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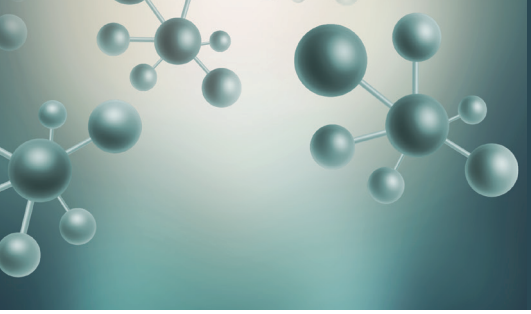

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PRISMA statement of preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 2009; 6(7): e1000097.) (http://www.prisma-statement.org/);

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Results: The detailed results of the study should be given and the statistical significance level should be indicated.

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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CONTENTS

Turk J Pharm Sci 2023;20(5):285-293
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ORIGINAL ARTICLE

Development of the Forskolin Microemulsion Formula and its Irritation Test on Rabbits

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ABSTRACT

Objectives: This study aimed to develop a microemulsion formula that can increase the solubility and stability of forskolin and its safety for topical use.

Materials and Methods: The materials used for the development of the microemulsion formula were triglyceride oil, non-ionic surfactants, and polyethylene glycol (PEG) for cosurfactants, which were selected on the basis of the results of the forskolin solubility test using high performance liquid chromatography (HPLC). The microemulsion was formulated by the phase titration method. Formula stability was determined by storage for 90 days in a refrigerator at room temperature, and an accelerated stability test was performed by determining globule size, forskolin concentration, and pH. The safety of using microemulsions was determined by skin irritation tests on albino rabbits.

Results: The optimum microemulsion formula consisted of Maisine® CC, polyoxyethylene sorbitan 20 (POE 20), and PEG 400 with a ratio of 4:25:5 *w/v*, which increased the solubility of forskolin the most, namely 2.19 mg mL^{-1}. Based on globule size (<50 nm), forskolin concentration (2 $mg.mL^{-1}$), and pH (6.0-6.35), the formula was stable in refrigerator storage and room temperature but unstable in the accelerated stability test (40 °C) starting on day 21. This optimum formula exhibits a primary irritation index (PII) of 0.11, which is categorized as feeble irritation and can be ignored.

Conclusion: The microemulsion prepared by the phase titration method containing Maisine® CC, POE 20, and PEG 400 (4:25:5, *w/v*) as a base and 0.2% forskolin was stable in refrigerator storage and at room temperature. This microemulsion is mild or negligible irritant with a PII: 0.11.

Key words: Microemulsion, forskolin, skin irritation test

INTRODUCTION

A microemulsion is a colloidal dispersion composed of water, oil, and a mixture of cosurfactants (Smix) that spontaneously occurs.[1] Microemulsion is a delivery system that can function as a solubility enhancer,[2,3] penetration,[4-7] stability, and diffusion and can dissolve lipophilic and hydrophilic active pharmaceutical ingredients (API).[8]

Each microemulsion component has a specific function. Oil can increase the hydrophilic API partition coefficient in the skin and change the conformation of lipids in the *stratum corneum*.[9] In contrast, surfactants can reduce surface tension, increase API solubility, and increase skin penetration.[10] The cosurfactant increases the density and membrane permeability of the microemulsion globule monolayer, which is formed together with the surfactant, so that the monolater membrane does not leak easily.

Forskolin is a secondary metabolite found in the roots of *Coleus forskohlii* Briq. This plant is often found in India, Nepal, Burma, and Thailand.[11] One of the pharmacological effects of forskolin is its lipolytic activity, where forskolin can activate the adenylate cyclase enzyme in adipose cells to stimulate the formation of cyclic adenosine monophosphate (cAMP). *In vitro*, forskolin can induce lipolysis by increasing the concentration of cAMP and forming protein kinase A.[12-14] Organoleptically; forskolin is in the form of a white powder with a molecular weight of 410.5

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grams.mol^{-1}.[15] The molecular structure of forskolin is shown in Figure 1. Forskolin is insoluble in water with log p = 3.89[15] but undergoes hydrolysis and isomerization at temperatures above 50 °C.[16]

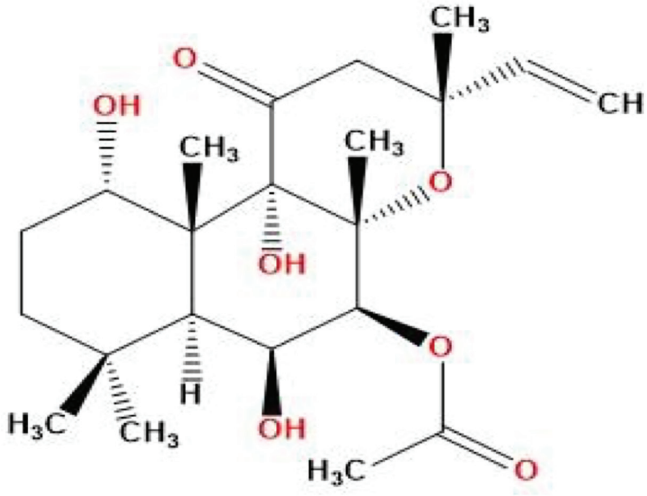

Figure 1. Molecular structure of forskolin

Forskolin is generally expressed in capsule dosage forms and is used orally as a slimming agent. The development of formulas in topical dosage forms can increase their usefulness. The microemulsion was chosen as a delivery system for forskolin because it can increase solubility by interacting with the microemulsion-forming material with forskolin. In addition, the microemulsion formulation uses low energy at room temperature, thereby avoiding the breakdown of forskolin, which is unstable at high temperatures.

Material selection can affect the success of microemulsion formulations. A high monoglyceride content in oil can increase the solubility of API with log p > 2.[17] Acharya et al.[2] found that a microemulsion containing triglyceride oil can increase the solubility of aceclovenac. In this study, a microemulsion was developed based on the length of the carbon chain of triglyceride oil. The oil used is Maisine® CC, which is a long-chain triglyceride oil of oleic and linoleic unsaturated fatty acids (C18:1, C18:2), and medium-chain triglyceride (MCT), which is a medium-chain triglyceride oil of caproic, caprylic, capric, and lauric saturated fatty acids (C6-C12).[18]

One of the undesirable effects of microemulsions on the skin is their irritating properties due to high concentrations of surfactants. Wang et al.[19] found that a rotigotine microemulsion containing 25.2% Smix did not irritate the skin. Soliman et al.[20] also found that celecoxib microemulsion containing 45.7% Smix did not irritate the skin.

Based on the description above, using Smix, which is quite high in microemulsions that can increase the solubility and stability of forskolin, is expected to produce a safe microemulsion formula.

MATERIALS AND METHODS

Materials

Forskolin was purchased from BOCSCI (USA), Maisine® CC oil was donated from Gattefosse (France), and MCT oil was purchased from Okusi Biotech Asia (Indonesia). The surfactants used were polyoxyethylene sorbitan 20 (POE 20) purchased from Croda (Singapore), polyoxyethylene sorbitan 80 (POE 80) purchased from Seffix (Singapore), and sorbitan monolaurate (Span 20) purchased from Sigma-Aldrich (USA). The cosurfactants used were polyethylene glycol (PEG) 400, purchased from Indokemika (Indonesia), and propylene glycol (PG), purchased from Dow Chemical Pacific (Singapore). Sodium hydroxide, potassium dihydrogen phosphate, ethanol absolute, and acetonitrile were purchased from Merck (Germany), and distilled water was purchased from IPHA Lab (Indonesia).

Methods

Solubility test

The solubility test was performed by gradually adding 5 mg of forskolin to 1 mL of each microemulsion-forming material (oil, surfactant, and cosurfactant) until saturated. The sample was stirred using an orbital shaker for 48 h at 25 °C at 100 rpm. Then, it was centrifuged at 6.000 rpm for 10 min and filtered through a 0.45 µm membrane (Sartorius).[21]

Forskolin concentration in the sample was determined by diluting the sample 20 times with ethanol. Then, it was injected into high performance liquid chromatography (HPLC) (Waters 2.487) system with column C18 (Cap Cell) and eluent water:acetonitrile (35:65).[15] The chromatogram was detected at a maximum λ of 210 nm with a retention time of 6.5 min.

Pseudoternary phase diagram construction

The pseudoternary phase diagrams were prepared at room temperature by mixing oil and Smix in a ratio of 1:9 to 9:1, then dripping water to form a clear microemulsion. Smix comparisons were made with surfactant: cosurfactant ratios of 1:1, 2:1, and 3:1. The pseudoternary diagram was created using the Ternary Diagram ProSim SA application version 1.0.3.0.

Microemulsion formulation and characterization

Microemulsions are prepared by mixing forskolin in oil and Smix. The aqueous phase was added slowly, while stirring with a magnetic stirrer at 300 rpm for 10 min at room temperature. The resulting microemulsion was characterized for its clarity by measuring light transmittance (T%) with a spectrophotometer (Beckman Coulter DU® 720 ultraviolet/visible) at a maximum λ of 630 nm,[22,23] globule size and homogeneity using a particle analyzer (Beckman Coulter DelsaTM Nano C), pH formula with a pH meter (SevenEasy Mettler Toledo), zeta potential using a zeta analyzer (Beckman Coulter DelsaTM Nano C), and phase separation test using the centrifugation method (Hettich EBA 200) at 3500 rpm for 30 minutes.[24]

Optimization of the forskolin concentration in microemulsions

Optimization of forskolin concentration in microemulsions was determined by mixing forskolin in the range of 1-5 mg.mL^{-1}

into the formula. The dissolved forskolin concentrations were determined by HPLC.

Characterization of the shape and size of microemulsion globules

The shape and size of the microemulsion globules were determined by transmission electron microscopy (TEM). Microemulsion was dropped on the preparation, then covered with a 400-mesh grid, and left for approximately 1 min to absorb. Next, uranyl acetate was dropped on the grid and left for 30 min to dry and read with TEM.

Microemulsion spectrum characterization

The spectrum of the microemulsion formula and the interactions between forskolin and the ingredients in the microemulsion formula were analyzed by fourier transform infrared-attenuated total reflectance (FTIR-ATR) (Agilent Technologies).

Microemulsion stability test

The stability test for liquid preparations refers to the International Council for Harmonisation (ICH) guidelines,[25] where the samples were stored in the refrigerator (5 ± 3 ˚C), room temperature [30 ± 2 ˚C and 65 ± 5% relative humidity (RH)], and in the climatic chamber (40 ± 2 ˚C and 75 ± 5% RH) (Hotpack model 317322, USA) for accelerated stability testing. The stability of the samples was tested at storage periods of 1, 7, 14, 21, 30, 60, and 90 days by measuring the globule size, forskolin concentration, and pH of the formula.

Irritation test

The safety of using microemulsion topically was determined through an *in vivo* skin irritation test on albino rabbits. The irritation test was approved by the Ethics Committee for the Use of Experimental Animals (KEPHP) of the Bandung Institute of Technology (no: 02/KEPHP/-ITB/10-2021.)

The irritation test refers to the guidelines for non-clinical toxicity tests *in vivo*.[26] The irritation test was performed on three New Zealand strain male albino rabbits. The animal's back was shaved with an area of about 10-15 cm^2, then divided into four locations with a size of 2 x 3 cm^2 each. At each location, 0.5 g of sample consist of microemulsion formula, microemulsion base, and 2 sites were applied as a control, which was not treated. The layout of the location of the formula application is given in Figure 2. Then, the application site is covered with gauze and covered with a bandage for 24 h, after which the gauze is removed. The appearance of erythema and edema was observed at 24, 48, and 72 h after the gauze was removed. The severity of the effect was scored between 0 and 4, with a score of 0: no erythema, 1: very little erythema, 2: well-defined erythema, 3: moderate erythema, and 4: severe erythema, as well as for edema.

