be synthesized by human, which through a desaturation process gives rise to arachidonic acid (20 carbons). Arachidonic acid is a precursor of prostaglandins, leukotrienes, thromboxanes and lipoxins, which in turn act as mediator of platelet function and of inflammatory, vascular, motor and sensory processes, among others.[11,12] Linoleic acid has also been shown to participate in cell proliferation and inflammatory process, where in the latter it plays a role as a mediator of leukocyte function having chemotactic and stimulatory effects on neutrophils.[13]

Free fatty acids (FFAs) are known to possess anti-*Staphylococcus aureus* (*S. aureus*) activity[14,15,16] and are important components of the innate immune system.[17] These FFAs, including oleic acid, can function as innate bactericides.[18] More importantly, it has been found that two FFAs (linoleic acid and dehydrocrepenynic acid) inhibit the bacterial drug resistance by decreasing the transfer frequency of the conjugal DNA.[15,19]

Skin is a biological interface with the environment, and is frequently and directly exposed to prooxidative stimuli, which are known to promote the generation of Reactive Oxygen Species (ROSs) and lipid peroxides.[20,21] Wounding is another condition that results in a decrease in antioxidants,[22,23] increase ROSs[24] and elevated lipid peroxide levels.[25] Antioxidants have been shown to promote wound healing[23,24,26,27] Their levels have been shown to be depleted in the healing cutaneous wounds in normal subjects; however, they partially or completely recovered during the remodelling phase of healing.[22]

Oxygen free radicals play an important role in delaying ischemic wound healing.[28] Tocopherols have antioxidant properties[29] and they are active as vitamin E, which makes them particularly important for human health.[3] Vitamin E, the term for a group of tocopherols and tocotrienols, is the major lipid soluble antioxidant in skin that protects the cell membranes from the peroxidative damage.[30] It has been used to treat almost every type of skin lesion, and is frequently used by the general population to treat burns, surgical scars[31] and enhances healing of the irradiated skin in rat and increases the healing rate of the patients with chronic post-thrombotic leg ulcers.[32,33] Vitamin E is an antioxidant that protects the body against the deleterious effects of free radicals.[32]

Antioxidant properties of *P. atlantica* might be an alternative medicine or beneficial herbal drug for the prevention delay wound healing and scar formation during skin healing. Considering the lack of data regarding its efficacy in treating wound healing, the present study was undertaken to fully evaluate the effects of topical application of the gel formulation of this fruit on cutaneous wound healing in an experimentally induced cutaneous wound model in rat.

EXPERIMENTAL

Animals

One hundred twenty adult male Sprague Dawley rats weighing 200 to 220 g were used in the experimets. The animals were housed under standard environmental conditions (23±1˚C, with 55±5% humidity and a 12 h light/dark cycle) and maintained with free access to water and *ad libitum* standard laboratory diet (70% carbohydrates, 25% proteins, 5% lipids).

Animal ethics

The study was approved by the local ethics committee of School of Veterinary Medicine, Shiraz University in accordance with the ethics standards of "Principles of Laboratory Animal Care and was also the recommendations of the European Council Directive (86/609/EEC) of November 24, 1986.

Oil extraction

The fruit powder was extracted with n-hexane (1:4 wt/vol) by mixing in a dark place at ambient temperature for 48 h. The solvent was evaporated *in vacuo* to dryness at 40˚C.[34]

Preparation of the gel

To prepare 5% gel, 1 g carbopol was added to 95 mL distilled water, and for 10% gel, 1 g to 90 mL distilled water; after 4-5 h, all the carbopol powder was dissolved and sodium hydroxide was added to make the gel base. In the next step, *P. atlantica* extracts (5%, and 10%) were added to gel base and used for all experiments.

Wound creation

The rats were weighed prior to the surgical procedure. The animals were anaesthetised by intramuscular injection of 10 mg/kg xylazine hydrochloride (HCl) (Xylazine 2%; Alfasan) as premedication, and 90 mg/kg ketamine HCl (Ketamine 10%; Alfasan) for anaesthesia. Carprofen was subcutaneously injected in all animals (4 mg/kg) just before the operation for pre-emtive analgesia and then every 24 h for 3 days postoperatively. A square-shaped 2x2 cm skin defect was created aseptically by surgical incision at the first thoracic vertebrae and 1 cm proximal to the first lumbar vertebrae. This method of wound

induction has been used previously to investigate cutaneous wound healing in rats.[35]

Experimental design

After wound creation, the animals were assigned into four groups of thirty rats and each group was divided into five subgroups.

Group I, untreated controls,

Group II, topically treated base gel,

Group III, topically treated 5% gel,

Group IV, topically treated 10% gel.

The animals of each subgroup (n=5) were sacrificed on the 3th, 7th, 10th, 14th and 21st day after wound creation and the entire wound with adjoining tissue was cut out.

Digital images of the ulcers were taken, *in vivo*, in a standardized manner, immediately after the surgical procedure (day zero) and on 3, 6, 9, 12, 18, and 21 postoperative days.

Samples from all these groups were collected for histopathological studies and biochemical analysis. On day 21, samples from all these groups were collected for histopathological studies, biochemical analysis and samples from the five remaining animals in each group were assessed for biomechanical investigation. Similar skin sample from the intact skin of a comparable area far from the site of the initial excision of the same animal was excised as intact control skin.

Sample collection

The injured area and a rim of the surrounding normal skin, including dermis, epidermis and subcutaneous, were carefully dissected for histopathological and biochemical evaluations. The harvested skin samples were then longitudinally bisected. The first half consisted of normal peripheral tissue and injured area (5x5 mm) was used for histopathologic and histomorphometric studies and the second half from the center of the lesion, was used for biochemical analysis (approximately 5x5 mm). On day 21, five rats in each group were used for biomechanical sampling. A 1.5x10 cm^2 rectangular skin sample with the injured area in the middle was harvested for biomechanical testing.

Histopathological evaluation

Skin samples from both the wound and comparable contralateral normal skin were fixed in 10% neutral-buffered formalin, dehydrated in graded ethanol, and cleared in xylol. The specimens were then embedded in paraffin, and sections of 5 mm in thickness were stained, using hematoxylin and eosin (H&E) and Masson green trichrome, and studied by a routine light microscope (Olympus, Tokyo, Japan). Histopathological examinations were performed in double-blind fashion. The criteria that were studied in histopathological sections consisted of hemorrhage, fibrin deposition, polymorphonuclear cell and mononuclear cell infiltration, reepithelialization, cornification of epithelium, fibroblast content, collagen content, revascularization, necrosis, presence of fibrocytes, maturation and organization of collagen, fibroblasts and blood vessels. Collagen content was measured on the basis of the connective tissue density measurement in the histopathological sections stained with Masson green trichrome.

The microphotographs were then recorded by a digital camera (Olympus, Tokyo, Japan) and transferred to the computer software (Photoshop CS-5; Adobe) for digital analysis. Five photomicrographs, equivalent to five microscopic fields from each tissue sample, were used for histopathologic and histomorphometric analysis.[36,37]

Biomechanical testing

The method used has been described previously.[37,38,39] The skin samples were harvested immediately after euthanasia and placed between sterile sponges soaked with 0.9% saline, to preserve the normal tissue hydration after harvesting. They were then placed in occlusive bags and stored at -70°C prior to testing. Biomechanical testing was performed 2 days after tissue sampling. Before tensile testing, the samples were thawed at room temperature (15-20°C). The samples were mounted between two cryoclamps. The cross-sectional area of the samples was calculated by approximating the area as a rectangle, multiplying the thickness and width of the skin samples, which were measured using a digital micrometer (Microtech; Samsung). Biomechanical testing was performed by testing to failure, using a tensile testing machine (Instron Tensile Testing Machine, Instron).

Each sample was loaded by elongating it at a displacement rate of 20 mms^{-1}. Load and crosshead displacement data were recorded at 1500 Hz, and a load-deformation curve was generated for each specimen, using Test Works 4 software (SUME Systems Corporation). The ultimate tensile strength, yield strength, stress and stiffness were measured. The stress value (N mm^{-2}) was calculated by dividing the ultimate strength (N) by the cross-sectional area (mm^{-2}). Stiffness (Nmm^{-1}) was calculated by fitting a linear regression line to the load-deformation data from 30% to 90% of the ultimate tensile strength on the deformation curve.

Determination of the collagen content

After thawing, the samples' pieces were dried in a hot-air oven at 60-70°C until a constant weight was achieved. They were then hydrolysed with 6N HCl for 2 hours at 120°C. The hydrolysed samples were adjusted to pH 7 and subjected to chloramine-T oxidation. The absorbance of the colored adduct formed with the aldehyde perchloric acid reagent at 60°C was recorded at 550 nm, after cooling for 5 min. The data were then multiplied by 7.46 to convert them from hydroxyproline content to collagen content.[35,40]

Glycosaminoglycans content

Content of Glucosamine (GLA) and galactosamine (GAA), as indices of tissue glycosaminoglycans (GAGs), were analysed according to the methods of Ohkuma et al.[41] Briefly, 10 mg of the freeze-dried tissue sample was diluted in 2 mL of 1N HCl and incubated at 120°C, to produce hydrolysed and dried tissue. Phosphate buffer saline (0.5 mL; pH 7) and 1 mL 4N NaCl were added to the hydrolysed sample. The hydrolysed samples were then converted from acetylated forms of hexoseamines to pyrrole derivatives, using 0.1 mL of the 6.3% acetylacetone solution. 0.8 mL Potassium tetraburate 0.7 M was added and the

final solution was boiled for 3 min. 10 g Dimethyl banzaldehyde was blended with 100 mL acetic acid, containing 12.5% 10N HCl, and 5 mL of this solution was added to the boiled sample. The final solution was incubated in a 37°C water bath and its absorbance rate was read and analysed at two wavelengths, 550 nm and 590 nm. The absorbance rate of 590 nm is related to GLA and that of the 550 nm belongs to the concentration of GAA in tissue. The concentrations of hydroxyproline, GLA and GAA in each sample were measured using the regression curve from the GLA and GAA standards (0.0, 0.5, 1.0, 2.0, 4.0 and 8.0 mg) and were reported as gr/100 g of tissue.

Statistical analysis

After testing to see if the data were normally distributed, quantitative data were compared, using one-way analysis of variance. The post-hoc Tukey test was used to determine differences between the groups. All statistical analyses were performed using SPSS software (v17.0; SPSS Inc.). Differences of $p<0.05$ were considered significant.

RESULTS

Observations during daily wound care

The surface area of wounds was calculated and expressed in cm^2 as shown in Table 1. There were no significant differences in wound closure between the Bene treated, control and base gel groups. The lesions in the Bene oil gel treated groups showed improved cosmetic results compared with the control and base gel groups (Figure 1).

Histopathological finding

The data from the histopathological analysis are shown in Table 2. Three days after injury, Bene 5% and 10% oil gels resulted in reduction of neutrophils and lymphocyte and increase in fibroblasts but the differences were not significant (Table 2). Seven days after injury, the total fibroblast count was significantly greater in the treated with 5% Bene oil gel, compared with the control and base gel groups. Treatment with 10% Bene oil gel increased fibroblast and fibrocytes counts compared with the control and base gel group but these differences were not significant. The neutrophils counts were significantly lower in the lesions treated with Bene compared with the base gel groups (Table 2).

Ten days after injury, treatment with Bene oil gels decreased total lymphocytes compared with the control and base gel groups but this reduction was only significant compared with the control group. Treatment with Bene oil gels also significantly decreased total neutrophils counts compared with the base gel groups ten days after injury (Table 2).

Fourteen days after injury, the total lymphocyte and neutrophils counts decreased and the total fibrocytes count was higher in the Bene oil gel treated groups compared with the control and base gel groups but this these differences were not significant (Table 2).

Compared with control and base gel groups, there was a reduction in the total lymphocyte count in both 5% and 10% Bene gel groups at twenty-one days after injury but this reduction was only significant in 10% Bene gel groups. Total fibrocytes count was significantly higher in the 10% Bene treated group compared with the 5% Bene, control and base gel groups (Table 2).

There was a significant increase in the number of blood vessels in the lesions treated with 10% Bene and these differences were significant with the base gel group on day 14 and with the control group on day 21.

As shown in Table 2, compared to those of the control and base gel treated lesions, fewer lymphocytes and neutrophils were infiltrated in the lesions of the treated animals on day 21 post injury.