The primary irritation index (PII) is calculated using the following formula:

PII = (A-B)/C..... (equation 1)

where A: sum of erythema and edema scores at all locations given the test microemulsion at observations at 24, 48, and 72 h divided by the number of observations, B: sum of erythema and edema scores for all control locations at 24, 48, and 72 h divided by the number of observations, and C: number of animals.

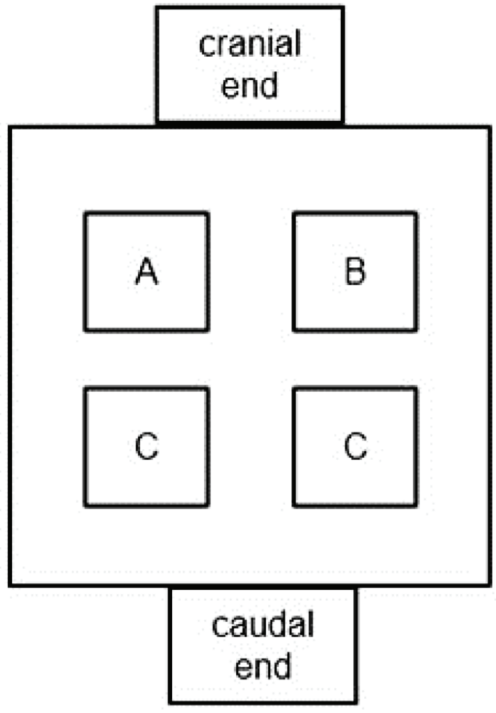

Figure 2. Sites of formula application in irritation test
A: Microemulsion, B: Microemulsion base, C: Untreated control

The level of irritation is determined on the basis of PII, that is, if PII: 0.0-0.4, the test sample is categorized as very mild irritation (negligible); 0.5-1.9 is a mild irritant (slightly); 2.0-4.9 is a moderate irritant, and 5.0-8.0 is a strong irritant (severe).

In this study, statistical data were not used.

RESULTS AND DISCUSSION

Solubility test

The solubility test showed that forskolin is dissolved easily in materials containing polyoxyethylene groups, as shown in Table 1. This group is found in surfactant and cosurfactant molecules. Based on its molecular structure, forskolin has one acetate ester, a cyclic ketone, and a tertiary alpha hydroxyl group, as well as three heterocyclic rings and a hydroxyl group.[27] The presence of ketone and ester groups on forskolin molecules tends to form hydrogen bonds with polyoxyethylene groups on surfactant and cosurfactant molecules.

The solubility of forskolin in oil displays that forskolin is more soluble in Maisine® CC than MCT. Maisine® CC is a triglyceride oil with long-chain fatty acids derived from oleic and linoleic acids (C18:1, C18:2). Meanwhile, MCT is a triglyceride oil consisting of medium chain saturated fatty acids (C6-C12). Based on the results of this solubility test, the interaction between forskolin and oil probably occurs in the fatty acid chains of the oil and forskolin molecules through van der Waals interactions.[28]

The solubility test of forskolin in surfactants showed that forskolin was more soluble in POE 80 (22.97 mg/mL) than

Table 1. Forskolin solubility in materials for microemulsion formulations

Materials	Forskolin solubility, $mg.mL^{-1}$ ± SD
MCT	3.24 ± 0.06
Maisine® CC	5.23 ± 0.22
POE 20	20.88 ± 0.73
POE 80	22.97 ± 0.19
Span 20	2.11 ± 0.04
PG	4.67 ± 0.10
PEG 400	23.08 ± 0.10

n: 3, SD: Standard deviation, MCT: Medium chain triglyceride, POE: Polyoxyethylene sorbitan, PG: Propylene glycol, PEG: Polyethylene glycol, Span: Sorbitan monolaurate

in POE 20 (20.88 mg/mL). However, in preliminary tests of formulation, it was found that POE 20 could mix with 4% Maisine® CC, whereas POE 80 could only mix with 2% Maisine® CC at the same surfactant concentration. This may be related to the fatty acid groups of the oil and surfactants.

The solubility test of forskolin in cosurfactant showed that forskolin was more soluble in PEG 400 (23.08 mg/mL) than in PG (4.67 mg/mL). PEG 400 consists of 8 ethylene molecules and one primary hydroxyl group, whereas PG is propane-1,2-diol with two hydroxyl groups in each molecule.[29] The presence of 8 ethylene molecules in PEG 400 allows it to form more hydrogen bonds to interact with forskolin molecules compared with PG. PEG 400 log P value is -4.8, whereas that of PG is -1.34.[30] This shows that PEG 400 is more hydrophilic than PG; therefore, it is easier to form hydrogen bonds in *o/w* microemulsions.

Based on the solubility test data and the preliminary formulation test, the microemulsion components used for the formulation were MCT, Maisine® CC, POE 20, and PEG 400.

Pseudoternary phase diagram construction

Pseudoternary phase diagrams were prepared to compare the microemulsion area formed between microemulsion-MCT (FMEA) and microemulsion-Maisine® CC (FMEB) with a ratio of Smix POE 20-PEG 400 at 1:1, 2:1, and 3:1 (*w/w*). Figure 3 illustrates that the Smix ratio shows no difference between the two types of microemulsions. However, there are differences between FMEA and FMEB. The figure shows that the interaction between MCT and Smix tends to form *w/o* microemulsions, whereas the interactions between Maisine® CC and Smix tend to form *o/w* microemulsions.

Microemulsion formulation

Microemulsions are colloidal dispersions similar to other drug delivery systems, which contain high concentrations of surfactants. Table 2a shows that FMEA1 was formed by MCT:POE 20:PEG 400 (2:27.5:10 *w/v*), whereas the FMEB1 formula was formed by Maisine® CC:POE 20:PEG 400 (4:25:5 *w/v*). Increasing the amount of oil in each formula increases

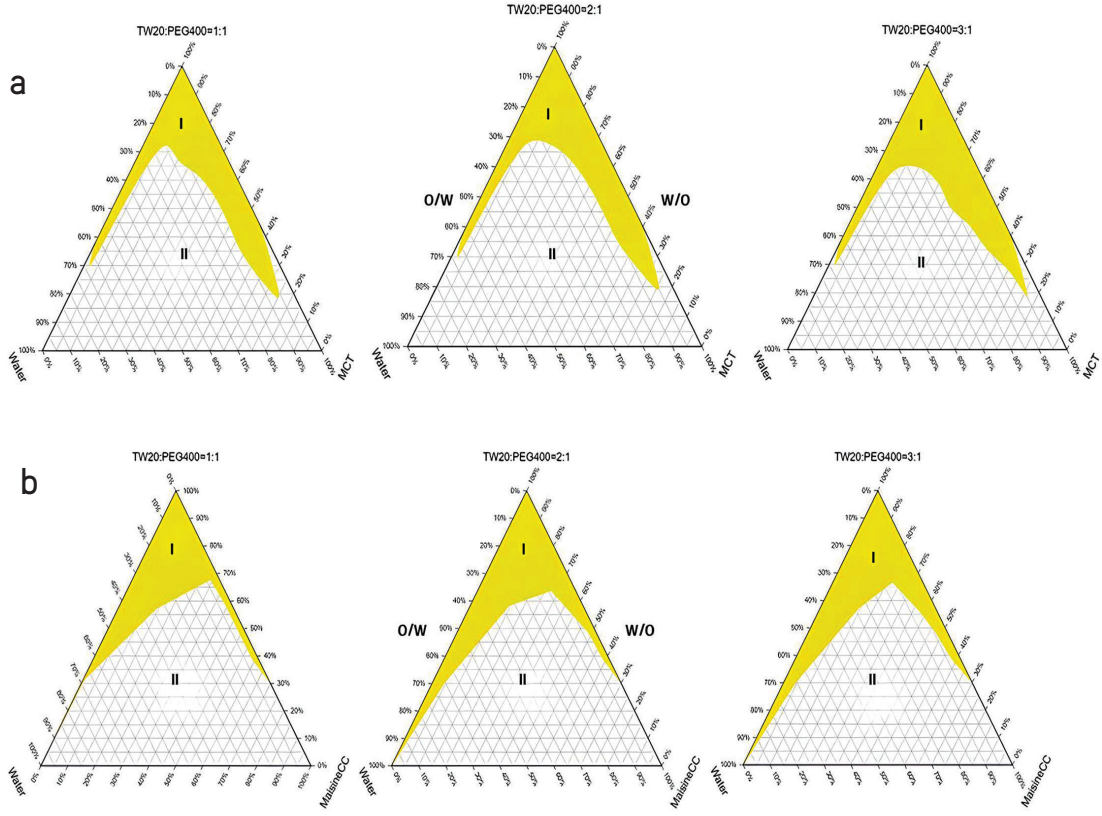

Figure 3. Microemulsion pseudoternary phase diagrams. (a) Pseudoternary phase diagrams with MCT oil, (b) pseudoternary phase diagrams with Maisine®, I: microemulsion area, II: emulsion area

turbidity and globule size and decreases the percentage transmission (T%) value.

In the FMEA1 formula, the interaction between POE 20 and MCT is unstable, which is presumably because the lipophilic part of POE 20 is difficult to interact with MCT, which is oil from saturated fatty acids (C6-C12). The interaction between POE 20 and Maisine® CC on FMEB1 shows that the system can form stable microemulsions. POE 20 tends to interact more easily with Maisine® CC oil, which has unsaturated fatty acid groups from oleic and linoleic acids, which can form *o/w* microemulsions. Hathout et al.[9] found that POE 20 can interact with oleic acid to form an *o/w* system in a testosterone microemulsion. Oleic acid and Maisine® CC are both unsaturated fatty acid (C18) oils.

In Table 2b, FMEB1, which comprises Maisine® CC:POE 20:PEG 400 at 4:25:5 *w/v*, is the optimum formula. This formula gives a T% value of 100.67%, which shows that the formula can transmit all light, giving a clear appearance. Other formulas provide a T% value of less than 90%. T% value can be used as a reference when selecting the microemulsion formula. The level of clarity of the formula is also related to the size of the globules. The FMEB1 formula has a globule size of approximately 28 nm and homogeneity value of approximately 0.3.

The zeta potential values of all formulations range from -1.40 *mV* to -1.74 *mV*, indicating that the formulas are non-ionic. The centrifugation test showed that only the FMEB1 formula was homogeneous, while the others were separated. Based on the test results, FMEB1 can be used in the next stage of testing.

Optimization of forskolin concentrations in microemulsions

Optimization of forskolin solubility in the FMEB1 formula is shown in Table 3, where FMEB1 can dissolve forskolin by 2.19 ± 0.05 mg/mL. The addition of forskolin concentration starting at 3 mg mL^{1} gave the appearance of cloudy microemulsions, large globule size and precipitate. This indicates that FMEB1 has been saturated; therefore, the next concentration of forskolin used is 2 $mg.mL^{-1}$ or 0.2%.

Characterization of the shape and size of microemulsion globules

TEM results (Figure 4) indicated globule-shaped droplets of FMEB1 with a size of approximately 30-50 nm.