Histopathological evaluation of each treatment group was blindly performed by a pathologist according to a modified

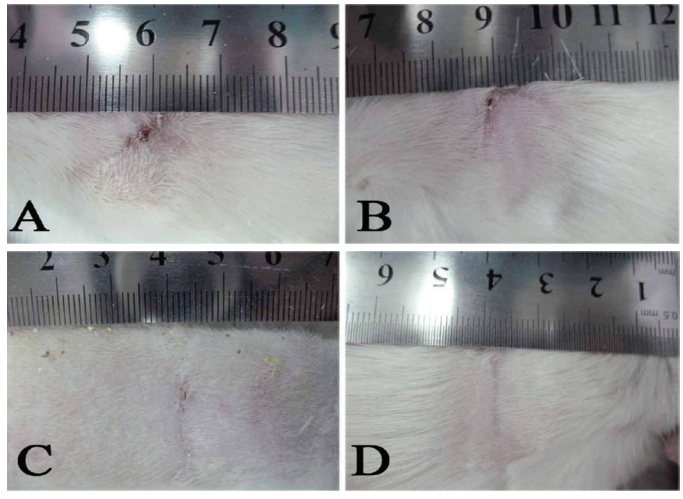

Figure 1. Cosmetic result [control (a), basal gel (b), 5% Bene oil gel (c) and 10% Bene oil gel (d)] on day 21 post injury

Table 1. Mean ± standard error of the mean of wound surface area (cm^2) in Bene oil gels treated, control and base gel groups on different days

Groups	Day 3	Day 6	Day 9	Day 12	Day 18	Day 21
Control[a]	2.23±0.11[c]	1.5±0.22	0.79±0.09	0.34±0.09	0.15± 0.04	0.13±0.03
Base gel[b]	2.00±0.04	1.38±0.11	0.71±0.07	0.28±0.02	0.11±0.03	0.00±0.00
5% Bene[c]	1.64±0.20[a]	1.24±0.04	0.52±0.11	0.1±0.05	0.00±0.00	0.00±0.00
10% Bene[d]	1.81±0.23	1.33±0.07	0.52±0.17	0.21±0.063	0.00±0.00	0.00±0.00

Characters ([a]=control, [b]=base gel, [c]=5% Bene oil gel, [d]=10% Bene oil gel) represent significant differences for that variable between the associated groups, at that time point

Table 2. Mean ± standard error of the mean of Histopathologic and histomorphometric analysis of Bene oil gels compare with control and base gel treated group on different days post injury

	Control	Base gel	5% Bene gel	10% Bene gel
Day 3				
Lymphocyte	43.2±6.76	27.1±1.82	21.2±5.72	19.2±0.66
Fibroblast	5.8±0.86	8.6±0.68	7.6±1.5	13±3.56
Fibrocyte	0	0	0	0
Neutrophil	41.4±15.45	37.2±0.86	20±6.78	8.4±3.26
Macrophage	6.6±0.98[b]	15.2±0.86[ac]	6±1.67[b]	9.4±3.04
Number of vessels	2.3±0.92	0.6±0.25	2.4±0.4	1.4±0.25
Day 7				
Lymphocytes	20.4±2.86	18.4±3.96	26.6±4.12	20.2±4.32
Fibroblasts	9.8±1.07[c]	8.8±2.35[c]	20.8±5.18[ab]	12.4±1.99
Fibrocytes	0	0	0	0
Neutrophils	29±1.51	72.4±1.2[cd]	13±4.06[b]	15.2±0.97[b]
Macrophage	5.6±1.36	11.6±1.2	7±1.67	8±1.22
Number of vessels	2.6±0.4	1.8±0.58	1.8±0.37	2±0.63
Day 10				
Lymphocytes	89.8±18.5[cd]	60±7.36	36.4±5.21[a]	19.8±1.93[a]
Fibroblasts	18±1.3[bd]	5±0.32[ac]	15.8±3.43[bd]	4.2±0.49[ac]
Fibrocytes	9.6±1.72	5.8±0.37	8.2±1.39	19.4±2.46
Neutrophils	43.8±1.44	84.8±1.92[cd]	15±3.4[b]	7.8±0.97[b]
Macrophage	8.6±1.44	14±1.92	11.4±2.71	7±1.38
Number of vessels	2.8±0.37	2.8±0.66	3.6±0.6	3.2±0.58
Day 14				
Lymphocytes	19.4±2.77	30±5.4	12±2.57	5.4±0.75
Fibroblasts	5.6±1.08	6.4±2.04	3.2±0.8	1.8±0.66
Fibrocytes	7.4±1.67	4.6±0.87	14.6±1.12	18.2±1.56
Neutrophils	9.4±0.81	21.6±0.8	3.2±0.37	3.8±1.11
Macrophage	5.6±0.81	5.8±0.8	5.2±0.86	3.2±1.11
Number of vessels	2.6±0.68	2.4±0.25[d]	4.4±0.51	5.2±0.49[b]
Day 21				
Lymphocytes	21.4±1.03	51.8±28.42[d]	12±2.28	7.4±1.29[b]
Fibroblasts	14.6±1.69[d]	11.2±0.86	7.2±2.56	3.2±0.37[a]
Fibrocytes	2.4±0.75[d]	9.2±0.58[d]	17.6±0.4[d]	38.4±16.03[abc]
Neutrophils	8.4±1.28	12±2.11	5.8±2.18	2.4±0.4
Macrophage	5.2±1.28	9.4±2.11	5.8±1.56	3.8±1.11
Number of vessels	1.6±0.4[a]	2.4±0.4	4±0.45	4.8±0.66[d]

Different letters ([a]=control, [b]= base gel, [c]=5% Bene oil gel, [d]=10% Bene oil gel) represent significant differences for that variable between the associated groups, at different days

scoring system for surgical wound healing (Table 3).[42] This scoring system was used to determine grade of healing in the lesion of each treatment group. This scoring system was based on the following repair indices: epithelialization, collagen deposition, inflammation, ulceration, and necrosis.

As has been shown in Figure 2, the mean score of the 5% and 10% Bene treated groups were respectively higher than those of the control and base gel groups. Tissue regeneration in the 5% and 10% Bene treated wounds got a better score than the control and base gel groups but the differences were not

significant. Figure 3 shows representative photomicrograph of skin sections stained with hematoxylin and eosin and Masson green trichrome which have been properly repaired in the treated group. On day 14, the 5% and 10% Bene treated groups revealed more developed reepithelialization and continuous stratum basalis with a mature granulation tissue, while the lesions in the control group showed necrosis, and immature granulation tissue (Figure 3m, 3n, 3o, 3p).

On day 21, the 5% and 10% Bene treated groups revealed full collagen deposition and full reepithelialization with a mature granulation tissue and properly developed adnexa (hair follicles and sweat gland) compared with control and base gel groups (Figure 3q, 3r, 3s, 3t). The collagen fibers showed a more organized pattern and the tissue alignment was greater as compared to the control and base gel treated groups at the same stage (Figure 3u, 3v, 3w, 3x).

There was no evidence of pus accumulation, fibrin deposition or edema in the lesions of treated animals during the 21 day treatment period with Bene oil gels.

Biomechanical findings

The biomechanical findings are presented in Table 4. Treatment with 10% Bene oil gel significantly increased the tensile strength, maximum stress, yield strength and stiffness of this group compared with the control and base gel groups after 21 days post injury ($p<0.001$). Treatment with 5% Bene oil gel increased the tensile strength, ultimate stress, yield strength and stiffness of this group compared with the control and base gel group but the differences were not significant at this period. The lesions of all groups showed significantly lower tensile strength, ultimate stress, yield strength and stiffness than the intact skin at this stage.

Biochemical findings

The biochemical findings are presented in Figure 4. There was no significant difference in the GLA content of the injured samples on day 21 after injury with the Bene oil treated gels and the control and base gel groups. 10% Bene oil gel significantly increased the galactoseamin content of the injured samples three days after treatment compared with the 5% Bene ($p=0.036$), control ($p=0.048$) and base gel ($p=0.039$) groups during the period of this experiment.

Treatment with 10% Bene oil gel significantly increased the Hydroxyproline content of the injured samples on day 10 post injury ($p=0.007$) compared with the control group, and the base gel on day 14 post injury ($p=0.032$).

Seven days after injury, treatment with 10% Bene oil gel significantly increased the collagen content of the injured samples compared with the control group ($p=0.013$).

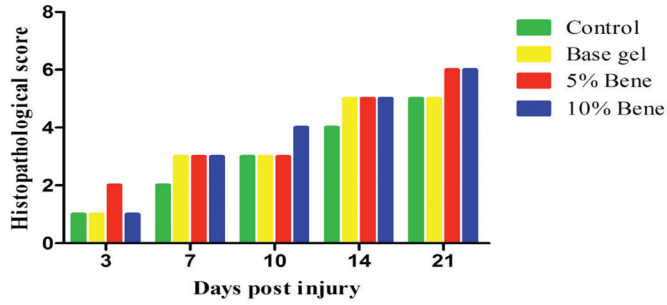

Figure 2. The mean score of the 5% and 10% Bene oil gel treated groups compared with the control and base gel treated groups

Table 3. Histopathological scoring of the cutaneous wound healing, This scoring system was used to determine grade of healing in the lesion of each treatment group

Score	Epithelialization	Collagen deposition	Inflammation	Neovascularization	Necrosis	Granulation tissue	Adenexa (hair follicle)
1	None	None	Moderate	None	Focal	Immature	Distraction
2	None	Partial	Mild	<3	None	Immature	Distraction
3	None	Partial	Mild	<3	None	Mild mature	Distraction
4	Partial	Partial	Mild	<5	None	Mod mature	Distraction
5	Complete, mature	Complete, regular	None	>5	None	Fully mature	Distraction
6	Complete, mature	Complete, regular	None	>5	None	Fully mature	Repair

Table 4. Mean ± standard error of the mean of biomechanical performance of Bene oil gels compare with control and base gel on different days post injury

	Control	Base gel	5% Bene	10% Bene	Intact skin
Maximum stress (kg/mm^2)	0.78±0.05^{de}	0.72±0.09^{de}	1.21±0.2^{e}	1.87±0.18^{abe}	4.72±0.21^{abcd}
Tensile strength (kg)	15.64±0.88^{de}	14.48±1.73^{de}	26.28±3.99^{e}	37.48±2.6^{abe}	94.4±4.3^{abcd}
Yield strength (kg)	10.43±0.58^{de}	9.65±1.16^{de}	17.52±2.66^{e}	24.99±2.4^{abe}	62.93±2.87^{abcd}
Stiffness (kg/mm)	9.29±0.48^{de}	8.69±1.04^{de}	15.77±2.4^{e}	22.49±2.16^{abe}	56.6±2.61^{abcd}

Different letters (^{a}=control, ^{b}= base gel, ^{c}=5% Bene oil gel, ^{d}=10% Bene oil gel and ^{e}= Intact skin) represent significant differences for that variable between the associated groups, at different days

DISCUSSION

These results suggest that topical application of Bene oil gels (especially 10% Bene) resulted in improved reepithelialization with continuous stratum basalis and a mature granulation tissue and adnexa (hair follicles and sweat gland) compared with control and base gel groups. Treatment with 10% Bene oil gel significantly enhanced the tensile strength, ultimate stress, yield strength and stiffness of this group compared with the control and base gel group at 21 days post injury. The collagen fibers showed a more organized pattern and the tissue alignment was greater as compared to the control and base gel treated groups at the same stage.

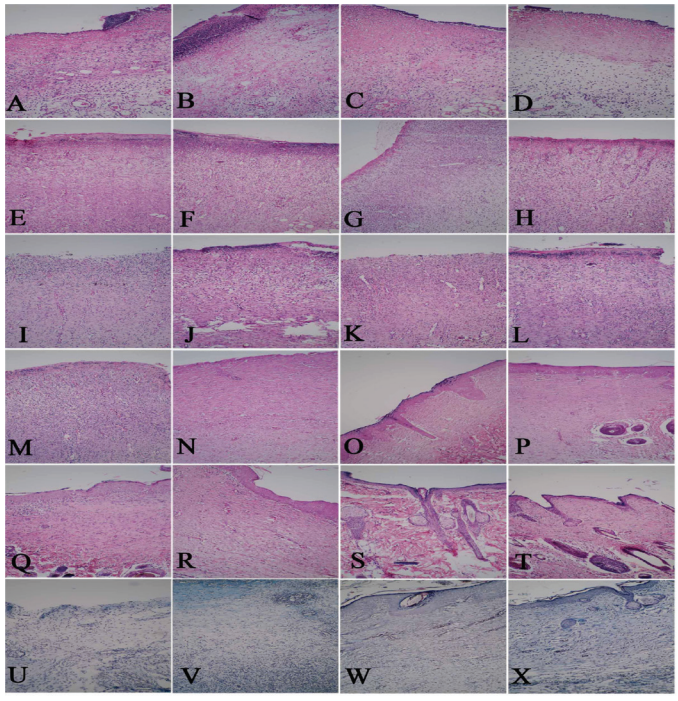

Figure 3. Tissue sections of the control (a, e, ı, m, q), basal gel (b, f, j, n, r), 5% Bene oil gel (c, g, k, o, s) and 10% Bene oil gel groups (d, h, l, p, t), on days 3, 7, 10, 14 and 21 post injury and Masson green thricrome of this groups on day 21 post injury (u, v, w, x)

However, to our knowledge, this is the first time that this reagent has been used on experimentally induced cutaneous wound defects in rats and except for the antioxidants potential of this reagent, its other beneficial effects, such as production of collagen and increased stiffness, its influence on mesenchymal cells and tissue maturity, are novel and suggest this reagent as a potent initiator of wound healing.

Consist with our finding Tanideh et al.[43] showed that a high dose of *P. atlantica* fruit oil extract, administered orally and rectally can improve colitis physiologically and pathologically in a rat model, and may be efficient for ulcerative colitis.

The balance between free radicals and antioxidants may be disrupted in many diseases.[44] Wounding is condition that results in reduction of the antioxidants.[22,23] Hamidi et al.[23] showed that excision of the wound leads to oxidative stress and decrease significantly level of superoxide dismutase, plasma glutathione peroxidase and catalase, and also increase level of malondialdehyde during wound closure in control groups and topical administration of *P. atlantica* gels causes remarkable changes in antioxidant parameter during wound closure

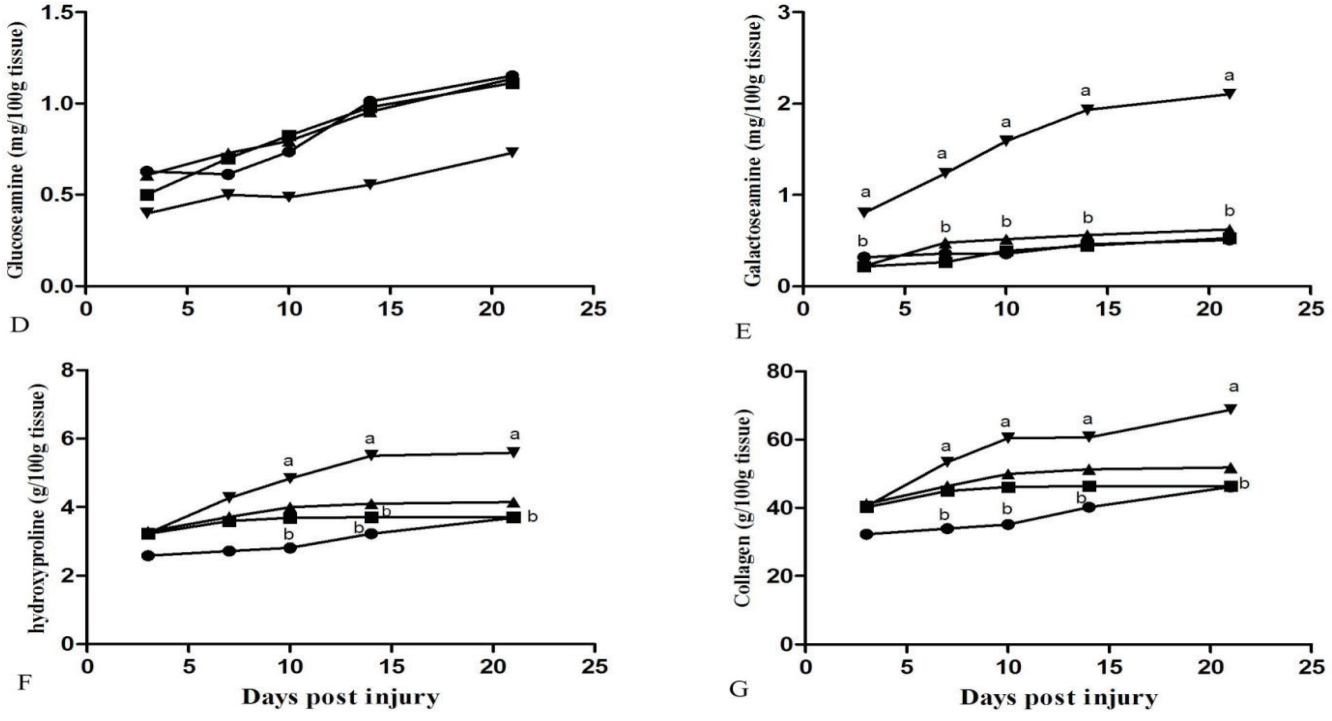

Figure 4. Pattern of changes in biochemical parameters in the lesions of the of the control (●), base gel (■), 5% Bene (▲) and 10% Bene (▼) rats at different time points (day), values are expressed as mean ± standard error of the mean a, b and c show significant differences ($p<0.05$) between groups at different days post injury are indicated

(especially gel 10%) via pro-oxidative and antioxidant activity which can improve oxidative stress.

Guo and DiPietro[45] suggested that vitamin E, an anti-oxidant, maintains and stabilizes cellular membrane integrity by providing protection against destruction by oxidation. We showed Bene oil gel has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in wounds. Consistent with our finding, Burgess[46] and Arnold and Barbul[47] showed that vitamin E also has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds.

Another study showed in acute rodent wounds, the levels of vitamin E, ascorbate and glutathione decreased by 60-70% as compared to normal skin and only the levels of glutathione recovered completely within 14 days after wounding.[22] In another study it has been shown that strong reduction levels of glutathione, Vitamin E and ascorbate were observed in wounded skin of immunosuppressed rats as compared to the immunocompetent animals.[48] In addition, it has been found that palm vitamin E extract, which contains a mixture of 60% tocotrienol and 40% tocopherol, enhances wound healing in diabetic rats and increases the activity of the GPx enzyme.[49] Consistent with our finding Arnold and Barbul[47] and Burgess[46] indicated that vitamin E supplementation is beneficial to wound healing,[50] and topical vitamin E has been widely promoted as an anti-scarring agent and Zampieri et al.[51] also showed that topical application of vitamin E could improve the cosmetic results of surgical incisions. Topical formulations of vitamin E have been promoted to help wound healing, presumably because they inhibit collagen synthesis and reduce both fibroblast proliferation and inflammation.[31,52]

Erhan et al.[53] suggest that both vitamin E and selenium promote wound healing activity by increasing the blood flow in the wound region. New evidences from the studies on diabetic mouse models point to an involvement of oxidative stress in diabetic wound healing and significantly improved wound healing by topical vitamin E.[54,55] Another finding showed vitamin E or α-tocopherol supplementation reduced the levels of lipid peroxides in the wound tissue of diabetic rats, and the wound healing process was enhanced, in particular in the Vitamin E treated animals.[56] Tsoureli-Nikita et al.[57] performed a clinical single, blind, placebo controlled study in which 96 atopic dermatitis patients were treated with either placebo or oral vitamin E (400 IE/day) for 8 months.

Fatty acids have important roles in immune and inflammatory responses. They are important components of the cell membrane.[58,59] In this experiment no evidence of pus accumulation at Bene treated group was seen. Chen et al.[18] showed that oleic acid (C18:1) exerts excellent antimicrobial activity against various *S. aureus* strains, including a hospital-acquired methicillin-resistant *S. aureus* strain (MRSA252) and a community associated-MRSA strain (USA300). It was also shown that oleic acid may have broad-spectrum antimicrobial activity against *S. aureus* bacteria and can suppress growth of gram-positive bacteria including *Staphylococcus epidermidis*, *Listeria monocytogenes* and *Bacillus anthracis.*

EFA, such as linoleic acid, have been employed for the prevention[60] and treatment of pressure ulcers.[61] The deficiency of EFA has impaired cutaneous wound healing in mice, rats[62,63] and infants.[64] Fatty acids have been shown to control the functions of neutrophils. These cells play a key role in the healing process by releasing various cytokines at first stage of healing. Therefore, the effect of fatty acids on neutrophils may play an important role in the healing process, in particular, on the control of angiogenesis and cell proliferation. Simpson and Ross[65] indicated that neutrophils are not essential for proper wound healing in the skin during wound healing. Studies using antibody-based methods to reduce neutrophils in mice suggest that neutrophils can deter healing, as depletion of neutrophils led to significantly faster reepithelialization rates compared to the control wounds.[66,67] We showed that treatment with Bene resulted in remarkable reduction in the number of neutrophils during the period of healing.

Pereira et al.[68] showed that oleic and linoleic acids cause marked changes in the wound during the inflammation period of the healing process. These fatty acids showed a pro-inflammatory effect on cutaneous wound healing. Cardosoa et al.[69] showed that linoleic and oleic acids can modulate the closure of surgically induced skin wounds.

Linoleic acid can be used for the biosynthesis of arachidonic and Prostaglandins in the epidermis.[68] Arachidonic acid and its metabolites are mediators of several events during wound healing, such as cell growth, angiogenesis and synthesis of extracellular matrix. Prostaglandins are involved in tissue repair, cell spreading and migration and epidermal cell proliferation.[70]

The nutritional deficiency of EFA has also been associated with deficient cutaneous wound healing as reported in mice, rats[62,63] and infants.[64] Fatty acid mixtures containing oleic and linoleic acids have been used for prevention and treatment of pressure ulcers.[60,71] Topical application of fatty acids also improves hydration, elasticity and prevents skin breakdown in patients under poor nutritional conditions.[10,61,62]

Although the role of vitamin E in the treatment and prevention of skin disorders seems to be effective[72,73] there are controversial data about the use of topical vitamin E to prevent and treat hypertrophic scars. Some authors have shown that topical applications of vitamin E do not have significant effects in the enhancement of the cosmetic results of surgical incisions. It seems that current evidence from the literature does not support the conclusion that the topical use of vitamin E cream can reduce scar formation. In fact, studies report some adverse effects related to its use.[74] Some reports indicate that vitamin E may impair collagen synthesis and wound healing in animals[52,75] whereas other authors report enhanced healing in irradiated rat skin and patients with post thrombotic leg ulcers.[76]

CONCLUSION

The results suggest that, there was a reduction in the total lymphocyte count in both 5% and 10% Bene gel groups at twenty one days compared with control and base gel groups after injury but this reduction was only significant in 10% Bene gel groups. Total fibrocyte count was significantly higher

in the 10% Bene treated group compared with the 5% Bene, control and base gel groups. topical application of Bene oil gels (especially Bene 10%) resulted in improved reepithelialization with continuous stratum basalis and a mature granulation tissue and adnexa (hair follicles and sweat gland) compared with control and base gel groups. The lesions in the Bene oil gel treated groups showed improved cosmetic results compared with the control and base gel groups during wound repair. Treatment with 10% Bene oil gel significantly enhanced the tensile strength, ultimate stress, yield strength and stiffness of this group compared with the control and base gel group at 21 days post injury. The collagen fibers showed a more organized pattern and the tissue alignment was greater as compared to the control and base gel treated groups at the same stage.

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REVIEW ARTICLE

Melatonin in Edible and Non-Edible Plants

Yenilebilen ve Yenilemeyen Bitkilerde Melatonin

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ABSTRACT

The concept of melatonin has become more important recently both in plants and in human who utilize plants for nutritional and health purposes. Melatonin, synthesized from L-tryptophan by enzyms, protects plants against difficult conditions. People have consumed these plants for their antioxidant, immunomodulator, antiinflammatory and anticancer effects. In parts of edible and non-edible plants, levels of melatonin are determined by cyclodextrin-modified micellar electrokinetic chromatography, enzyme-linked immuno sorbent assay, radioimmunoassay, high-performance liquid chromatography, liquid chromatography with electrochemical detection, liquid chromatography with fluorimetric detection, liquid chromatography-mass spectrometry, and liquid chromatography-ultraviolet spectrophotometry. In this review, biosynthesis of melatonin in both animal and plants, function of melatonin in plant kingdom, especially in medicinal/edible and nonedible plants, and detection of phytomelatonin content in those plants are presented.