Table 2a. Composition of microemulsion formulations

Materials, *w/v* %	Formulations			
	FMEA1	FMEA2	FMEB1	FMEB2
Forskolin	0.2	0.2	0.2	0.2
MCT	2	3	-	-
Maisine® CC	-	-	4	5
POE 20	27.5	27.5	25	25
PEG 400	10	10	5	5
Water	60.5	59.5	66	65

FMEA: Forskolin microemulsion with MCT oil, FMEB: Forskolin microemulsion with Maisine® CC oil, *w/v*: Weight/volume

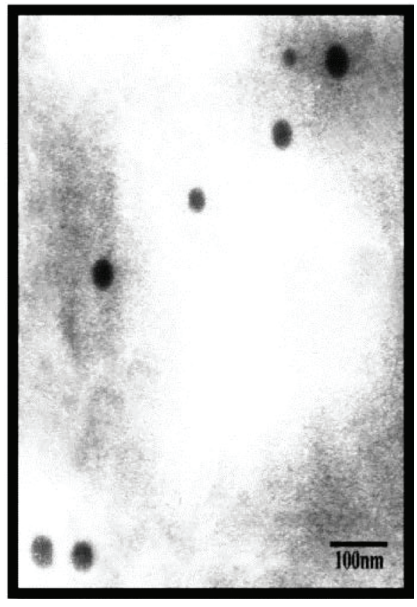

Figure 4. TEM results from FMEB1 at 40.000x magnification

TEM: Transmission electron microscopy, FMEB: Optimum microemulsion formula

Table 2b. Characteristics of microemulsion formulations

Test	Formulations			
	FMEA1	FMEA2	FMEB1	FMEB2
Appearance	Bluish	Cloudy	Transparent	Bluish
T, % ± SD	63.9 ± 0.15	nd	100.67 ± 0.26	85.27 ± 0.66
Globules size, nm ± SD	76.67 ± 5.82	nd	28.80 ± 2.85	74.11 ± 6.00
PI ± SD	0.30 ± 0.05	nd	0.31 ± 0.17	0.40 ± 0.02
pH ± SD	6.34 ± 0.02	nd	6.23 ± 0.06	6.25 ± 0.01
Zeta potential, mV ± SD	-1.40 ± 1.22	nd	-1.73 ± 1.87	-1.74 ± 0.76
Centrifuge	Separation	nd	Homogenous	Separation

FMEA: Forskolin microemulsion with MCT oil, FMEB: Forskolin microemulsion with Maisine® CC oil, T: Transmittance, PI: Polydispersity index, nd: No detected, SD: Standard deviation

Microemulsion spectrum characterization

The FTIR spectra of FMEA1 and FMEB1 microemulsions are shown in Figure 5a, and the spectra of the microemulsion ingredients and forskolin interactions in FMEB1 are shown in Figure 5b.

As shown in Figure 5a, there is no significant difference between the FMEA1 and FMEB1 spectra. These data indicate the length of the fatty acid chains in the triglyceride oils used in FMEA1 and FMEB1 does not show any difference in FTIR spectrum.

Figure 5b shows the same spectrum of FMEB1 and MEB at 3,768 cm^{-1}: OH stretching of a water molecule; 1,638 cm^{-1}: stretching of the C=O functional group due to the conjugation effect of the aldehyde group on Maisine® CC, POE 20, and forskolin; 1,082 cm^{-1}: stretching of the C-O group due to the interaction between POE 20 and PEG 400.[31] From the spectrum of that figure, there are no new bands indicating a strong interaction between molecules or it can be concluded that the interactions that occur in the microemulsion system are weak interactions such as van der Waals forces or hydrogen bonds.

Microemulsion stability test

The size of FMEB1 globules is shown in Table 3. The sizes of the FMEB1 globules stored in the refrigerator (16-17 nm) and RT (17-27 nm) are still within the microemulsion requirements of < 50 nm.[32] In contrast, in the accelerated test, there was an increase in globule size from the 21st day at the end of the test with (115-942 nm).

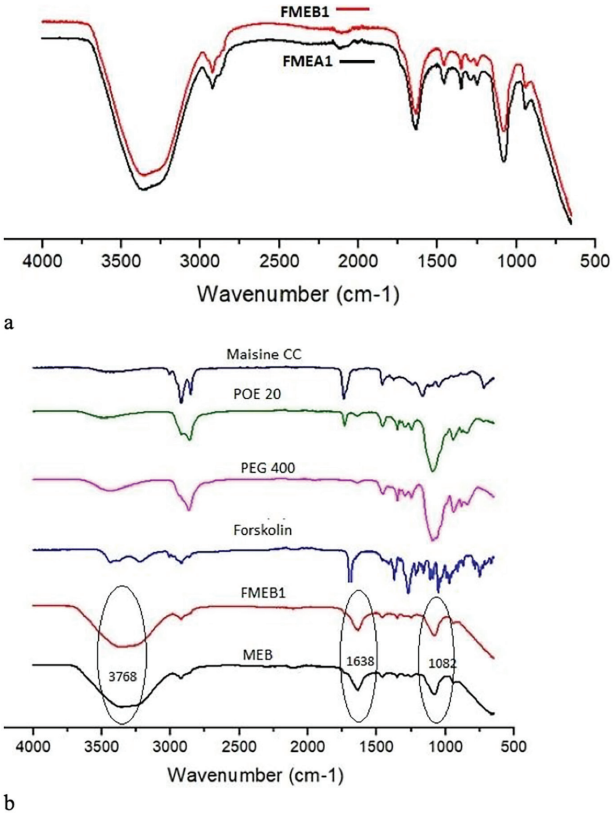

Figure 5. (a) Comparison of the FTIR spectrum pattern between FMEA1 and FMEB1 formula. (b) FTIR spectrum of forskolin and microemulsion constituent in FMEB1

FMEA1: Microemulsion with MCT oil, FMEB1: Microemulsion with Maisine® CC oil, FTIR: Fourier transform infrared, POE: Polyoxyethylene sorbitan, PEG: Polyethylene glycol, MEB: Microemulsion base

The instability of the globule size in the accelerated test was probably due to the increase in temperature, which caused stretching of the polyoxyethylene groups of POE 20, resulting in reduced hydrogen bonding between the ethylene groups of the surfactants and water. This condition causes a decrease in the hydrophilic nature of POE 20, so it cannot dissolve in water.[33] This phenomenon causes POE 20 to be unable to reduce the surface tension, which causes the microemulsion monolayer membrane to break, resulting in coalescence and phase separation in the microemulsion.

The concentration of forskolin in FMEB1 determined in the stability test can be seen in Figure 6 and pH in Figure 7.

Weng and Huang[16] investigated the kinetics of forskolin degradation in an aqueous environment and found that the stability of forskolin was affected by pH and storage temperature. Forskolin is relatively stable at pH 3.5-5 and temperature < 50 °C. At pH > 6.5 and temperature > 50 °C, forskolin tends to hydrolyze to 7-diacetyl forskolin (forskolin-D), which then undergoes isomerization to isoforskolin. Yamamura et al.[34] developed a forskolin emulsion with a globule size of 204 nm, which was stable for 30 days, and found that forskolin was incorporated into the monolayer membrane to protect it from hydrolysis. The finding of stable levels of forskolin in FMEB1 indicates that forskolin is thought to be incorporated into the monolayer membrane formed by Smix used in this study.

Irritation test

The results of the irritation test are shown in Table 5 and Figure 8.

In Table 5, PII FMEB1 is classified as a very weak and negligible irritant (PII: 0.11), where erythema only occurs in 1 rabbit at T24 with a score: 1, then disappears. These data indicate the use of 25% POE 20 and 5% PEG 400 as Smix, both of which are composed of non-irritating polyoxyethylene groups. The results of this test agree with Rhein et al.[35] Rhein found that the use of 1-30% *w/w* non-ionic surfactants or cosurfactants that had polyoxyethylene groups did not cause swelling.

Swelling is a condition in which the *stratum corneum* is hydrated, which can absorb 5 to 6 times its weight in water[36] and can cause edema as one of the effects of irritation. Swelling occurs due to ionic interactions between water molecules in the skin and surfactant hydrophilic groups and the interaction of surfactant alkyl chains with corneocytes. The type of hydrophilic groups in the surfactants is a determining factor for swelling. POE 20 used in FMEB1 is non-irritating. This is due to the interaction between the polyoxyethylene groups in POE 20 and water molecules in the skin only forming weak interactions in the form of hydrogen bonds so that these interactions do not cause swelling.[35,37] PEG 400 is a hydrophilic molecule with a distribution coefficient of 0.000015[30]. This makes it difficult for this molecule to interact with the *stratum corneum* and only acts

Table 3. Optimization of forskolin concentration in FMEB1 formula and globule size

Forskolin concentration in FMEB1, mg/mL	Forskolin solubility, mg/mL ± SD	Globule size, nm ± SD	PI ± SD	Appearance
1	0.98 ± 0.01	26.06 ± 3.44	0.27 ± 0.01	Transparent
2	1.89 ± 0.02	24.03 ± 4.11	0.15 ± 0.03	Transparent
3	2.25 ± 0.03	19.63 ± 0.67	0.28 ± 0.09	Bluish (precipitate)
4	2.18 ± 0.03	3220.37 ± 492.85	0.76 ± 0.18	Cloudy
5	2.14 ± 0.03	3147.80 ± 408.98	0.81 ± 0.12	Cloudy
Ā	2.19 ± 0.05	-	-	-

n: 3, Ā: Mean of FSK solubility in FMEB1, SD: Standard deviation, PI: Primary irritation, FMEB: Forskolin microemulsion with Maisine® CC oil

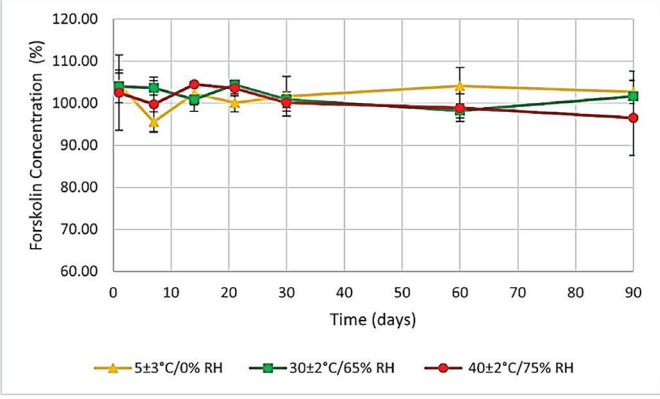

Figure 6. Stability study of forskolin concentration test in FMEB1, n: 3

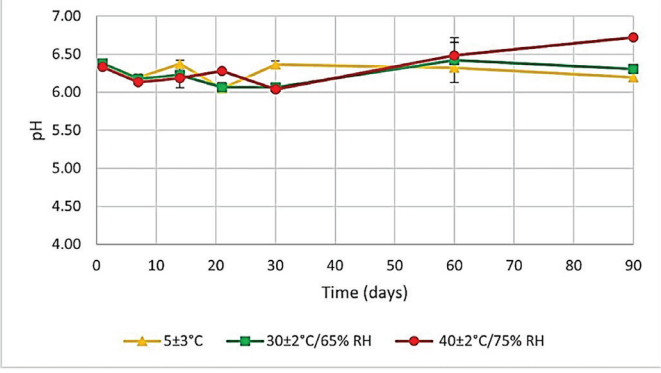

Figure 7. Stability study of pH in FMEB1, n: 3

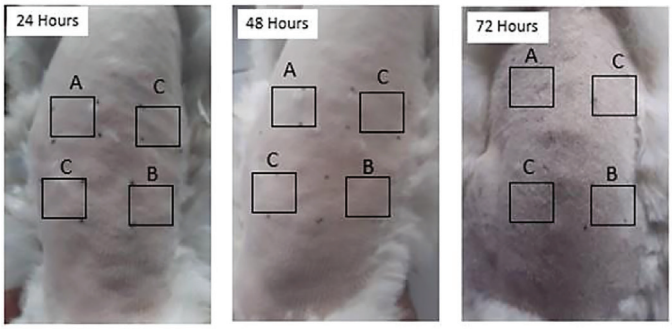

Figure 8. Results of irritation test on the skin of albino rabbits, A: FMEB1, B: MEB, and C: untreated control

FMEB1: Microemulsion with Maisine® CC oil, MEB: Microemulsion base

Table 4. Stability study of globule size in FMEB1

Time (days)	Globule size (nm) at storage condition		
	5 °C/0% RH	30 °C/65% RH	40 °C/75% RH
1	16.70 ± 1.59	21.73 ± 6.05	21.63 ± 4.09
7	16.63 ± 1.10	24.28 ± 6.70	21.74 ± 2.44
14	17.13 ± 2.93	19.73 ± 2.21	21.94 ± 1.05
21	17.00 ± 1.32	21.88 ± 2.33	115.37 ± 11.79
30	17.82 ± 0.52	21.40 ± 2.96	108.6 ± 8.00
60	17.30 ± 2.92	17.9 ± 2.36	409.10 ± 68.03
90	17.53 ± 1.65	27.00 ± 2.46	942.63 ± 28.92

n: 3, RH: Relative humidity

Table 5. Skin irritation test result

Group	Time (hours)	Total score		PII
		E	O	
FMEB1	24	1	0	0.11
	48	0	0	
	72	0	0	
MEB	24	0	0	0
	48	0	0	
	72	0	0	
Untreated control	24	0	0	-
	48	0	0	
	72	0	0	

n: 3 (number of rabbits), E: Erythema, O: Oedema, PII: Primary irritation index, FMEB1: Optimum microemulsion formula, MEB: Microemulsion base

as a carrier in the delivery system.[38] Therefore, the POE 20 and PEG 400 used in this study are not irritants.

CONCLUSION

Forskolin can be expressed as a microemulsion using the phase titration method. The optimum formula consisted of Maisine® CC, POE 20, and PEG 400 (4:25:5 *w/v*) with a forskolin

concentration of 0.2%. Microemulsions are stable when stored in the refrigerator and at room temperature, and are very mildly irritating and can be ignored with PII: 0.11.

With the successful preparation of stable forskolin microemulsion, there is an opportunity to use forskolin topically as a candidate for lipolysis. For further research, *in vitro* diffusion tests, lipolysis effects, histopathology, and cytotoxicity tests will be performed to ensure the efficacy and safety of the formula.

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Ethics

Ethics Committee Approval: This research received ethical approval number: 02/KEPHP/-ITB/10-2021 from the Ethical Commission for the Use of Experimental Animals, School of Pharmacy, Institute of Technology Bandung.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: R.N., Y.C.S., S.T.D., M.I.I., Design: Y.C.S., S.T.D., M.I.I., Data Collection or Processing: R.N., S.T.D., Analysis or Interpretation: R.N., Y.C.S., S.T.D., Literature Search: R.N., M.I.I., Writing: R.N., S.T.D., M.I.I.

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

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ORIGINAL ARTICLE

Evaluation of Biodegradable Microparticles for Mucosal Vaccination Against Diphtheria Toxoid: Nasal Efficacy Studies in Guinea Pigs

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ABSTRACT

Objectives: In this study, poly-(ε-caprolactone) (PCL) and poly-(lactic-co-glycolic acid) (PLGA) microparticles encapsulating diphtheria toxoid (DT) were investigated for their potential as a mucosal vaccine delivery system.

Materials and Methods: Antigen-containing microparticles were prepared using the double emulsion (*w/o/w*) solvent evaporation method.

Results: The average geometric diameter of the particles was found to be between 7 and 24 µm, which is suitable for uptake by the antigen-presenting cells in the nasal mucosa. Although the differences were insignificant, the PLGA polymer-containing formulations exhibited the highest encapsulation efficiency. Microparticle formulations, prepared with both PLGA and PCL polymers, were successfully produced at high production yields. The *in vitro* release profile was presented as a biexponential process with an initial burst effect due to the release of the protein adsorbed on the microsphere surface, and the subsequent sustained release profile is the result of protein diffusion through the channels or pores formed in the polymer matrix. DT-loaded microparticles, DT solution in phosphate-buffered saline (PBS), and empty microparticles (control) were administered *via* nasal route and subcutaneously to guinea pigs. The antibody content of each serum sample was determined using an enzyme-linked immunosorbent assay (ELISA).

Conclusion: Absorbance values of the ELISA test showed that PLGA- and PCL-bearing microparticles could stimulate an adequate systemic immune response with intranasal vaccination. In addition, PLGA and PCL microparticles resulted in significantly increased IgG titers with intranasal administration as a booster dose following subcutaneous administration. PCL polymer elicited a high immune response compared with PLGA polymer ($p < 0.05$).

Key words: Diphtheria toxoid, immunity, intranasal, microparticulate formulations

INTRODUCTION

The effects of emerging technologies have shown not only the success of the correlation between vaccination and immunogenic components of vaccines but also the effectiveness of delivery systems. Therefore, studies on an effective vaccine formulation or delivery system are of great importance for developing modern vaccines. Most important viral and bacterial infections occur through mucosal membranes such as the respiratory, intestinal, tear, or urogenital tracts.[1] Adjuvants have been developed to facilitate and improve the immune response obtained after mucosal immunization.[2] Particulate adjuvants or antigen delivery systems are considered an alternative to other immune stimulating adjuvants.[3] The concept of a polymeric delivery system has emerged for targeting specific regions of proteins or antigens. This approach for developing mucosal vaccine delivery systems has become mandatory, especially for protecting proteins against fragmentation of antigens in the mucosal environment and for increasing their uptake by the immune system. Polymeric particles are adsorbed at higher efficiency rates compared with soluble molecules in mucosal epithelial tissues and thus, high antigen concentrations are provided in the area requiring an immune response.[4,5] Biodegradable and biologically compatible polyesters and poly-lactic-co-glycolics (PLG) are some of the most preferred

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polymeric particulate systems because they have been used in humans for many years on surgical suture materials, as well as controlled-release drug systems.[6]

Diphtheria is a bacterial disease caused by a toxin produced by *Corynebacterium diphtheria* and causes clinical symptoms.[7] Because the ability to create long-term immunological memory of the vaccine applied is parenterally weak, booster doses should be administered intermittently. Booster vaccination is recommended as a strategy to reduce the disease's health and economic burden in these populations.[8] The reason for not providing a formation of permanent immunological memory is thought to arise from the inability to stimulate local humoral immunity in the respiratory tract.[9,10] Because mucosal immunization stimulates both systemic and mucosal immunity, it is considered an alternative route to parenteral administration. In addition, side effects are minimized with the application of mucosal vaccines, which is an easier route than the parenteral route. Therefore, mucosal immunization holds notable importance when the vaccination needs to be repeated at certain intervals, as in diphtheria.[11] The nasal mucosa is an important part of the mucosal immune system. It is the first point of contact for antigens inhaled into the organism. Thus, pathogen neutralization occurs at the first point at which pathogens enter the organism with nasal vaccination. As a result, both systemic and mucosal immune responses could be accomplished.[7,12] Recent studies have shown that the nasal route is more sufficient for the systemic transport of low-molecular-weight polar drugs, peptides, and proteins than the mucosal delivery routes. Traditional drugs applied nasally are used for treating local diseases such as nasal allergy, nasal congestion, and nasal infection.[13,14]

The purpose of this study was to develop microparticulate vaccine systems against diphtheria with long-term immunological memory. One of our main objectives was to eliminate repetitive doses to improve patient comfort. In addition, alternative intranasal administration was studied instead of parenteral root, which is more difficult and painful.

MATERIALS AND METHODS

Materials

Poly(lactide-co-glycolide) (PLGA, 50:50) was obtained from Boehringer Ingelheim. Poli-(ε-caprolactone) (PCL, M.A. 14 Kda) was purchased from Sigma. Polyvinyl alcohol (PVA; 87-89% hydrolyzed, molecular weight 13,000-23,000) was purchased from Aldrich Chemical Company (USA). Diphtheria toxoid (800 Lf/ampoule, Japanese Lot) was provided by the Refik Saydam National Public Health Agency (Türkiye) as a standard. Dichloromethane (DCM) was purchased from Sigma-Aldrich (Germany). The other agents were all special reagent grade.

Preparation of the DT-loaded microparticles

Microparticles were prepared by double emulsion-solvent evaporation technique as follows:[15] 250 µL of diphtheria toxoid in ultrapure water was emulsified with 2.5 mL of 5% (*w/v*) PLGA or 5% (*w/v*) PCL in DCM using an Ultraturrax model T 25 (IKA Laboratory Technology, Staufen, Germany) at 8,000 rpm for 5 min or Sonicator model Sonopuls HD 2070 (Bandelin Electronics GmbH&Co, Germany) at 60 W for 30 s. The resulting water-in-oil emulsion (2.5 mL) was then emulsified at 8,000 rpm for 5 min in a Eurostar mechanical stirrer (IKA Laboratory Technology, Staufen, Germany) with 50 mL of 5% (*w/v*) polyvinyl alcohol to produce a water-in-oil-in-water emulsion. This emulsion was stirred magnetically overnight under pressure at room temperature to allow evaporation of the organic solvent and the formation of microparticles. Microparticles were isolated by centrifugation (10 min at 4,000 x g), washed three times in 10 mL of ultrapure water, and freeze-dried. The prepared formulations are given in Table 1.

Characterization and quantification of DT-loaded microparticles

Particle appearance and particle size analysis

Photomicrographs of microparticles were taken using a Leica DM 4000B microscope. Lyophilized microparticles were dispersed in purified water and analyzed using (Sympatec Helos H0728) particle size analyzer.

The surface morphologies of the microspheres were also observed using an environmental scanning electron microscope (ESEM) (FEI Quanta Model 200 FEG, Tokyo, Japan). A small aliquot of the microspheres was mounted onto metal stubs using double-sided adhesive tape for sample preparation. After being vacuum coated with a thin layer (100-150 A°) of gold, the microspheres were examined by ESEM operated at 5 kV accelerating voltage. The photomicrographs were then taken at a magnification of 10,000.

Table 1. The description of the formulations

Formulation code	Diphtheria toxoid (mg)	Route of administration	Polymer used	1st step of homogenization
D1	75.8	Intranasal	PLGA	Ultraturrax
D2	75.8	Intranasal	PLGA	Sonicator
D3	75.8	Intranasal	PCL (70-90 kDA)	Ultraturrax
D4	75.8	Intranasal	PCL (70-90 kDA)	Sonicator
D5	75.8	Intranasal	PCL (14 kDA)	Ultraturrax
D6	75.8	Intranasal	PCL (14 kDA)	Sonicator

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

In vitro release of protein from PLGA and PCL microparticles

In vitro drug release was determined by the statical method.[16] Microparticles were suspended in pH 7.4 PBS contain 0.01% NaN_3. The samples were retained in a water bath at 37 ˚C and shook at 40 rpm. At appropriate time intervals, the release medium was completely withdrawn after centrifugation at 7,500 rpm and replaced with fresh buffer. The diphtheria toxoid concentration in the supernatant was determined by the mBCA technique. In addition, all release data (n: 3) were calculated (with SPSS) according to the percentage released amount of diphtheria toxoid to determine the release kinetics.

Diphtheria toxoid loading in microparticles

The protein contents of the microparticles were assayed by the digestion technique. For this technique, a NaOH/SDS solution (1 N NaOH, 5% *w/v* SDS) was added to the microparticles and dissolved.[3] The mixture was neutralized with 1 N HCl and centrifuged. The diphtheria toxoid content was determined using the microbicinchoninic acid (mBCA) (Molecular Devices, $SPECTRA_{max}$ 190 pc) total protein assay at 560 nm.

Stability studies

D1 and D5 formulations were stored in a refrigerator at 2-8 ˚C and in a climate cabinet at 25 ± 2 ˚C, 60 ± 5% relative humidity for 3 months. The diphtheria toxoid content was determined by mBCA assay. At the end of 3 months, non-reduced-PAGE analysis was performed to understand whether the protein integrity of the diphtheria toxoid was preserved.

Polyacrylamide Gel Electrophoresis

Diphtheria toxoid calibration samples and formulations that remained at 2-8 ˚C for three months were subjected to PAGE gel under non-reducing conditions using an electrophoresis system. Samples and reference materials for diphtheria toxoid were run on discontinuous gels prepared by stacking and separating in a sample buffer.

Immunization studies

The immunization program we followed included intranasal vaccination and triple repeat blood sample collection from animals; thus, guinea pig was selected as the most suitable experimental animal, and ethical approval was obtained from the Refik Saydam Hıfzıssıhha Center Presidency Scientific Committee and the Ethics Committee (06.01.2011/14886). For *in vivo* studies, weights ranging from 300 to 350 g of 54 male albino guinea pigs (7-8 years old) were used. The morphology of the D1, D2, D3, D4, D5, and D6 coded formulations, the sizes and distribution of particles, the encapsulation and production efficiency, and *in vitro* release results were considered. For the application of D1 and D5 coded formulations, *in vivo* studies were conducted. In the control group, diphtheria toxoid with PBS (pH 7.4) was preferred.