Key words: Melatonin, phytomelatonin, activity of melatonin

ÖZ

Bitkilerde melatonin kavramı, son yıllarda, hem bitkiler hem de beslenme ve sağlığı koruma amacıyla bitkileri kullanan insanlar için oldukça önemli olmuştur. Enzimler aracılığıyla L-triptofandan sentezlenen melatonin bitkiyi zorlu koşullara karşı korumaktadır. İnsanlar antioksidan, immünomodülatör, antienflamatuvar ve antikanser etkilerinden dolayı bu bitkileri kullanmaktadır. Yenilebilen ve yenilemeyen bitki kısımlarında siklodeskstrinle modifiye edilmiş miseller elektrokinetik kromatografi, enzim bağlı immünosorban deneyi, radyoimmün test, yüksek performanslı sıvı kromatografisi, elektrokimyasal algılamalı sıvı kromatografisi, florometrik algılamalı sıvı kromatografisi, sıvı kromatografisi-kütle spektrometrisi ve sıvı kromatografisi-ultraviyole spektrofotometri yöntemleri ile tespit edilmiştir. Bu derlemede, melatoninin hem hayvanlarda hem de bitkilerde biyosentezi, özellikle tıbbi/yenilebilen ve yenilemeyen bitkilerde melatoninin fonksiyonu ve bu bitkilerde fitomelatonin içeriği sunulmuştur.

Anahtar kelimeler: Melatonin, fitomelatonin, melatonin etkisi

INTRODUCTION

Melatonin (*N*-acetyl-5-methoxytryptamine) means melanophore-contracting hormone (Greek: μαύρος=black; τάσης=tension) firstly was isolated from bovine's pineal gland in 1958.[1,2] It is a neurohormone secreted by the pineal gland and a derivative of serotonin.[3] Serotonin is a monoamine neurotransmitter and one of the precursors (Figure 1), whereas L-tryptophan, like serotonin is the common precursor of melatonin biosynthesis.[4,5] Both have many influences on health of animal and human being, such as serotonin is used against depression[6] and also affects behaviours and inward.[7] Secretion of melatonin increases in the dark on the contrary of light, seasonal and physiological alteration effect levels of melatonin[8,9] for that reason that has been studied for its hormon like effects and its biological activities for decades.

Although melatonin was described in organisms such as bacteria, fungi, algae, and vertebrates[10] it was notified in plants at the end of 1994.[11,12] Increasing number of studies have proved that there was melatonin in different parts (seed, fruit, leaf, root etc.) of plants and in so much as medicinal herbs.[13] A major role of melatonin in plants have been discovered that protects plants against damages of changing climate.[14]

Biosynthesis of melatonin

Melatonin is synthesised not only in bone marrow cells[15] but also in retina.[16,17] Thus it is both a hormone and tissue factor.[10] The presence of melatonin was detected in egg, biological fluids like plasma, milk, by developed methods, such as liquid chromatography (LC) with fluorimetric detection, and LC-tandem mass spectrometry (LC-MS/MS).[18,19] Biosynthesis of melatonin is explained enzymatically from the essential amino acid precursor tryptophan to melatonin. The synthesis includes four different enzymes. The first one is tryptophan hydroxylase (TPH), which forms 5-hydroxytryptophan from tryptophan; the second is aromatic amino acid decarboxylase which forms

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serotonin from 5-hydroxytryptophan; the third is arylalkylamine *N*-acetyl-transferase (AANAT), which forms *N*-acetylserotonin from serotonin; and the last one is *N*-acetylserotonin *O*-methyltransferase (ASMT), which forms the final step to melatonin (Figure 2). AANAT and ASMT is considered that they were speed limiting enzymes.[4,20]

Biological activity of melatonin

A major role of melatonin is the antioxidant function with free radicals (reactive oxygen species) and reactive nitrogen species scavenging activity[21-25] thus has protective effect against ultraviolet (UV) radiations induced damages.[26] Consequently, melatonin can be used for healing of muscle diseases, Parkinson and Alzheimer's due to antioxidant and neuroprotective affects.[27-31] Melatonin is widely used for sleep disorders such as jetlag and insomnia.[32] Its administration can relieve daytime and overnight sleep.[33,34] Clinical and *in vivo* studies showed that melatonin decreased symptoms of depression[35-37] moreover has immunomodulator function.[38,39] It regulates immuno fuctions by means of production interleukin (IL)-2, IL-6, IL-12 and interferon gamma.[40,41,42] An *in vivo* study showed that melatonin have potential anticonvulsant activity.[43] Melatonin effects vasculer system.[44] Studies showed that melatonin suppress proliferation of cancer cell line and induces apoptosis tumor cell and also it is promising for the treatment of prostat cancer, and breast cancers.[25,45-53] A study has also emitted that melatonin can be effective on malaria.[54]

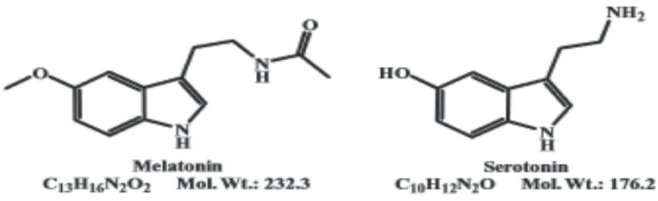

Figure 1. Structure of melatonin and serotonin

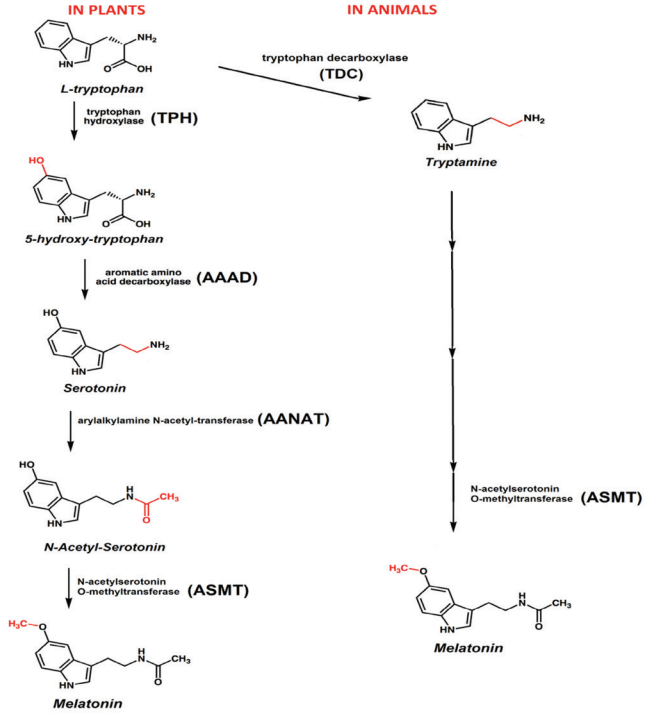

Figure 2. Modified synthesis of melatonin from tryptophan

TPH: Tryptophan hydroxylase, TDC: Tryptophan decarboxylase, AAAD: Aromatic amino acid decarboxylase, AANAT: Arylalkylamine *N*-acetyl- transferase, ASMT: *N*-acetylserotonin *O*-methyltransferase

Melatonin in plants-phytomelatonin

First evidence of the presence of melatonin in organisms was obtained in *Lingulodinium polyedrum* (syn. *Gonyaulax polyedra*) and *Pyrocystis acuta*, which were unicellular organisms. Scientists detected melatonin metabolite 5-methoxytryptamine and the melatonin analogue *N*, *N*-dimethyl-5-methoxytryptamine in those living organisms.[55-57] By following studies melatonin was determined in the members of alga, bacteria, fungi, plant families. Level of melatonin, although differs from plant to plant, that was observed higher than level of melatonin in animal blood.[58-60] Melatonin level varies both from plant to plant and also tissues/organs of same plant, moreover, temperature, pH, effects of present metal ions's, sensitivity of analytics and extraction methods cause these diversities. For example, melatonin of *Datura metel* L. (devil's trumpet) differed from flowers and leaves. In addition, melatonin of *Lycopersicon esculentum* Mill. varied by region.[11,61-64] Presence of melatonin in different plants were shown in Table 1.

Biosynthesis of phytomelatonin

Plant melatonin biosynthesis pathway firstly was determined owing to *Hypericum perforatum* L. (St John's wort).[4,59] Synthesis in plants is complicated on the contrary in animals (Figure 2). Initial enzyme is tryptophan decarboxylase (TDC) instead of TPH. TDC forms tryptamine from essential amino acid tryptophan. The last enzyme is ASMT (Figure 2).[65,66] Plants take melatonin also by their roots apart from biosynthesis.[67,68] Although its biosynthetic pathway and metabolic mechanisms are unclear, the presence of melatonin in plants is a wide concept.[69]

Functions of phytomelatonin

Melatonin has roles in plants similar to animals, that protects plants against extreme conditions such as temperature change, UV exposure, environmental pollution, toxins, drought oxidative and (a) biotic stress. Exogenous melatonin applied to *Arabidopsis* (thale cress) leaves has demostrated preservative potency against high salinity, cold and dryness, additionally plant has developed tolrerance biotic and abiotic stresses.[70] Corn embriyo proteome was improved due to exogenous melatonin.[71] Moreover, harmful effects of salt diminished by melatonin in faba bean.[72] Conservation aspects of melatonin were studied in a variety of plants such as wheat, oat, barley, canary grass, tobacco, Chinese liquorice, soybean, cucumber, tomato.[14,67,73-79] The studies also has shown that melatonin has regulatory role in growth of thale cress, specially growth of flowers and fruits.[80] Reports, which investigated effect of exogenous melatonin on both tomato's and maize's seeds, have confirmed this case too.[78,81] Melatonin plays an important role to maintain the vitality of the plants.[82]

Table 1. Levels of melatonin in plants with the detection methods

Family	Latin name	Part	Quantity		Method	Ref.
			ng/g	pg/g		
Actinidiaceae	*Actinidia chinensis Planch.*	Fruit		24	RIA	61
Amaranthaceae	*Basella alba L.*	Leaf		39	RIA	61
Amaryllidaceae	*Allium cepa L.*	Bulb		32	RIA	61
Amaryllidaceae	*Allium fistulosum L.*	Bulb		86	RIA	61
Anacardiaceae	*Pistacia lentiscus L.*	Leaf		581	ELISA	96
Anacardiaceae	*Pistacia lentiscus L.*	Whole fruit		536±129	ELISA	96
Anacardiaceae	*Pistacia palaestina Boiss.*	Leaf		498	ELISA	96
Apiaceae	*Angelica keiskei Koidz.*	Leaf and stem of leaf		624	RIA	61
Apiaceae	*Apium graveolens L.*	Seed	7		HPLC-ECD	97
Apiaceae	*Coriandrum sativum L.*	Seed	7		HPLC-ECD	97
Apiaceae	*Daucus carota L.*	Root		55	RIA	61
Apiaceae	*Foeniculum vulgare Mill.*	Seed	28		HPLC-ECD	97
Apiaceae	*Pimpinella anisum L.*	Seed	7		HPLC-ECD	97
Arecaceae	*Phoenix dactylifera L.*	Whole fruit		469	ELISA	96
Asparagaceae	*Asparagus officinalis L.*	Shoot		10	RIA	61
Asparagaceae	*Ophiopogon japonicus (L.f.) Ker Gawl.*	Whole plant	198		HPLC-FD-MS	90
Asteraceae	*Glebionis coronari (L.) Cass. ex Spach*	Leaf		417	RIA	61
Asteraceae	*Dendranthema morifolium (Ramat.) Tzvelev*	Whole plant	160		HPLC-FD-MS	90
Asteraceae	*Helianthus annuus L.*	Seed	29		HPLC-ECD	97
Asteraceae	*Petasites japonicus F. Schmidt*	Shoot		50	RIA	61
Asteraceae	*Silybum marianum (L.) Gaertn.*	Seed	2		HPLC-ECD	97
Araceae	*Colocasia esculenta (L.) Schott*	Tuber		55	RIA	61
Araceae	*Peltandra virginica (L.) Raf. ex Schott*	Whole plant	585		HPLC-FD-MS	90
Brassicaceae	*Arabidopsis spp.*	Leaf	548±26		SPE, CD-ME-KC	98
Brassicaceae	*Brassica campestris L.*	Leaf		657	RIA	61
Brassicaceae	*Brassica hirta Moench*	Seed	189		HPLC-ECD	97
Brassicaceae	*Brassica nigra (L.) W. D. J. Koch*	Seed	129		HPLC-ECD	97
Brassicaceae	*Brassica oleracea L.*	Leaf		107	RIA	61
Brassicaceae	*Raphanus sativus L.*	Whole plant	485		HPLC-FD-MS	90
Brassicaceae	*Raphanus sativus L.*	Root		113	RIA	61
Bromeliaceae	*Ananas comosus (L.) Merr.*	Fruit		36	RIA	61
Caprifoliaceae	*Lonicera etrusca hort. ex Tausch*	Leaf		521	ELISA	96
Caprifoliaceae	*Lonicera etrusca hort. ex Tausch*	Seed		403	ELISA	96
Caprifoliaceae	*Lonicera japonica Thunb.*	Whole plant	140		HPLC-FD-MS	90
Caprifoliaceae	*Viburnum tinus L.*	Leaf		613	ELISA	96
Cucurbitaceae	*Cucumis sativus L.*	Fruit		25	RIA	61
Ephedraceae	*Ephedra campylopoda C. A. Mey.*	Leaf		178	ELISA	96
Ephedraceae	*Ephedra campylopoda C.A.Mey.*	Seed		379	ELISA	96
Fabaceae	*Glycyrrhiza uralensis Fisch. ex DC.*	Whole plant	112		HPLC-FD-MS	90
Fabaceae	*Lupinus albus L.*	Seed (Cotyledone)	1.28±0.06		HPLC-FD	99, 100
Fabaceae	*Medicago sativa L.*	Seed	16		HPLC-ECD	97
Fabaceae	*Trigonella foenum-graceum L.*	Seed	43		HPLC-ECD	97
Juglandaceae	*Juglans nigra L.*	Fruit	3.5±1.0		HPLC-ECD	101