Many formulations containing diphtheria toxoids are 25-30 Lf in the first vaccination and booster dose application for the pediatric population, whereas it contains 2 Lf and above diphtheria toxoids for adults.[17] A formulation of 25 Lf/50 µL (25 µL/nostril) was applied to the nostrils of male guinea pigs using a micropipette without using anesthetics for intranasal vaccination.[18] With the application to both nostrils, the membrane area and, thus the absorption of formulations were aimed to increase. Guinea pigs were kept still for approximately 2 min to avoid any loss after administration of the vaccine into the nose. For subcutaneous vaccination, 25 Lf/500 µL of formulation was injected into male guinea pigs without anesthetics.

For *in vivo* studies, formulations containing diphtheria toxoid were applied in certain ways and days to nine groups (n: 6) of male guinea pigs (Table 2). To obtain information about whether the experimental animals had ever experienced diphtheria toxoid or not, blood samples were taken from each animal a week before the first vaccination. In this way, exposure to stress conditions of experimental animals was prevented and the best response after immunization was aimed to be obtained. To determine the changes in immune responses of guinea pigs, 1 mL of blood samples from each one was taken from their hearts, when immunoglobulin A (IgA) and IgG antibody formation took place on the 20th and 42nd days.[19] During blood collection, guinea pigs were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg). The collected

Table 2. Groups of guinea pigs immunized with adjuvant-free, microencapsulated diphtheria toxoid and control (n: 6)

Group code	Formulation	Polymer used	Administration route	Dose (Lf)		
				1st Immunisation 0 day	2nd Immunisation 7th day	3rd Immunisation 21th day
Control 1 (F1)	Dtxd-solution	-	*i.n.*	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)
Control 2 (F2)	Placebo MP	PLGA	*i.n.*	50 µL (*i.n.*)	50 µL (*i.n.*)	50 µL (*i.n.*)
Control 3 (F3)	Placebo MP	PCL	*i.n.*	50 µL (*i.n.*)	50 µL (*i.n.*)	50 µL (*i.n.*)
MP-D1 (F4)	Dtxd-MP	PLGA	*i.n.*	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)
MP-D5 (F5)	Dtxd-MP	PCL	*i.n.*	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)	25 Lf/50 µL (*i.n.*)
MP-D1 (F6)	Dtxd-MP	PLGA	*s.c.* + *i.n.*	25 Lf/500 µL (*s.c.*)	-	25 Lf/50 µL (*i.n.*)
MP-D5 (F7)	Dtxd-MP	PCL	*s.c.* + *i.n.*	25 Lf/500 µL (*s.c.*)	-	25 Lf/50 µL (*i.n.*)
Control 4 (F8)	Dtxd-solution	-	*s.c.* + *i.n.*	25 Lf/500 µL (*s.c.*)	-	25 Lf/50 µL (*i.n.*)

Dtxd: Diphtheria toxoid, MP: Microparticle, *s.c.*: Subcutan, *i.n.* : Intranasal

blood samples were centrifuged at 10,000 rpm for 10 min after waiting 1 h at room temperature and then another 1 h in the refrigerator (2-8 ˚C). The serum samples were collected from the supernatant and kept at -20 ˚C before analysis. Immune responses in collected serum samples were evaluated using an ELISA kit (total IgG guinea pig).

Anti-DT antibody assays

DT-specific IgG antibodies were measured by ELISA. The immunization chart is given in Table 2. In this assay, the IgG in the sample reacts with the anti-IgG antibodies that have been adsorbed on the surface of polystyrene microtiter wells. After removing unbound proteins by washing, anti-IgG antibodies conjugated with horseradish peroxidase were added. These enzyme-labeled antibodies form complexes with previously bound IgG. Following another washing step, the enzyme bound to the immunosorbent is assayed by adding a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of IgG in the tested sample; thus, the absorbance at 450 nm is a measure of the concentration of IgG in the test sample. The quantity of IgG in the test sample can be interpolated from the standard curve constructed from the standards and corrected for sample dilution.

RESULTS

Preparation and characterization of DT-loaded PCL and PLGA microparticles

The microparticles prepared using the *w/o/w* solvent evaporation method had a regular morphology and a smooth surface. The particle size of the formulations was between 7 and 34 µm (Figure 1). D5 formulation was also observed under scanning electron microscopy (SEM) with a smooth spherical shape (Figure 2).

DT loading

DT-loaded microparticles were prepared using different polymers (PCL and PLGA) by variation in the weight of the polymer dissolved in DCM to investigate the eventual modifications of the particle size, protein loading, and efficiency of entrapment (Table 3). It was indicated that the type of polymer used also affected the encapsulation efficiency. It was determined that formulations prepared with PLGA polymer showed higher encapsulation efficiency than those prepared with PCL polymer.[20,21]

Stability studies

D1 and D5 formulations were stored for 3 months at 2-8 ˚C and 25 ± 2 ˚C (60 ± 5% RH). Bands of digestion and calibration samples were obtained as shown in Figure 3. According to the bands of integrity, we can conclude that diphtheria toxoids maintained their stability during the formulation process. The number of changes during stability studies under different conditions for the active substance and encapsulated DT are given in Table 4.

To examine the stability of diphtheria toxoid, it was kept in refrigerated conditions for multiple months. It has been stated that DT remains stable for years when stored at 2-8 ˚C.[22]

PAGE electrophoresis

In our previous study, the structural integrity of the diphtheria toxin was analyzed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Our standard diphtheria toxin has a broad band around 66 kDa, with two fragments (A fragment around 45 kDa, and B fragment around 29 kDa). Preservation of the integrity and stability of the diphtheria toxin in 3 months under both conditions did not show any significant differences in the non-denaturing environment (p >0.05). Samples extracted from microparticles under both stability conditions were applied to the polyacrylamide gel under non-denaturing conditions (Figure 3). There was only one band around 66 kDa. This shows that the antigen encapsulated in the formulations remains intact.

In vitro release studies

Rapid drug release from polymeric particles, called "burst release" were examined in all formulations (Figure 4). This rapid initial release is attributed to the fraction of the drug adsorbed

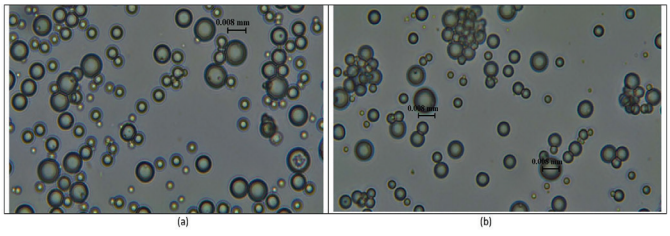

Figure 1. Optical micrograph of (a) PLGA and (b) PCL (90 kDa) microparticles entrapping BSA produced by the standard double emulsion solvent evaporation method

PLGA: Poly-(lactic-co-glycolic acid), PCL: Poly-(ε-caprolactone), BSA: Bovine serum albumin

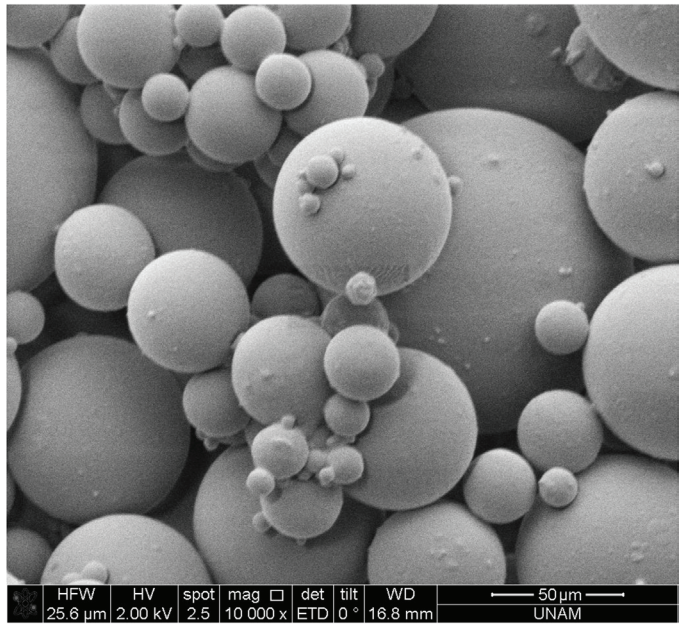

Figure 2. Environmental scanning electron microscopy images of D5 microparticles

Table 3. Characterization and physicochemical properties of the DT-loaded microparticles

Code	Type of polymer	1st step of homogenization	Antigen (mg)	Encapsulation efficiency (%) ± SD*	Yield (%)	Geometric diameter (μm) ± SD*
D1	PLGA	Ultraturrax	75.8 (144 g protein)	60.6 ± 0.39	88.38	6.93 ± 0.45
D2	PLGA	Sonicator	75.8 (144 g protein)	53.7 ± 1.53	87.23	7.98 ± 0.83
D3	PCL (70-90 kDa)	Ultraturrax	75.8 (144 g protein)	46.8 ± 1.97	73.57	22.47 ± 0.31
D4	PCL (70-90 kDa)	Sonicator	75.8 (144 g protein)	52.6 ± 1.08	74.00	34.4 ± 1.22
D5	PCL (14 kDa)	Ultraturrax	75.8 (144 g protein)	55.7 ± 0.86	88.30	8.88 ± 0.02
D6	PCL (14 kDa)	Sonicator	75.8 (144 g protein)	45.9 ± 1.68	88.96	12.6 ± 0.19

SD: Standard deviation, *n: 6, DT: Diphtheria toxoid, PLGA: Poly-(lactic-co-glycolic acid), PCL: Poly-(ε-caprolactone)

Table 4. Diphtheria toxoid integrity during stability studies (n: 3)

Formulation code	Condition	Remaining amount of diphtheria toxoid (%) ± SD		
		Initial	1st month	3rd month
DT alone	5 ± 3 °C	99.85 ± 0.27	99.78 ± 0.18	99.44 ± 0.33
D1	5 ± 3 °C	60.6 ± 0.39	58.7 ± 1.30	57.5 ± 1.54
D5	5 ± 3 °C	55.7 ± 0.86	54.63 ± 1.53	52.48 ± 1.69
D1	25 ± 2 °C	60.6 ± 0.39	57.3 ± 0.99	52.39 ± 1.72
D5	25 ± 2 °C	55.7 ± 0.86	52.8 ± 1.15	46.63 ± 0.57

SD: Standard deviation, DT: Diphtheria toxoid

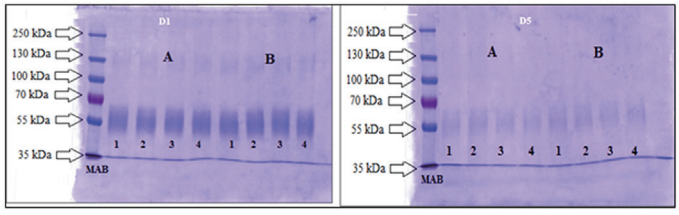

Figure 3. Bands obtained by polyacrylamide gel electrophoresis for D1 and D5 coded formulations (A) 2-8 °C, (B) 25 ± 2 °C, 1) initial, 2) 1st month, 3) 2nd month, 4) 3rd month

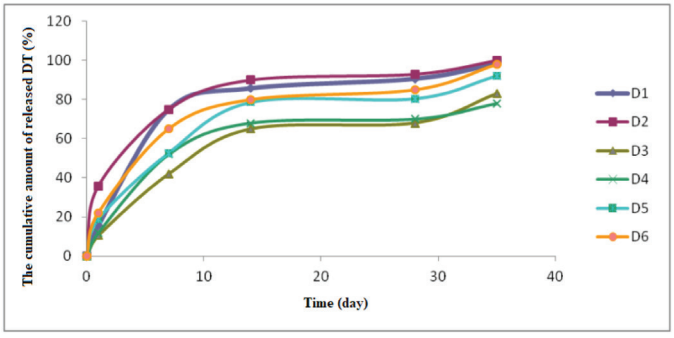

Figure 4. *In vitro* release profiles of diphtheria toxoid from the formulations

or weakly bound to the surface of the microspheres. Similar to our previous investigation, we obtained triphasic release profiles generally found in formulations prepared with PLGA and PCL polymers.[23,24] It was observed that D1 and D2 coded formulations prepared with the PLGA polymer had a faster release than those prepared with PCL polymers. At the end of five weeks, the PLGA polymer used formulation released all active ingredients. On the other hand, PCL formulations (coded as D3, D4, D5, and D6) could not release all the active ingredients in 5 weeks. In addition, previous studies have revealed that the release rates of particulate systems prepared with PLGA are higher than those of particulate systems prepared with PCL.[25] Furthermore, all of the prepared formulations were compatible with the Higuchi kinetics, which is an expected outcome in matrix systems. In addition, silver staining electrophoresis was applied to *in vitro* release samples, and no extra band formation was observed (data not shown). The obtained single band indicates that the structural integrity of the diphtheria toxoid is preserved during microparticle formation.