Table 1. Continue						
Lamiaceae	*Salvia miltiorrhiza Bunge*	Whole plant	187		HPLC-FD-MS	90
Lauraceae	*Laurus nobilis L.*	Leaf		8331	ELISA	96
Lauraceae	*Laurus nobilis L.*	Whole fruit		3710	ELISA	96
Lauraceae	*Laurus nobilis L.*	Seed		6060	ELISA	96
Lauraceae	*Laurus nobilis L.*	Pulp		1820	ELISA	96
Liliaceae	*Asparagus aphyllus L.*	Leaf		142	ELISA	96
Liliaceae	*Ruscus aculeatus L.*	Leaf		954	ELISA	96
Liliaceae	*Smilax aspera L.*	Leaf		443	ELISA	96
Linaceae	*Linum usitatissimum L.*	Seed	12		HPLC-ECD	97
Meliaceae	*Melia azedarach L.*	Leaf		1579	ELISA	96
Meliaceae	*Melia azedarach L.*	Whole fruit		585	ELISA	96
Moraceae	*Morus alba L.*	Leaf	1510		HPLC-FD-MS	90
Moraceae	*Morus spp.*	Leaf		990	ELISA	96
Moraceae	*Ficus carica L.*	Leaf		12.915	ELISA	96
Moraceae	*Ficus carica L.*	Whole fruit		3963	ELISA	96
Myrtaceae	*Feijoa sellowiana (O. Berg) O. Berg*	Leaf		1529	ELISA	96
Myrtaceae	*Myrtus communis L.*	Leaf		291	ELISA	96
Myrtaceae	*Myrtus spp.*	Leaf		490	ELISA	96
Oleaceae	*Olea europaea L.*	Leaf		4306	ELISA	96
Oleaceae	*Olea europaea L.*	Pulp		532	ELISA	96
Oleaceae	*Phillyrea latifolia L.*	Leaf		6337	ELISA	96
Oleaceae	*Phillyrea latifolia L.*	Seed		439	ELISA	96
Oleaceae	*Phillyrea latifolia L.*	Pulp		589	ELISA	96
Papaveraceae	*Papaver somniferum L.*	Seed	6		HPLC-ECD	97
Poaceae	*Avena sativa L.*	Seed		1796	RIA	61
Poaceae	*Avena sativa L.*	Seed	90.6±7.7		HPLC-ECD	102
Poaceae	*Hordeum vulgare L.*	Seed		378	RIA	61
Poaceae	*Hordeum vulgare L.*	Seed	82.3±6.0		HPLC-ECD	102
Poaceae	*Hordeum vulgare L.*	Seed	0.09±0.01		HPLC-FD	99
Poaceae	*Hordeum vulgare L.*	Seed	0.58±0.05		HPLC-FD	99
Poaceae	*Oryza sativa L. subsp. japonica Shig. Kato*	Seed	1006		RIA	61
Poaceae	*Phalaris canariensis L.*	Seed	26.7±2.2		HPLC-ECD	102
Poaceae	*Triticum spp.*	Seed	124.7±14.9		HPLC-ECD	102
Poaceae	*Triticum spp.*	Seed	2		HPLC-UV	102
Poaceae	*Triticum spp.*	Seed	4		HPLC-UV	102
Poaceae	*Zea mays L.*	Seed		1366	RIA	61
Poaceae	*Zea mays L.*	Seed	0.011*10-9-2.034*10-9		HPLC	103
Resedaceae	*Ochradenus baccatus Delile*	Leaf		474	ELISA	96
Resedaceae	*Ochradenus baccatus Delile*	Whole fruit		488	ELISA	96
Rhamnaceae	*Rhamnus alaternus L.*	Leaf		306±75	ELISA	96
Rhamnaceae	*Rhamnus palaestina Boiss.*	Whole fruit		907	ELISA	96
Rhamnaceae	*Rhamnus palaestina Boiss.*	Seed		547	ELISA	96

Table 1. Continue

Rhamnaceae	*Rhamnus palaestina Boiss.*	Pulp		409	ELISA	96
Rhamnaceae	*Ziziphus jujuba Lam.*	Whole plant	146		HPLC-FD-MS	90
Rhamnaceae	*Ziziphus jujuba Mill. var. spinosa (Bunge) Hu ex H. F. Chou*	Whole plant	256		HPLC-FD-MS	90
Rhamnaceae	*Ziziphus spina-christi (L.) Willd.*	Leaf		1324	ELISA	96
Rosaceae	*Crataegus aronia (Willd.) Bosc*	Leaf		341	ELISA	96
Rosaceae	*Crataegus azarolus L.*	Leaf		435	ELISA	96
Rosaceae	*Fragaria magna Thuill.*	Fruit		12	RIA	61
Rosaceae	*Malus domestica Borkh.*	Fruit		48	RIA	61
Rosaceae	*Prunus amygdalus Stokes*	Seed	39		HPLC-ECD	97
Rosaceae	*Prunus avium L.*	Fruit (harvested arround middle May-'Burlat')	0.224±0.012		HPLC-MS	104
Rosaceae	*Prunus avium L.*	Fruit (harvested 6 days after 'Burlat')	0.027±0.024		HPLC-MS	104
Rosaceae	*Prunus avium L.*	Fruit (harvested 31 days after 'Burlat')	0.006±0.007		HPLC-MS	104
Rosaceae	*Prunus avium L.*	Fruit (harvested 33 days after 'Burlat')	0.06±0.02		HPLC-MS	104
Rosaceae	*Prunus avium L.*	Fruit (harvested 37 days after 'Burlat')	0.115±0.033		HPLC-MS	104
Rosaceae	*Prunus avium L.* http://www.ipni.org/ipni/idPlantNameSearch.do;jsessionid=3F8C9196D5F394AC6484CACBAF1C2FEE?id=160672-3&back_page=%2Fipni%2FeditSimplePlantNameSearch.do%3Bjsessionid%3D3F8C9196D5F394AC6484CACBAF1C2FEE%3Ffind_wholeName%3DPrunus%2Bavium%26output_format%3Dnormal	Fruit (harvested 44 days after 'Burlat')	0.048±0.022		HPLC-MS	104
Rosaceae	*Prunus cerasus L.*	Fruit	1.07±0.35-2.18±0.26		HPLC-ECD	105
Rosaceae	*Prunus cerasus L.*	Fruit	5.57±0.38-19.59±2.76		HPLC-ECD	105
Rosaceae	*Prunus cerasus L.*	Fruit (Montmorency frozen)	12.3±2		HPLC-MS	106
Rosaceae	*Prunus cerasus L.*	Fruit (Balaton frozen)	2.9±0.6		HPLC-MS	106
Rosaceae	*Prunus cerasus L.*	Fruit (Balaton individually quick frozen powder)	1.7±0.5		HPLC-MS	106
Rosaceae	*Prunus cerasus L.*	Fruit (Montmorency individually quick frozen powder)	7.5±0.9		HPLC-MS	106
Rosaceae	*Rubus idaeus L.*	Whole plant	387		HPLC-FD-MS	90
Rosaceae	*Rubus sanctus Schreb.*	Leaf		805	ELISA	96
Rubiaceae	*Rubia tenuifolia d'Urv.*	Leaf		905	ELISA	96
Rubiaceae	*Rubia tenuifolia d'Urv.*	Whole fruit		339	ELISA	96
Rubiaceae	*Rubia tenuifolia d'Urv.*	Seed		539	ELISA	96
Santalaceae	*Osyris alba L.*	Leaf		844	ELISA	96
Schisandraceae	*Schisandra chinensis (Turcz.) K. Koch*	Whole plant	86		HPLC-FD-MS	90
Scrophulariaceae	*Scrophularia nodosa L.*	Whole plant	342		HPLC-FD-MS	90
Solanaceae	*Lycium barbarum L.*	Seed	103		HPLC-ECD	97

Table 1. Continue

Solanaceae	*Lycium barbarum L.*	Whole plant	530		HPLC-FD-MS	90
Solanaceae	*Lycopersicon esculentum Mill.*	Fruit		32	RIA	61
Solanaceae	*Solanum elaeagnifolium Cav.*	Whole fruit		7895	ELISA	96
Solanaceae	*Solanum elaeagnifolium Cav.*	Seed		5604	ELISA	96
Solanaceae	*Solanum elaeagnifolium Cav.*	Pulp		7392	ELISA	96
Solanaceae	*Solanum nigrum L.*	Whole fruit		323±46	ELISA	96
Styracaceae	*Styrax officinalis L.*	Leaf		4069	ELISA	96
Theaceae	*Camellia sinensis (L.) Kuntze*	Leaf	386±21		CD-MEKC	98
Tiliaceae	*Tilia cordata L.*	Leaf	410±16		CD-MEKC	98
Verbenaceae	*Lantana camara L.*	Leaf		389	ELISA	96
Xanthorrhoeaceae	*Aloe vera (L.) Burm. f.*	Whole plant	516		HPLC-FD-MS	90
Zingiberaceae	*Elettaria cardamomum Maton*	Seed	15		HPLC-ECD	97
Zingiberaceae	*Zingiber officinale Roscoe*	Rhizome		584	RIA	61

RIA: Radioimmunoassay, ELISA: Enzyme linked immunosorbent assay, HPLC: High performance liquid chromatography, ECD: Electrochemical detection, FD: Fluorescence detector, MS: Mass spectrometry, SPE: Solid phase extraction, CD: Cyclodextrin, MEKC: Micellar electrokinetic chromatography, UV: Ultraviolet

Phytomelatonin in diets

The most popular drinks, which are tea, coffee, beer and wine contain melatonin. Not only melatonin but also its isomers (tryptophan-ethylester) were determined in wine and bread.[83-85] A study reported that regular coffee consumption remarkably decreases the prevalence of human prostate cancer.[86-88] Scientists introduced that melatonin in wine besides the other secondary metabolites, had protective effect against heart injury.[89] Melatonin was determined high amount in Chinese medicinal herbs. Some of them were *Viola philippica* Cav., Uncaria rhynchophylla Miq., *Morus alba* L. and *Phellodendron amurense* Rupr.[90] In Mediterranean diet, melatonin was found in some foods. It'is thought that melatonin can have positive effects on health via synergic effects with other compounds.[91] Dietary suplement/melatonin supplement preparations have been consumed for different purposses by people mostly in Europe and the United States than the other countries.[92]

Determination of phytomelatonin levels in plants

Melatonin has been detected in fruits, leaves, roots, and seeds of a considerable variety of plant species. Various methods, such as cyclodextrin-modified micellar electrokinetic chromatography, enzyme-linked immuno sorbent assay, radioimmunoassay (RIA), high-performance LC (HPLC), HPLC-electrochemical detection, HPLC-fluorescence detector, HPLC-MS and HPLC-UV spectrophotometry (UV) can be applied in order to determine melatonin levels in plants.

The first step in determining the levels of melatonin in plants is to find the right extraction method, which have been tried by different authors. The first identification method of melatonin in plants was described by Van Tassel et al.[93] in a congress communication in 1993. The authors had detected melatonin in tomato fruits (*Solanum lycopersicum* L.) by using RIA and gas chromatography attached with MS, but the results were not published extensively until 1995.[94]

Nowadays, most of the researchers have been utilizing liquid nitrogen treated-plant tissue, which were extracted with organic solvents such as methanol, chloroform, or ethyl acetate. Analysis of these extracts by LC and identification by MS are the most used and recommended techniques for the detection and quantification of melatonin in plants. Due to the developed technology of LC coupled to time-of-flight/MS has also been applied for the melatonin detection in recent years.[95]

Biotechnology

A biotechnologic study showed that transgenic plant rich on account of melatonin had more antioxidative activity and higer yield than regular plants.[107-109] When activity of ASMT enzyme-catalyzed from *N*-acetylserotonin to melatonin and isolated firstly from rice in plants- was increased by overexpression, the level of melatonin has also increased.[110,111] A study demonstrated that since 6-hydroxymelatonin was not determined in rice, melatonin 2-hydroxylase has been dominant enzyme in melatonin production.[112]

CONCLUSION

Melatonin has been studied to treat some symptoms and diseases in human over the years. Melatonin supplements have proven significant results for treating insomnia and other circadian rhythms caused sleep disorders, morever, jet lag and shift work, headache, various cancers, gallbladder stones, tinnitus, rheumatoid arthritis, Alzheimer's disease, and psychiatric disorders have also tried to be eased with melatonin. Besides, it is known that melatonin is a powerful antioxidant and it improves the immune system. According to recent research, melatonin has also a great anti-aging effect.

Melatonin is a hormone that naturally produced by pineal glad in human brain especially at night-time, hovewer, smoking, using

alcohol, excessive coffee consumption, some medications and disorders can suppress the production of the melatonin. Therefore melatonin should be taken externally such as synthetic melatonin supplements, or from natural resources which produce or contain melatonin. Furthermore, taking nutrients, which contain tryptophan, can increase the secretion of melatonin in the body. For instance, eating strawberries, apples, cherry/juice, rice, pistachios, almonds, spinach, cabbage, onions, tomatoes, cucumber, linseed and sunflower seeds, thistle, fenugreek and mustard; drinking teas such as fennel and anise tea.

In this study, our aim was to bring attention to melatonin in plants, which has important roles in plants as well as in animals. Many scientists have laboured to identify and quantify the levels of melatonin in plants. Although there are numbers of studies were completed in plants still more studies have been needed to analyse the levels and their absorption and efficiency of melatonin directly from plants, teas and pharmaceutical preparations.

Conflict of Interest: No conflict of interest was declared by the authors.

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REVIEW ARTICLE

A Review on: Phase '0' Clinical Trials or Exploratory Investigational New Drug

Klinik Faz '0' Testleri ya da Yeni İlaç Keşif Araştırmaları Üzerine Bir Derleme

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ABSTRACT

In a move to speed up the development of new medicines, the Food and Drug Administration announced in January 2006 the creation of the exploratory Investigational New Drug (IND), the so-called phase '0' clinical trials. This guidance is intended to clarify what preclinical and clinical approaches, as well as chemistry, manufacturing, and controls information, should be considered when planning exploratory studies in humans, including studies of closely related drugs or therapeutic biological products, under an IND application (21 CFR 312). Existing regulations allow a great deal of flexibility in the amount of data that needs to be submitted with an IND application, depending on the goals of the proposed investigation, the specific human testing proposed, and the expected risks. The agency believes that sponsors have not taken full advantage of that flexibility and often provide more supporting information in INDs than is required by regulations. This guidance is intended to clarify what manufacturing controls, preclinical testing, and clinical approaches can be considered when planning limited, early exploratory IND studies in humans.

Key words: IND, preclinical testing, clinical trials, phase 0

ÖZ

Yeni ilaçların gelişimini hızlandırmak amacıyla Gıda ve İlaç İdaresi, Ocak 2006'da faz '0' klinik testleri olarak adlandırılan keşif amaçlı Yeni Araştırılan İlaç (YAİ) rehberinin oluşturulduğunu açıkladı. Kimya, üretim ve kontrol bilgilerinin yanı sıra klinik öncesi ve klinik yaklaşımların açıklığa kavuşturulması için tasarlanan bu rehber YAİ uygulaması altında, yakından ilişkili ilaçlar veya terapötik biyolojik ürünler ile ilgili çalışmalar da dahil olmak üzere insanlarda keşif çalışmaları planlanırken göz önüne alınmalıdır (21 CFR 312). Mevcut düzenlemeler, önerilen araştırmanın amaçlarına, önerilen özgül insan testlerine ve beklenen risklere bağlı olarak YAİ başvurusu ile gönderilmesi gereken veri miktarında büyük bir esneklik sağlar. Ajans, sponsorların bu esneklikten tam olarak yararlanmadığına ve genellikle YAİ'lerde düzenlemeler için gerekli olanlardan daha fazla destekleyici bilgi sağladığına inanmaktadır. Bu rehber, insanlarda sınırlı, erken keşif YAİ çalışmalarını planlarken hangi imalat kontrollerinin, klinik öncesi testlerin ve klinik yaklaşımların düşünülebileceğini açıklığa kavuşturmayı amaçlamaktadır.

Anahtar kelimeler: YAİ, klinik öncesi testler, klinik testler, faz 0

INTRODUCTION

The phase '0' clinical trials have tremendous scope in near future. It is also called exploratory Investigational New Drug (IND) or "micro dosing method". The purposes of this guidance the phase exploratory IND study is intended to describe a clinical trial that;

- Is conducted early in phase 1,
- Involves very limited human exposure, and
- Has no therapeutic or diagnostic intent (e.g., screening studies, micro-dose studies).

Such exploratory IND studies are conducted prior to the traditional dose escalation, safety, and tolerance studies that ordinarily initiate a clinical drug development program.[1,2] The duration of dosing in an exploratory IND study is expected to be limited (e.g., 7 days). This guidance applies to early phase 1 clinical study of IND and biological products that assess feasibility for further development of the drug or biological product.[3,4] The major objectives of phase '0' trials is to interrogate and refine a target or biomarker assay for drug effect in human samples implementing procedures developed and validated in preclinical models. Data cleaned from a phase '0' trial are beneficial not only in prioritizing promising compounds but also in allowing the modification of phase 1 study design before initiation. Phase '0' trials provide an opportunity to generate essential human pharmacokinetic and pharmacodynamics (PD) data earlier in the drug development process.[5] With the help of phase '0' clinical trial only most promising compounds get into subsequent study,

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due to which sponsors reduces the excessive cost, time and human volunteers.[6]

BACKGROUND

In its March 2004 Critical Path Report, the Agency explained that to reduce the time and resources expended on candidate products that are unlikely to succeed, new tools are needed to distinguish earlier in the process those candidates that hold promise from those that do not.

Traditional phase 1 approach

Typically, during pharmaceutical development, large numbers of molecules are generated with the goal of identifying the most promising candidates for further development. These molecules are generally structurally related, but can differ in important ways. Promising candidates are often selected using *in vitro* testing models that examine binding to receptors, effects on enzyme activities, toxic effects, or other *in vitro* pharmacological parameters.[7]

These tests usually require only small amounts of the drug. The candidates that are not rejected during these early tests are prepared in greater quantities for *in vivo* animal testing for efficacy and safety. Commonly, a single candidate is selected for an IND application and introduction into human subjects, initially healthy volunteers in most cases. If this phase 1 get passed then drug goes towards phase 2 and then phase 3 but drug get eliminated then again take its analogue or other drug and again repeat this procedures. Upto 250 drug candidates involved in preclinical study from that only 1-2 drug molecules get into market. Upto 67% fail in phase 1, 45% in phase 2 and 8% in phase 3 this percentage are on the basis of those drugs get passed from animal models and evaluated in the clinical phases [United State Food and Drug Administration (FDA), Center for Drug Evaluation and Research, 2006].[8]

Exploratory Investigational New Drug approach

Exploratory IND studies can help to identify important essential promising drugs for further development and if not can eliminate such type of drug candidate. As a result, exploratory IND studies may help to reduce the number of human subjects and resources, including the amount of candidate product and need to identify important promising candidate. The clinical trials studies involve dosing a very small and limited number of subjects (patients) with a very small and limited range of doses for a specific period of time.[9]

Need of phase '0' clinical trials

Anthony[10], and Kummar and Rubinstein[11], in this research paper discussed the need and use of phase '0' clinical trials as fallows.

- For getting pharmacokinetics (PK) and PD data early in phase 1, this would be helpful in further assessment or for evaluation of compound.
- Time period can be reduced by examining only capable drug candidate for further study.
- Costs of New Drug Development process get reduced, by studying only most promising compound in further study.
- % drug into market get increased, because unwanted compounds get eliminated in the phase '0' clinical trials.

Criteria for phase '0' clinical trials

Anthony[10], Kummar and Rubinstein[11], and James[12], have explained the criteria for phase '0' clinical trials.

- Before phase 1 study,
- Fill the exploratory IND application,
- Volunteers are healthy human or patient,
- Less than 15 volunteers,
- Duration 7 days,
- Dosing 1/100th or 100 µg or 30 nanomole,
- Only one dosing cycle for study,
- In anti-cancer IND pre and post tumor biopsies done if required.

Micro-dosing approach in phase '0' clinical trials

Micro-dosing approach is adopted for evaluating PD (Mode of Action) and PK (volume of distribution, binding and clearance) in the human being for predicting or selecting the most promising drug candidate or its analogue for further study. Due to it unnecessary drug candidate separated from phase 1 or from further study and investigator save unnecessary wastage of money, time and risk concerned with human volunteers. By using only a very tiny amount of active substance, one can establish the more appropriate pharmacological dose and with this estimate the first dose for phase 1 clinical trials study. With addition, micro-dosing can select the better animal species and model for toxicological studies mainly for long term studies from micro-dose metabolite profiling data. Ultrasensitive methods are required for evaluation of very small amount of drug substance in the human body. Concentration 10^{-12} to 10^{-15} gm should be the range of method which should be employed for evaluation of micro-dose. There are two methods which having such abilities of evaluation of micro-dose. These techniques rely on radioisotopes incorporation.[12,13]

Two methods used and this technique was explained by Hung[9] and Rani and Naidu[14].

Accelerator Mass Spectrometry: This technique is used for evaluation of pharmacokinetic data mainly. In this technique radioisotope C^{14} is used for the evaluation of drug or micro-dose. The half-life of this radioisotope is 5740 years.

Positron Emission Tomography: This is mainly used for evaluation of PD data. For evaluation of PD data C^{11} radioisotope is used which having 20 min half-life.

Phase '0' trials in oncology study

Kummar and Rubinstein[11], had conducted the first phase '0' clinical trial for cancer study with sub therapeutic and therapeutic drug candidate under the IND guidance of the FDA. It was first

time in human (First in Human) study of the poly(adenosine diphosphate-ribose)polymerase (PARP) inhibitor code name given (ABT-888) in volunteers with advanced malignant cancer. Promising candidate ABT-888 was administered as a single oral dose of 10, 25 or 50 mg to identity and determines the dosage regimen with duration of time for which drug candidate (ABT-888) inhibits PARP activity in tumour samples and peripheral blood cells as well as estimate candidate (ABT-888) PK profile. Blood samples and tumour biopsies were obtained before and after drug administration for evaluation of PARP activity and PK. A novel statistical approach was developed and utilized to study PD modulation as the primary end point for trials of limited sample size.[5,7,11]

Thirteen patients with advanced malignancies received the study drug; nine patients undergone paired tumour biopsies. ABT-888 demonstrated good oral bioavailability and was well tolerated. Statistically significant inhibition result was observed in tumour biopsies and peripheral blood cells at their respective dose level particularly at 25 and 50 mg dose levels. They obtained very important biochemical and pharmacokinetic data within 5 months of study that have guided the design of phase 1 trials of candidate ABT-888 in combination with DNA damaging agents. In addition to accelerating the development of ABT-888, the immediate finding of this trial explained the feasibility of conducting phase '0' trials as part of an alternative program for early drug development in cancer study.[15]

Role of FDA in phase '0' trials

Lorusso[16], Vijayraghavan and Kumar[17], and Singh[18], were explained the role of phase '0' trials in FDA.

According to the FDA a phase '0' is designed to carry out before in phase 1, it has very limited human exposure receiving only sub-therapeutic dose and this means the volunteer produces a response (Pharmacological Action) than the toxic effect with less risk compared to conventional clinical trials in phase 1 in which administration continues if clinical benefit which means even phase '0' trials don't have any therapeutic intention. With the ultra-sensitive accelerator mass spectrometry (AMS) it was possible to carry out clinical trials in human using small dose to obtained pharmacokinetic data.[16,17,18]

Limitation for micro-dosing techniques

Rani and Naidu[14], Hermann[19], and Bertino et al.[20], had discussed the limitation for micro dosing techniques as fallows.

- It is new technique hence database is small,
- Micro-dose is sophisticated for the evaluation,
- Positron emission tomography (PET) has short half-life i.e. 20 min so on time testing is required,
- Both assay i.e. PET and AMS have limited specificity so test results are also having some time trouble due to this limited specificity,
- Micro-dose may not predict the behaviour of therapeutic or clinical dose.