Immunization studies

The first vaccination experiment was performed to investigate, whether DT-loaded microparticles can induce a systemic immune response following nasal administration to guinea pigs. The results of guinea pig serum antibody (IU/milliliter) are summarized in Figure 5.

By comparing the groups of F2 and F4, the formulation coded D1 increased the IgG titer on the 42nd day, when administered intranasally. When the IgG titers of group F6 were examined, increased concentrations were observed after intranasal application. Although this group first applied subcutaneously, continuous intranasal administration showed increased titers. According to these results, we concluded that formulation D1 can be a good candidate for a booster (rappel) formulation.

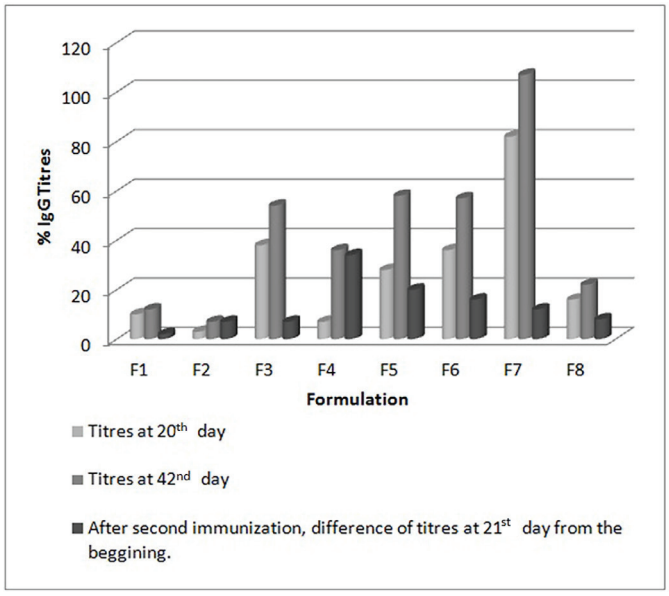

Figure 5. IgG titers at specified intervals
IgG: Immunglobulin G

When the groups of F3 and F5 were compared, D5 formulation administered intranasally was found to have an increment effect on the IgG titer on the 42nd day. When the blood concentrations of group F7 were examined, similar increasing titers were observed as formulation D1.

When D1- and D5-coded formulations were compared with each other, D5-coded formulation was observed to stimulate antibody formation stronger than the D1-coded formulation, and the difference was statistically evaluated by the *t*-test and was determined to be significant ($p < 0.05$). The reason is a result of the PCL polymer. In the formulation in which PCL polymer alone is applied intranasally (group F3), there is a significant increase in immune response obtained from the F1 group. This increase in IgG is thought to play an active role in the stimulation of systems forming the immune response of PCL polymer. Murillo et al. observed the same adjuvant effect, when they applied the PCL polymer to animals *in vivo*.[26] However, we concluded that the PLGA polymer did not show an adjuvant effect with nasal application.

The immune response of the F4 and F5 groups in which only intranasal application was performed was higher than those of the F6 and F7 groups in which subcutaneous injection followed intranasal application. In the first and second immunizations, the reason why the immune response obtained after intranasal application is higher than the others is thought to be from a disease that maintains the upper respiratory tract. This finding supports the request of World Health Organization (WHO) to encourage mucosal immunization with diseases that affect the upper respiratory tract like diphtheria.[22]

DISCUSSION

The purpose of this study was to develop microparticulate systems containing diphtheria toxoid as the model antigen and to evaluate their intranasal administration. New approaches to diphtheria vaccination that generate immune responses equal to or surpass those generated by traditional vaccines must have an increased safety profile. In this study, we selected mucosal vaccination because of capacity to stimulate both systemic and mucosal immune responses. It has been shown that using the nasal route, most soluble antigens can induce immune responses.[11] Conversely, it should not be ignored that these antigens can induce poor immunogenicity with immunological tolerance.[1-3] In our previous study, we found that nasal administration of diphtheria toxoid alone induces very weak immune responses.[27] In contrast, the use of polymeric-DT microparticulate formulations considerably enhance their immunogenicity for intranasal immunization of guinea pigs. These microparticulated formulations containing diphtheria toxoid were prepared using the double emulsion (*w/o/w*) solvent evaporation method. PLGA and PCL polymers were used in microparticulate formulations because of their biodegradable and biocompatible properties. Furthermore, recent studies have shown that PLGA copolymers significantly affect the stability and biological activities of active substances, especially depending on the hydrophobicity of the polymer and the presence of acidic degradation products.[28]

Sayin et al.[19] established that chitosan can enhance immune responses *via* the nasal route. They studied medium molecular weight chitosan, which could successfully enhance the absorption of diphtheria toxoid nasally in rat models. In the nasal cavity, biodegradable chitosan increases the residence of the antigen and can open tight junctions between the cells, thereby promoting absorption into the nasal cells.[19] On the other hand, organic solvent residues in the formulation, while preparing chitosan solutions can cause the lower stability of these molecules against proteolysis and lower absorption when administered intranasally.[9] We also used similar organic solvents while preparing microsphere formulations. However, in order to remove the organic solvent residue, during the preparation step, the solvent was allowed to evaporate overnight under pressure, and the washing procedure was applied under high-speed centrifugation three times. The encapsulation yields and immune response of the formulations show that we eliminated this negative condition.

Isaka et al.[7] previously demonstrated that immunization of mice with alum-adsorbed DT was considerably enhanced by booster immunization *via* the same route. Immunization of the mice in this way generated similar IgG and neutralizing antibody titers in parenterally immunized mice, and generated high levels of local IgA.[10] The development of an alternative microparticulate formulation for mucosally delivered diphtheria toxoid booster vaccines may eliminate some of the side effects associated with the conventional vaccine with aluminium salts and may also be more acceptable for frequent boosting against diphtheria. Similar results were obtained using our previous *in situ* gel formulations.[27] When the release kinetics were compared, it was shown that the microsphere formulation was more effective than the *in situ* gel formulations. In addition, it is one step ahead of other formulation designs because of the adjuvant effect of

the PCL polymer, which is used for microsphere formulations.

The morphological properties, particle size, encapsulation efficiency, and production yield of microparticles were investigated during pre-formulation studies. According to the obtained results, formulations were optimized and certain amounts of diphtheria toxoid were added to the formulations (D1, D2, D3, D4, D5, and D6). For these formulations; morphological properties, particle size, and distribution, encapsulation efficiencies, production yields, drug release profiles, and stability of microparticles were investigated. Formulations coded D1 and D5 were considered to be superior to other formulations given in the *in vitro* tests (*e.g.*, encapsulation efficiencies, particle size and distribution, particle size and distribution). Therefore, these formulations were tested in guinea pigs to determine immune responses that would be produced following intranasal and subcutaneous administration. Absorbance values of the ELISA test showed that formulations coded D1 and D5 could stimulate adequate systemic immune response with intranasal vaccination.

Additionally, D1 and D5 formulations exhibited a significant increment in IgG titers with intranasal administration as a booster dose following subcutaneous administration. PCL polymer elicited a high immune response compared with PLGA polymer (p <0.05). However, the PCL polymer, when used alone, was also found to have an adjuvant effect.

In this study, we developed intranasal vaccines as an alternative to parenteral formulations. Therefore, it was investigated for any inflammation, edema, or other side effects that occurred in the injection and nasal area for all guinea pigs within 42 days. The absence of adverse effects was also positive for our formulations for mucosal administration.

In our previous study, *in situ* gel formulations improved the residence time of the diphtheria toxoid in the mucosa for its therapeutic efficacy. [27] Although these gel formulations can be applied easily by droplet into intranasal way, both Kruskal-Wallis and Wilcoxon tests indicated that the microparticulated formulation (D1 and D5) resulted in an increased systemic immune response according to the *in situ* gel formulation (F3 and F7). This may have been caused by the late migration of DT in gel formulations.

CONCLUSION

This study demonstrates that microparticles of PCL and PLGA encapsulated with diphtheria toxoid can be produced using a modified double emulsion (*w/o/w*) technique. The morphology, size, and distribution of particles, encapsulation, production efficiency, and *in vitro* release of the formulations were investigated, and two candidate formulations were applied to the nostrils of male guinea pigs.

The immune response of formulations applied through the intranasal route was higher in groups, in which the subcutaneous injection followed the intranasal application. This finding supports the recommendation of WHO to encourage mucosal immunization with diseases that affect the upper respiratory tract, such as diphtheria.

The biodegradable property of the PLC polymer, its hydrophobicity, and its resistance to acidicu pH indicated make this delivery system a potential carrier for mucosal intranasal vaccines. Compared with PLGA microparticles loaded with the same amount of toxoid, this hydrophobic polymer (PCL) has a high immune response. In addition, PCL formulations without diphtheria toxoid have an immune response because of their adjuvant effect.

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Ethics

Ethics Committee Approval: The Refik Saydam Hıfzıssıhha Center Presidency Scientific Committee and the Ethics Committee (06.01.2011/14886).

Informed Consent: Not applicable.

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Authorship Contributions

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ORIGINAL ARTICLE

Cell Therapy and Investigation of the Angiogenesis of Fibroblasts with Collagen Hydrogel on the Healing of Diabetic Wounds

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ABSTRACT

Objectives: A diabetic ulcer is a common disease in patients with diabetes. Because of antibiotic resistance, new therapeutic alternatives are being considered in diabetic foot patients to reduce complications and mortality. This study aimed to evaluate the effect of collagen hydrogel on the wound-healing process in diabetic rats.

Materials and Methods: Diabetic wounds were induced with streptozotocin in all 42 male Wistar rats. The rats were divided into four groups: (a) treated with fibroblast cells, (b) collagen hydrogel, (c) collagen cultured with fibroblast cells, and (d) a control group. Microscopic and histological (hematoxylin and eosin staining and Mason trichrome staining), measurement of wound surface with image J, skin density and thickness by the ultrasound probe, and skin elasticity with cytometer tool were used to evaluate wound healing at days 14 and 21 after the treatment.

Results: The results showed that treating diabetic wounds with fibroblasts cultured in collagen hydrogel greatly reduces inflammatory responses in the skin tissue and significantly accelerates the healing process. In addition, 21 days after the start of treatment, skin elasticity, thickness, and density were higher in the collagen + fibroblast group than in the control group.

Conclusion: In addition, the results of the present study show that diabetic wound dressing can significantly reduce the inflammatory phase in the wound healing process by increasing the speed of collagen synthesis, skin density and elasticity, and angiogenesis.