Difference in between phase '0' and phase 'i' in oncology study

James[12], Anthony[10], Murgo et al.[21], Takimoto[22] and Kummar et al.[23] have explained the differences of phase trials described in Table 1 for oncology study.

Goals of phase '0' clinical trials

Anthony[10], Lorusso[16], and Lappin and Garner[24] had discussed the goals of phase '0' clinical trials as fallows.

- Define the Mode of Action (MOA) in non-clinical models achieved in human,

Table 1. Difference in between phase '0' and phase 1 clinical trials

Phase '0'	Variable	Phase 1
Less required	Preclinical toxicological study	Full IND required
Target/biomarker analytical assays validated in preclinical models	Pre-clinical target/biomarker validation studies	Not consistently performed
Establish a dose-range	Primary objective	Establish dose limiting toxicities
Limited dosing (e.g., 1-7 days) one cycle only	Limited dosing (e.g., 1-7 days) one cycle only	Repetitive; multiple cycles until disease progression or unacceptable toxicity
None	Evaluation for therapeutic benefit	Tumor response routinely evaluated
Required (pre- and post) to evaluate drug effect	Tumor biopsies	Almost always optional
Integrated into the trial to establish MOA and target/biomarker analytical assay validation in subject tissue samples	PD/target effect assays	Not consistently performed; commonly use assay methods that are not validated or standardized
SOP's validated first in *in vivo* preclinical models and applied to phase '0' human samples	SOP's for tissue acquisition, handling, and processing	Generally not validated or standardized
Performed in "real-time"	PK/PD analysis	Samples usually batched and analyzed at a later time point, generally after completion of the trial

IND: Investigational New Drug, MOA: Mode of Action, PD: Pharmacodynamics, SOP: Standard operating procedure, PK: Pharmacokinetics

• Define a biomarker assay by using human tumor tissue,

• Develop the novel imaging probe for evaluation of binding characteristics, bio-distribution and target effect,

• Evaluate PK and PD of drug and its analogue for selecting most promising candidate,

• Provide PK and PD data for further study i.e. phase 1 and further.

Advantages

Phase '0' clinical trials have several advantages before starting other phases of clinical trials. Vijayraghavan and Kumar[17] and Lappin and Garner[24] explained these advances as fallows.

• The time periods get reduced with the help of earlier testing of pharmacological action of new test candidate.

• It helps to select desirable promising targeting compounds for further extensive study before the conventional phase 1 trials.

• They help in overall acceleration in the process of drug development by focusing only the promising compounds.

• They avoid unnecessary exposure of the participants in the trial to the not so promising compounds.

• They possess less risk of human toxicity with low dose of the test candidate and less time duration for exposure. Moreover, a very limited number of subjects are involved. Also, such trials mostly involve a single dose administration as compared to a dose escalation study in the traditional phase 1 trials, there by further minimizing the risk.

• Pre-clinical safe site compared to conventional phase 1 clinical trials.

• Less number of animals is used.

• Overall time of drug development is reduced.

• This methodology helps in early selection of the smarter and more promising lead molecules hence, the more effective drugs reach to the market earlier.

• Small quantity of the test drug is required.

• All test drugs prepared according to principles and procedure of Good Laboratory Practices (GLP) as well as Good Manufacturing Practices for conventional phase 1 clinical trials.

• Any route of administration is possible.

• The drug canbe studied in sensitive patient like renal impairment, women in their reproductive age, cancer patients etc.

• This approach can help in studying the test drug for its modulator effects on the targets in a tumour.

• This approach is useful in the discovery of endogenous biomarkers for evaluating the quantitative effects of the test drug.

• The not so promising molecules can be eliminated earlier, thereby saving costs.

• They are helpful in obtaining the nearby therapeutic dose so determining the first dose for the further phase 1 study.

• The PK data can be obtained in only near about six month as compared to nearly 18 months in case of conventional phase 1 studies.

• They may help in selecting the best animal species for the long term toxicological studies based on the inference drawn from the micro-dose metabolite profiling data.

Disadvantages

Vijayraghavan and Kumar[17] and Eliopoulos et al.[25], had explained disadvantages of clinical trials phase '0' as given below.

• There is lack of any therapeutic as well as diagnostic intention.

• It may be difficult to motivate the volunteers to become a part of the trial because no therapeutic intent.

• Participation in the phase '0' trials may reduce the overall load of the subjects who become a part of the conventional phase 1 trials having therapeutic intention.

• Very few validated biomarkers are available for predicting the anti-cancer activity.

• There is requirement of ultra-sensitive and high tech equipment's like AMS and PET which are scarcely available.

• Since the technique of micro-dosing is still in its infancy, before applying this methodology precaution needs to be exercised to the drugs showing complex/non-linear kinetics.

• Since certain drugs dissolve readily at low dose but exhibit limited solubility at higher doses, it may be difficult to predict the absorption characteristics at the micro-dose levels.

• Phase 1 still needs to be done hence phase '0' unnecessarily prolongs the process and inflates the expenditure.

Content of exploratory investigational new drug submissions

FDA in 2006 explained and discussed the different documents for the submission for clinical trials phase '0'. Some of the important documents list as fallows.[26,27]

Clinical information;

• Introductory statement and general investigational plan,

• Types of studies,

Chemistry, manufacturing, and controls information;

• General information for the candidate product,

• Analytical characterization of candidate product,

Safety program designs;

• Clinical studies of PK or imaging,

• Pharmacological proper dosing needed for clinical trials,

• Clinical studies of MOAs related to efficacy,

• GLP compliance.

Volunteers recruitment in phase '0' clinical trials

Volunteer's recruitment is important part in all phases of clinical trials. Anthony[10], Fabio et al.[28] and Lexchin[29], had explained the recruitment of volunteers in phase '0' clinical trials.

Potential barriers to patient *enrollment* in phase '0' clinical trials because:

- No therapeutic intent or chance of benefit,
- Tissue biopsies before and after treatment,
- Other clinical trials or therapies deletion and delay study.

But plus points are:

- Low risk,
- Avoid biopsies if possible,
- Washout period is shorter.

CONCLUSION

The FDA has undertaken and initiated various approaches to reduce time, money and other factors in early drug development on such products that may be successful in near future. The detail guidance issued by FDA entitled "Exploratory IND Studies" described exploratory approaches exist in USA that are consistent with regulatory requirements currently but that will enable sponsors to move ahead more efficiently for successful product development of initial promising future candidate with needed human subject protections.

The preclinical testing programs for phase '0' or exploratory IND studies may be less extensive compare to conventional IND studies. This is because for the approaches described under this guidance, which maintain the administration of doses of promising candidate with minimum risk to subject compare to conventional phase 1 clinical trials. In near future the micro-dose study will place a remarkable event in drug development for all first in human studies in clinical trials. It is ethical to expose human subjects unnecessarily to a pharmacological dose of potential drug that has poor PK/PD properties, whose development is terminated as a result, when the same information could have been obtained in a micro-dose study. The micro-dose approach used in the exploratory IND trial will make a contribution to smarter drug development by enabling early human data to be obtained. Drug selection as a result will become more human based and therefore more predictive.

Phase '0' clinical trials are advantageous for the investigation sponsor, volunteers and public. Due to phase '0' clinical trials increased the chances of investigational drug into the market and reduce the unnecessary time, cost and volunteers in new drug development process by eliminating the unnecessary drug candidate prior to the phase 1.

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REVIEW ARTICLE

Flavin Containing Monooxygenases and Metabolism of Xenobiotics

Flavin İçeren Monooksijenazlar ve Ksenobiyotiklerin Metabolizması

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ABSTRACT

This review summarizes recent information concerning the pharmacological and toxicological significance of the flavin-containing monooxygenases (FMOs). FMOs are a family of microsomal enzymes involving in the oxygenation of certain xenobiotics and drugs containing nucleophilic heteroatoms. The activities of FMOs in drug metabolism and their relationships with diseases are the areas of research requiring further exploration. Future studies on FMOs may provide considerable information about the pathophysiology of diseases and the information related to this enzyme family may be important for drug designs in future.

Key words: Flavin monooxygenase, microsomal enzymes, phase 1 reactions

ÖZ

Bu derlemede flavin-içeren monooksijenazların (FMO) farmakolojik ve toksikolojik önemi ile ilgili güncel bilgiler özetlenmiştir. FMO'lar nükleofilik hetereatom içeren ksenobiyotiklerin ve ilaçların oksidasyonuna katılan mikrozomal bir enzim ailesidir. FMO'ların ilaç metabolizmasındaki etkinlikleri ve hastalıklarla olan ilişkileri, daha fazla araştırılması gereken alanlardır. FMO'lar üzerine yapılacak olan araştırmalar hastalıkların patofizyolojisi hakkında önemli bilgiler sağlayabilir ve bu enzim ailesi ile ilgili bilgiler gelecekte, ilaç tasarımları için önemli olabilir.

Anahtar kelimeler: Flavin monooksijenazlar, mikrozomal enzimler, faz 1 reaksiyonları

INTRODUCTION

All organisms are exposed unavoidably to chemicals (or xenobiotics such as drugs, pesticides, industrial chemicals, pollutants) with increasing industrialization and changing living conditions. Water-soluble of these chemicals are eliminated via the kidneys directly, whereas some chemical compounds are not suitable for renal excretion and lipophilic chemicals are converted to more hydrophilic forms by biotransformation. Increasing hydrophilicity of chemicals makes their excretion more efficient. Enzymes in catalyzing biotransformation reactions are generally divided into two groups, called phase 1 and phase 2. Cytochrome P450 (CYP450) and flavin-containing monooxygenases (FMOs) are the most important enzymes of phase 1 reactions.[1]

FMO enzymes are believed to protect organisms against xenobiotics in early environment such as CYP450 enzyme family.[2] Mammalian FMO enzymes from pig liver microsomes were first described in the 1970s by Ziegler and Poulsen.[3] Biochemical properties of FMOs were described largely by Ziegler and Poulsen.[3] As a result, for some time, this enzyme was known as Ziegler's enzyme. In the 1980s, pulmonary and hepatic forms of FMOs were exhibited distinct differences with regard to enzyme properties and substrate specificity.[4] Researchers have been knowledged about the structural, functional and regulatory aspects of FMOs since 1990.[5] Despite this progress, knowledge about FMOs is still insufficient.

Catalytic Mechanism of flavin-containing monooxygenases

FMO is a flavoprotein containing a single flavin adenine dinucleotide (FAD), as a prosthetic group. The microsomal nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen-dependent FMOs catalyze the oxidative metabolism of a wide variety of xenobiotics, including nucleophilic nitrogen-, sulfur-, phosphorous-, and selenium-heteroatoms. FMOs displayed activity toward a broad range of substrates consisting of phosphines, hydrazines, sulfides, selenides, iodide, boron-containing compounds and primary,

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secondary, and tertiary amines.[6] FMOs are responsible for about 6% of all the phase 1 reactions, and therefore, about 2.5% of all metabolic reactions.[7,8] These enzymes provide an efficient detoxification system for xenobiotics, by converting the lipophilic xenobiotics into polar, oxygenated, and readily excreted metabolites.[6,9]

FMOs catalyze the oxidative metabolism clinically important drug, including morphine, cocaine, nicotine, tamoxifen, fluoxetine, methimazole, chlorpromazine, imipramine, benzydamin, itoprid and clozapine; environmental toxins, including phorate, fonofos, aldicarb; endogenous substrates, including triamine, trimethylamine, catecholamines, cysteamine and methionine.[10-13]

FMO catalytic activity is quite different from the catalytic activity of CYP450 enzymes. Unlike CYPs, C (4a) hydroxy-peroxide, which is a stable intermadiate, is produced during the catalytic cycle of FMOs. This intermediate product can remain stable for hours at 4˚C and can be observed as spectral. This product is considered to be the form in which FMO exists in the cell.[10,13] The catalytic cycle of FMO are shown in Figure 1. In step 1 of FMO catalytic cycle, NADPH cofactor binds to the enzyme and FAD is reduced by two electrons from NADPH. In step 2, the reduced FAD reacts rapidly with molecular oxygen and formation of flavin hydroxy-peroxide. Subsequently, the nucleophilic substrate is coupled with this intermadiate products. One oxygen atom of molecular oxygen which reacts in the second step binds to substrate and the oxidized form of the substrate leaves the cycle. The other oxygen atom incorporated into H_2O-FMO is a monooxygenase. In the final step of FMO catalytic cycle, followed by a slow reaction, $NADP^+$ is released from the enzyme. Disintegrated reaction of falcarindiol and released reaction of $NADP^+$ are thought to be the rate-limiting steps in the catalytic cycle of FMOs. In either case, substrate binding has no effect on Vmax.[13]

Comparison of flavin-containing monooxygenases and CYP450

These two enzyme families which take part in the oxidization stages of phase 1 reactions resemble each other with respect to needing NADPH and molecular oxygen for catalytic activities, adding oxygen to the substrate during the reactions, having similar molecular weights, being localized in the endoplasmic reticulum in the cell, having the highest concentration in the liver and substantial amount in the entry and excretion organs for xenobiotics in the body such as lungs, skin, stomach, intestines and kidneys, and having the similar chemicals as substrates.[13]

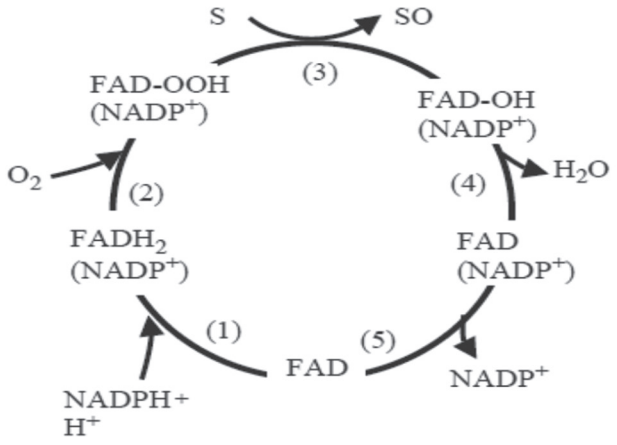

Figure 1. Catalytic cycle of flavin-containing monooxygenases[13]
NADPH: Nicotinamide adenine dinucleotide phosphate, FAD: Flavin adenine dinucleotide

In contrast to CYP450 gene family, FMO genes are expressed with less number of genes.[14] While most of the CYP450 enzymes take part in synthesis and catabolism reactions, the synthesis and catabolism reactions of FMOs are not clear except for the xenobiotic metabolisms. Catalytic cycles of FMOs and CYPs are significantly different from each other. For example, there is no real binding between the FMO and the substrate during the catalytic cycle of the FMO.[13] Despite using the same substrates, these enzymes usually create different products. In general, FMO enzymes produce less toxic metabolites compared to the CYP enzymes.[15] There is a specific inhibitor for almost each CYP450 enzyme, but there are no identified mechanism based inhibitors for FMOs.[16] FMO enzymes are more vulnerable to heat than CYPs and they are active in higher optimal pHs.[17] Unlike CYPs, FMOs are not easily induced with xenobiotics and do not become inhibited.[15] Despite not all, but for most drugs and xenobiotics, FMOs most probably have a secondary role compared to CYPs. While it is known that the number of FMOs participating in the drug metabolism is less than that of CYPs, the contribution of FMOs to the metabolism of drugs and other xenobiotics is still not clearly described.[13]

Nomenclature

It is proposed that the emergence of FMO gene family is due to a sequence of independent gene duplication. Human FMO genes are located in two clusters in chromosome 1. The first cluster is on the long branch of the chromosome 1 (q23-25) and the second cluster lies on the other region of the chromosome 1; however this second region contains pseudogenes. Up to now, a total of 5 FMOs, all of which are active, have been identified in the mammals.[18] FMOs with sequence similarities of more than 40% are classified inside the same family such as FMO1, FMO2 etc.[19] The sequence similarity for the functional human FMO forms (FMO1-FMO5) is in the 52-60% range. As an exceptional case, there is a sequence similarity of 71% between FMO3 and FMO6.[20,21] The translation products of FMO genes have a length of 532-558 amino acids. These products contain highly protected FAD and NADPH regions.[22] FMOs, which have been named as flavin-mixed functional monooxygenases or Ziegler's enzyme, are now named in accordance with their protein structures and FMO prefix is used in order to indicate the genes.

Localization and expression of flavin-containing monooxygenases

Among the FMO genes identified for bacteria to humans, the research is mostly concentrated on those for mammals.[23] The presence of FMO has been shown in most species and tissues mainly in liver, lung, kidney, intestine and brain tissue; and the studies are focused specifically on the liver and kidney where

xenobiotics are metabolized. In recent years, brain tissue has become prominent in FMO research. The presence of FMO has been detected in the human[24], monkey[25], rat[26], and rabbit[27] brains; and although low activity is reported for the brain FMOs, it has been pointed out that for specific substrates and regional concentrations, they have an important effect in the metabolism of xenobiotics.[28] Moreover, despite FMOs existing only in microsomal fractions in the liver, they are also found in the other intracellular fractions in the brain, besides microsomes.[29] The tissue specific expressions of FMOs are quite diverse between species. Hence studies on the rat brain FMOs are not easily compatible for the understanding of the human brain FMO enzyme activities and expressions.[22]

In some tissues, the FMO concentration is very high. For example, FMOs constitute more than 10% of the total microsomal proteins in the rabbit lungs. Considering the effect of FMOs on the metabolism, this high concentration is essential in terms of toxicological evaluations.[30]

FMO1 is the most common FMO in adult human kidney. Fetal liver and small intestine expression of FMO1 is approximately by 10-14 times less than the FMO1 expression in the adult kidney. The lung FMO1 expression is approximately 2.8% of the expression of FMO1 in kidney. The FMO1 expression in the brain is less than 1% of FMO1 in kidney, and there is suppression for the FMO1 expression in the brain after birth.

FMO2 is the dominant FMO type in the lungs and the FMO2 expression in the kidney is around 7 times less than that of the lung. Liver FMO2 expression is approximately 2% of the expression of FMO2 in lung. The FMO2 expression in the brain is less than 1% of that of the lung, and the amount of FMO2 in the brain is approximately the same for all age groups.

FMO3 is the most important FMO type in the liver and the level of FMO3 in the lungs, kidney, fetal liver and small intestines are 4.5%, 3.7%, 2.1% and 1% of the FMO3 level in the liver, respectively. The FMO3 expression in the brain is less than 1% of FMO3 in liver and does not change with age.

FMO4 is most commonly present in the liver and kidneys, and the FMO4 expression in the fetal liver, small intestine and lungs are approximately 10.9%, 10.8% and 7% of the liver FMO4 expression, respectively. The FMO4 expression in the brain is less than 1% of that of the liver and does not change with age.

FMO5 is highly abundant in the adult liver and the amount of FMO5 in the fetal liver, small intestine, kidney and lung is 18.8%, 12.8%, 9.8% and 4% of the amount of FMO5 in the liver, respectively. The amount of FMO5 in the brain is less than 1% of the liver FMO3 amount and does not change with age.[22] Contrary to the previous studies on the human FMO, it has been reported that the hepatic FMO5 expression is equal to or larger than FMO3 expression, and it is the dominant form of FMO in the adult liver.[31]

Factors affecting flavin-containing monooxygenases

The results obtained from animal modeling and human studies indicate that FMO can be regulated by physiological factors such as age, gender, pregnancy, hormones, diet.[32-35] Aside from physiological factors, co-factor supply and external factors that can affect the enzyme also have roles in the regulation of FMO enzymes.

Age

In populations, the expressions of specific genes change with the age and this in turn makes the understanding of the impact of genes on aging and illnesses more essential. On the other hand, it can be shown that a gene which increases in abundance as the population ages can be protective over the metabolism. For example, it has been shown that among FMO types, human brain FMO1 genes have a different profile.[36] In a study where the expressions of different FMO expression were investigated and the sample was divided into five groups based on age, it has been reported that the brain FMO1 expression is lower than that of other tissues, that there is a significant difference observed between the prenatal 17-21 week old samples and other three postnatal samples (0-8 months, 10-39 years, 40-70 years old), and that the expression of FMO1 in the brain is suppressed after birth.[37] However, no significant difference has been encountered before and after birth for the cases of other FMO forms aside from FMO1 (FMO2, FMO3, FMO4 and FMO5).

Gender

The effect of gender on the human FMO function has not been clearly identified. In some of the studies conducted, this factor has been investigated, however with the existing knowledge it does not seem possible to determine the impact of this factor on the FMO function. In a study based on the own accounts of sample individuals, it has been reported that trimethylaminuria caused by FMO3 enzyme deficiency is observed more in woman individuals.[38] In the study, it has been additionally reported that the reason for this case is the emergence of a temporary trimethylaminuria during menstruation in some women, although not in all women, induced by functional changes in FMO3.[39]

Diet

The gastrointestinal system naturally functions with the direct intake of food. If nutrition happens through direct infusion to the systemic circulation, some pathophysi-ologic changes occur due to bypassing of processes (e.g. intestinal absorption, etc.) in the system. Enzymes that are active in the xenobiotic metabolism are also affected by these changes. A study shows that in the rats that have been fed intravenously show 25% decrease in CYP450 content and 40-55% decrease in the CYP functional activity compared to the rats that have been fed orally.[40] In another study, it has been revealed that the FMO4 immunoreactivity is increased in rats that have been fed intravenously.[34]

It is known that FMOs, which are one of the enzymes active in xenobiotics metabolism, in general can be affected by diet, and with more research data on the subject, the effect of diet can be more clearly understood.

The relationship of flavin-containing monooxygenases and diseases

FMO enzymes are highly polymorphic, and this aspect is important on the metabolic events that the enzymes are active in. Polymorphisms in the FMOs can change the enzyme activity. Therefore, the FMOs can be associated with diseases via metabolism of chemicals. The primary disease associated directly with FMO is "Fish Odor Syndrome" (trimethylaminuria). Trimethylamine smells like rotten fish, and it is excreted after being transformed to an odorless *N*-oxide form in the human liver by FMO3. Some polymorphisms in the FMO3 gene causes functional loss and malfunctions occur in the trimethylamine metabolism. In patients with this syndrome, especially sweat, urine and breath have the rotten fish odor.[13,38] There is yet no cure for the syndrome; however, the strength of the odor can be reduced by avoiding certain types of food such as fish, eggs, etc. and with antibiotics treatment.

Another disease associated with FMOs is hypertension. Especially the results of studies conducted on fish indicate that FMO can have a role in regulating the blood pressure by participating in the metabolism of organic osmolites or substances that take part in regulating the blood pressure.[38]

In the studies where it is argued that there is a correlation between FMO enzymes and diabetes, it has been shown that the hepatic FMO activity in animal models increases in insulin dependent and non-insulin dependent diabetes. In this respect, it has also been reported that increase in FMO activity can be observed in obese or overweight individuals.[41,42]

FMO enzymes are also associated with other diseases such as hemochromatosis,[43] gastrointestinal system diseases and colon cancer;[44] and it has been reported that there is a significant relation between smoking and FMO enzymes.[22,45]

Role of flavin-containing monooxygenases in drug design

The effect of FMOs on the metabolism of drugs and other xenobiotics are still not clearly defined. Especially in the phase 1 reactions in drug metabolism, CYP450 enzymes have a significant dominance.

The number of FMO mediated drug metabolisms is far less than CYPs, and these enzymes are less stable than CYPs in the lack of NADPH and at high temperatures. Despite these disadvantages, the fact that FMO is not easily induced and inhibited by environmental chemicals may be essential in terms of drug reactions. On the other hand, the number of adverse drug-drug interactions can be reduced by increasing the contribution of FMO in the drug metabolism. By adding functional groups that are oxidized by FMO to the produced drugs can both decrease the dependency on CYP450 metabolism and reduce the number of adverse drug-drug interactions. Furthermore, while there are specific inhibitors for each CYP450 enzyme, there are only limited number of inhibitors for FMOs, which can pose as an advantage in drug manufacturing.

Oxidation metabolism of a specific drug depends on the enzyme activity and relatively on the amount in the tissue or organ in which the metabolism takes place. Moreover, even if the activities of CYP2D6 and FMO3 enzymes against a drug are the same, taking the amounts in the liver into consideration, the contribution of FMO3 in the metabolism of that drug is considered to be higher. However, under *in vivo* conditions, it is extremely difficult to determine precisely the contribution of the relative contribution of FMO to the metabolism of drug.[13]

CONCLUSION

In recent years, the number of studies on the metabolism of xenobiotics has substantially increased. By revealing the unknown properties of FMO, one of the phase 1 enzymes, more information about the biotransformation of xenobiotics can be obtained. In addition, when compared to CYP450 in terms of drug design, FMO enzymes may provide substantial advantage since they are not easily affected by chemicals, and there are low number of adverse drug-drug interactions due to their genetic polymorphisms and inhibitions.

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