Key words: Fibroblast, collagen hydrogel, burn wound healing, diabetic rat

INTRODUCTION

A diabetic ulcer is a common disease in patients with diabetes. Between 15% and 25% of people with diabetes develop diabetic foot ulcers. The 5-year mortality rate for diabetic wounds that require resuscitation varies between 39% and 80%. In addition, new therapeutic alternatives are being considered in patients with diabetes. There has been much research in recent years on the effects of collagen hydrogels and fibroblasts, but research in this area is still of interest to scientists. Stem cells can proliferate and are transformed into different cell types.[1-3] Collagen hydrogel is used for several skin diseases by increasing blood flow.[4-6] Huang et al.[7] reported that stem cells heal skin wounds by increasing the thickness and elasticity of the skin. Other research has shown that hydrogels cultured with stem cells facilitate rapid healing of diabetic skin ulcers through angiogenesis and collagen deposition.[1,4,8] In contrast, recent research has shown that mesenchymal stem cells (MSCs) interfere with healing diabetic wounds. Despite significant findings regarding the positive effects of collagen hydrogels,[8,9] the results of some studies in this area could be better.[10] There is conclusive evidence of the effects of collagen hydrogels on the healing of diabetic skin ulcers, which clearly indicates wound healing after the use of fibroblasts derived from and collagen hydrogels. Based on this and considering that

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common treatments are very complex,[1,11] in this regard, the use of collagen hydrogels cultured with fibroblasts derived from circumcised skin may be effective. In this study, the effects of collagen hydrogel were investigated using cell culture and histology methods. The results of this study can be used in the design of treatment methods to heal skin wounds.

MATERIALS AND METHODS

All study procedures were performed according to the local guidelines for the care of laboratory animals of the Faculty of Pharmacy and Pharmaceutical Sciences, Tehran Medical Sciences, Islamic Azad University, Iran (IR.IAU.PS.REC.1399.148).

Preparation of collagen hydrogels

Bovine collagen (40 mg, Biomaterials Company) was dissolved in a Falcon tube containing 25 mL of deionized sterile water. Two milliliters of the phosphate-buffered saline buffer and 8 mL of the HEPES buffer were added to the Falcon tube and placed at room temperature for one day. The obtained hydrogel was dewatered using an acetone solution and sterilized by ultraviolet (UV) irradiation. The morphology of the hydrogel was examined and photographed using an Olympus BX61 Research Slide Scanner Microscope.[12]

Isolation of fibroblasts

First, the foreskin of a human infant was collected and stored in a HEPES buffer at 4 °C. The skin sample was cut into small pieces using surgical forceps in a bacteriological Petri dish and washed to prevent microbial contamination in a Falcon tube containing 10 mL of 70% alcohol. In the next step, the dermis was separated from the epidermis and incubated with disease enzyme and the HEPES buffer for 24 h. Dermal samples were then incubated with collagenase (Sigma-Aldrich, USA) at 0.1% for 2 h. The contents of the dish were transferred to a 50 mL Falcon tube containing 5 mL of Dulbecco's modified Eagle medium (DMEM)-high glucose (HG) culture medium (GIBCO, USA) and 10% FBS and piritaged. The contents of the Falcon were passed through a mesh filter with 70 µm pores to separate the cells from the tissue fragments. The cells were centrifuged for 5 min at 200 g and 14 °C. The supernatant was drained and serum-free DMEM-HG culture medium was added to 5 mL of cell sediment. Finally, the cells were centrifuged at 200 g at 14 °C for 5 min.[13,14]

Fibroblasts characterization and count

Immunocytochemical staining was performed to confirm the identification of the isolated fibroblasts. To stain the nucleus, the slide was immersed in hematoxylin for 1 min and then rinsed with water. Dehydration and clarification were performed by placing the slides in 50, 70, and 96% alcohol. Cells were counted using a Fisher Scientific hemocytometer and an Olympus IX 70 light microscope. PI-acridine staining was applied to evaluate cell viability according to the manufacturer's instructions. Cells were cultured, isolated, washed, and incubated with annexin V and 7-AAD PE grafted in annexin V (BD Biosciences) binding buffer for 15 min. Finally, the percentage of living and dead cells was examined using a Beckman-Coulter Navios flow cytometer.[15,16]

Culture of fibroblasts in collage hydrogel

First, the hydrogel was prepared on the desired plate. After reaching the desired density, the fibroblasts were trypsinized and transferred to hydrogels. The cells containing hydrogel were placed in an incubator and observed daily under a microscope. Fibroblast cell culture was observed in hydrogels using an inverted microscope (Olympus, IX 70) and a scanning electron microscope (SEM, Hitachi, S-3000 N, Japan). After 14 days, collagen disks cultured with fibroblasts were used for wound dressing.[17,18]

Hydrogel cytotoxicity assessment

To measure cell viability, 1 x 10^5 fibroblasts were cultured in RPMI medium in a 96-well plate. After 24 h of incubation, the cells with hydrogel were treated. After 48 h of exposure of the cells to the hydrogel, 20 µL of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added (0.05 mg/well) to the plates in the dark. After 5 h of incubation of the plates, 100 uL of dimethyl sulfoxide solution was added to each well, and after 10 min of stirring, the resulting color intensity was measured by the Elisa reader at 570 nm. After culturing with glutaraldehyde, drying in room air, sectioning, coating with gold, the cultured cells were subjected to SEM imaging.[16-23]

Animal experiments

Male Wistar rats (42) weighing 200-220 g were purchased from Pasteur Institute (Tehran, Iran). The animals were kept in metal cages with access to water and food. Rats were exposed to 12 h of darkness and 12 h of light at 23 °C for 1 week under special conditions in terms of light and temperature. The rats were divided into 4 groups (6 in each group): (a) control group (regular saline intake group), (b) fibroblast-treated cells (fibroblast group), (c) collagen hydrogel-treated (collagen group), and (d) collagen cell-treated group + fibroblast group (collagen + fibroblasts). Diabetes was induced by injecting a dose of 50 streptozotocin (STZ, 40 mg/kg, Aladdin company) intraperitoneally into rats. Symptoms of diabetes, such as binge drinking and urinary incontinence, appeared in rats three days after the injection. Glucose levels in blood samples taken from the tail were measured using a glucometer 4 days after injection. Rats with blood glucose levels greater than 246 mg/dL were considered diabetic rats. The animals were anesthetized with 2 mg of intraperitoneal injection of ketamine, and 0.2 mg of xylazine was completely rubbed on the back of the animal. The wound was created using a sterile punch. The dressings were fixed to the wound using Vaseline gauze and transparent adhesive tape. Images of the wound area were captured on the days 7, 14, and 21 after the wound using a digital camera (S9 +, Samsung, South Korea). ImageJ was measured on a mm scale.

Histological examination

Animals were exterminated by the spinal method on the days 7, 14, and 21, and the skin tissue, including the wound area and the surrounding skin area, was removed by 2 cm and placed in 10%

formalin solution. Fixed samples in 10% formalin were stained with hematoxylin and eosin (H&E) (Thermo Fisher Scientific Co., Ltd., Shanghai, China) dyes for tissue analysis and Mason trichrome for cellular changes. Fixed textured slides were photographed using a digital camera (Olympus IX 70).

Biometric analysis

The thickness and density of the skin were assessed at the wound site using 75 MHz ultrasound imaging (digital ultrasound imaging system DUB Skin the Scanner 75, tpm taberna pro medium GmbH, Germany) on days 7, 14, and 21. Skin elasticity [net elasticity (R2), gross elasticity (R5), and post-recovery deformation (R7)] were examined using a catheter (Courage & Khazaka electronic GmbH, Germany) on the days 7, 14, and 21 after treatment in the wound area.

Statistical analysis

Data were analyzed using the SPSS statistical analysis software. One-Way ANOVA and the Tukey method were then applied for comparisons between groups. In the statistical study, the significance level was considered $p < 0.05$.

RESULTS

Microstructure and morphology of collagen

The color of the collagen hydrogel was clear and the results showed that UV light was sufficient for 1 h to sterilize the collagen and hydrogel samples (Figure 1A). The microstructure of the collagen samples exhibited a relatively rough surface with a spongy composition and appeared smooth and uniform (Figure 1B).

Isolated cell viability and cell count

The results indicated that a high percentage of fibroblasts were alive on the day of isolation, whereas a small number of them died, and this method was suitable for isolation (Figure 2).

Macroscopic observation and wound area measurement

The results revealed that the wound section in the group receiving fibroblasts and collagen cells compared with the control group and groups receiving each factor alone showed the highest improvement on the days 0, 7, 14, and 21 (Figure 3). Furthermore, with time, the amount of wound shell thickness in this group was more than that in other groups, and the wound diameter decreased more and, therefore, was faster the wound healing process.

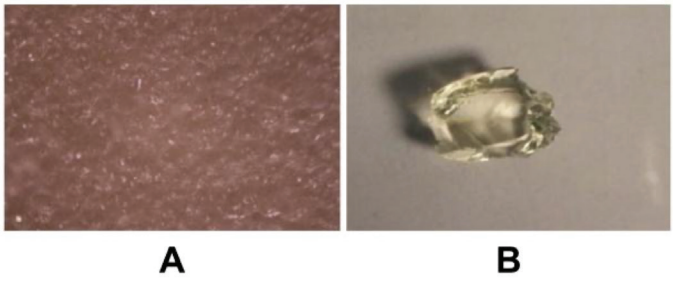

Figure 1. Digital images of (A) hydrogel samples and (B) microstructure of collagen (scale in nm)

Microscopic examination of the wound

The results showed that the neutrophil count reached its maximum on the third day after wounding and then decreased. This rate was almost the same in all 4 days of the study. Therefore, it can be concluded that all 4 groups do not stimulate the immune system, cause an inflammatory reaction, and increase the number of neutrophils at the wound site. At 21 days after the treatment, the number of macrophages decreased in all groups, and in the group treated with collagen, the hydrogel was significantly lower than in the other groups. The epithelial tissue was observed on the 7th day after treatment and increased for 14 days. On the 7th day, angiogenesis in the collagen hydrogel group was significantly less than that in the three experimental groups (Figure 4).

Biometric analysis

The results showed that the thickness of skin (epidermis + dermis) in the groups receiving fibroblast stem cells, the group receiving collagen hydrogel, and the group receiving fibroblast stem cells + collagen hydrogel increased significantly compared with the control group ($p < 0.001$). In addition, the amount of skin thickness in the group receiving fibroblast stem cells + collagen hydrogel significantly differed from the group receiving fibroblast stem cells ($p < 0.05$). Finally, in the group receiving fibroblasts + collagen hydrogel stem cells, a significant difference was observed with $p < 0.01$ compared to the group receiving collagen hydrogel (Figure 5).

The percentage of skin density in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel significantly increased compared with that in the control group ($p < 0.001$, $p < 0.05$). Finally, the percentage of skin density in the group receiving fibroblast stem cells + collagen hydrogel significantly increased compared with that in the groups receiving fibroblasts and collagen hydrogel ($p < 0.001$, Figure 6).

Gross skin elasticity (R2) significantly increased in the group receiving fibroblasts + collagen hydrogel compared with the control group and the group receiving collagen hydrogel ($p < 0.01$). In addition, the net skin elasticity (R5) increased

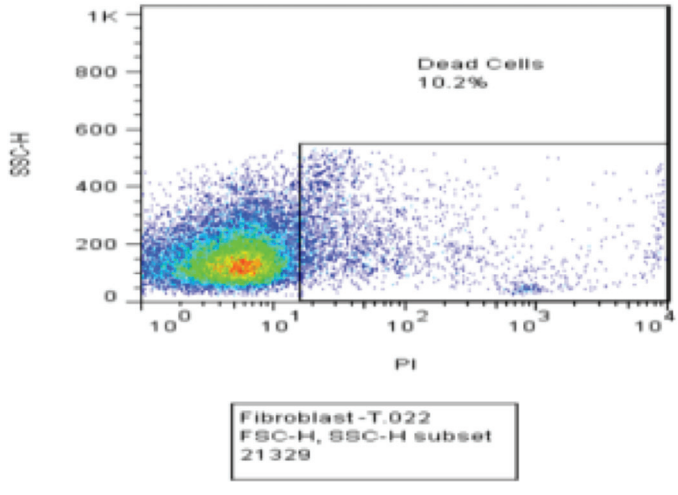

Figure 2. Viability of fibroblast cells isolated from the foreskin on the day of isolation

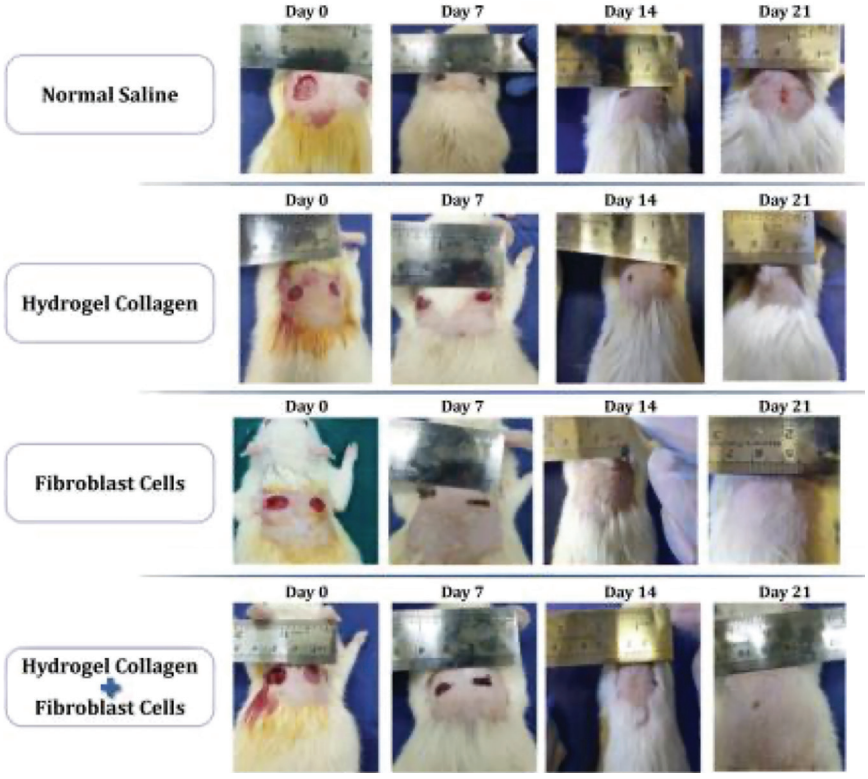

Figure 3. Wound closure on the days 0, 7, 14, and 21 in control and experimental rats

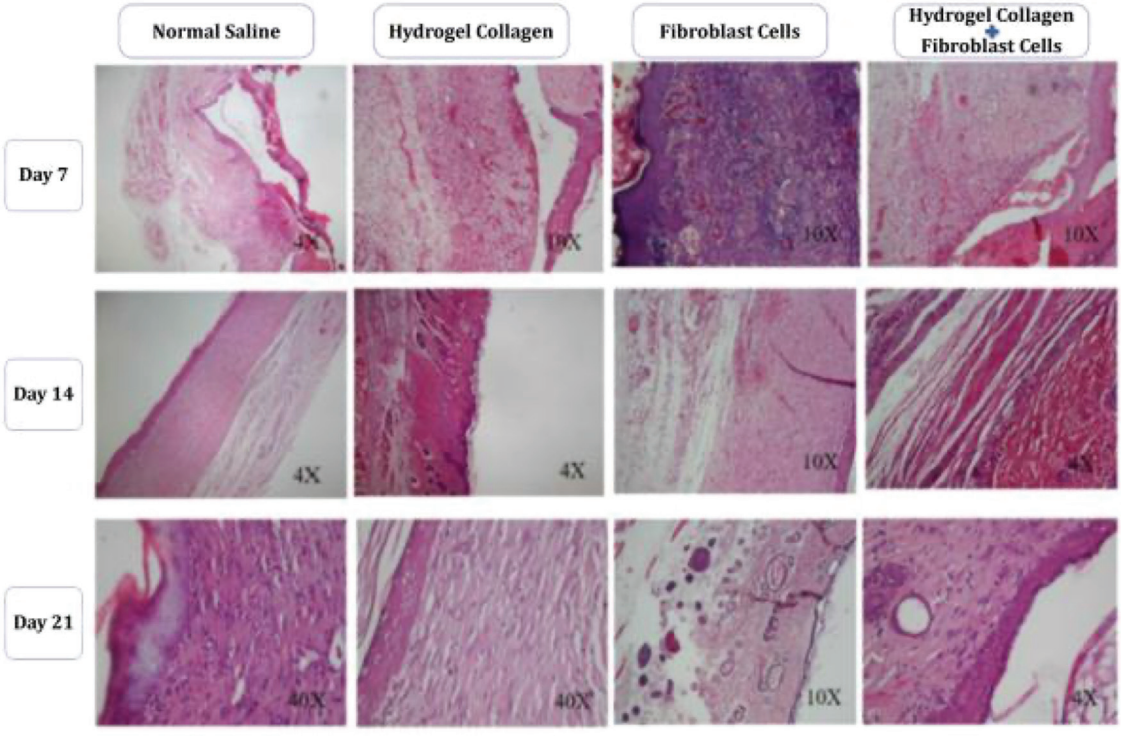

Figure 4. Histological sections of harvested wound area specimens on the days 7, 14, and 21 in the control and experimental groups

significantly in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel compared with the control group ($p < 0.01$, $p < 0.001$). The rate (R7) of skin recovery in the group receiving fibroblast stem cells and fibroblast stem cells + collagen hydrogel increased significantly compared with the control group ($p < 0.001$) (Figures 7-9).

The results obtained from ultrasound imaging showed that the wound thickness increased on the day 21 in all groups compared

with the days 7 and 14, which increased primarily in the group receiving fibroblasts + collagen hydrogel, and then the collagen group had the highest amount (Figure 10).

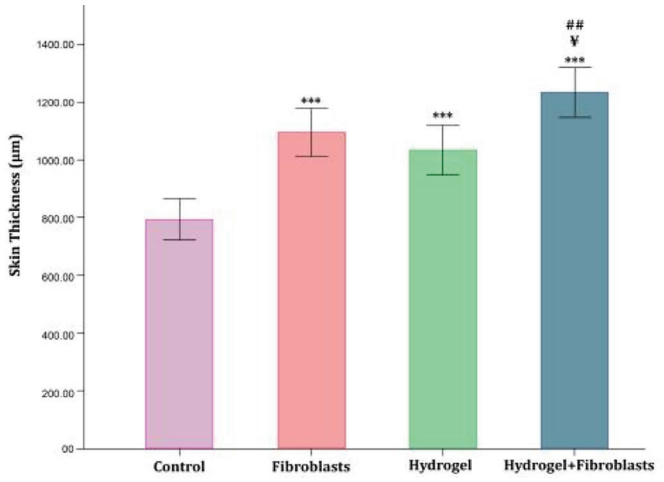

Figure 5. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in the studied groups on skin thickness during the healing of diabetic wounds in an animal model

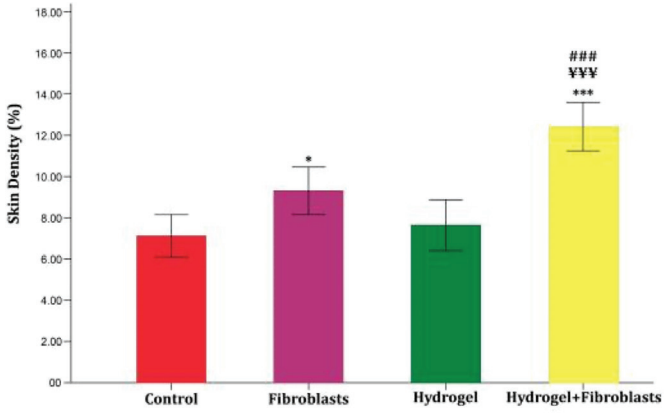

Figure 6. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in the studied groups on the percentage of skin density in the healing of diabetic wounds in an animal model

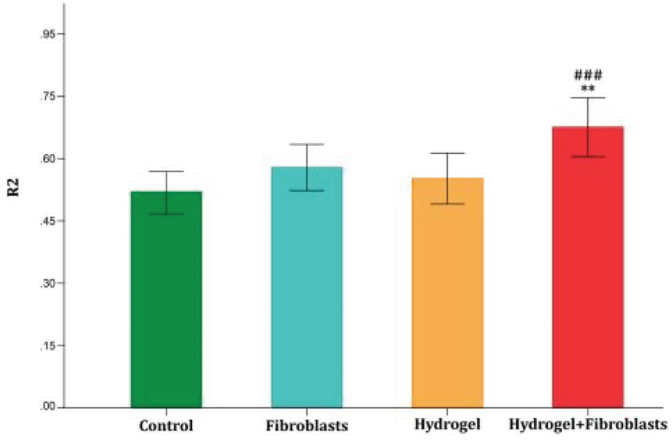

Figure 7. Data from the measurement of the effects of fibroblasts and collagen hydrogels in the studied groups on the amount of gross skin tension (R2) during the healing of diabetic wounds in an animal model

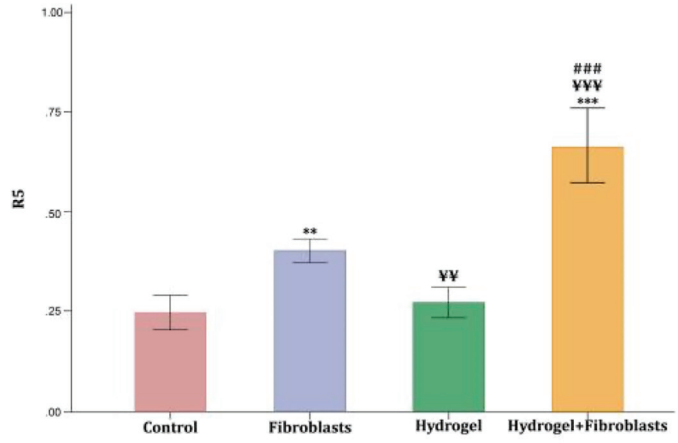

Figure 8. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in wounds in an animal model the studied groups on the amount of skin elasticity (R5: net elasticity) in the healing of diabetic wounds

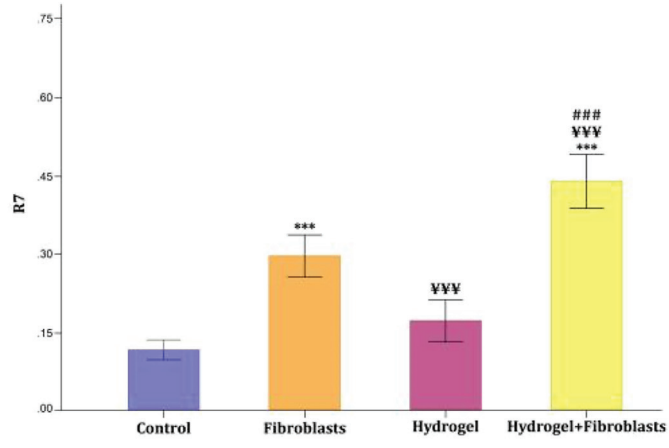

Figure 9. Data obtained from measuring the effects of fibroblasts and collagen hydrogels in the studied groups on the amount of skin elasticity (R7: recovery after deformation) during the healing of diabetic wounds in an animal model

DISCUSSION

Although many studies have shown that collagen hydrogels and fibroblasts are effective in wound healing,[1,4] the effect of cultured collagen hydrogels with circumcised fibroblasts on healing skin wounds is still challenging. It is the most interesting research topic. Based on the present study, using histological and microscopic methods, the effects of collagen hydrogel transplantation cultured with circumcised skin fibroblasts have been investigated to show that transplantation of cultured collagen hydrogels with fibroblasts can heal the wound at the right time and by what mechanisms it does this repair. According to the results, collagen hydrogel did not exhibit significant cytotoxic effects on neonatal foreskin-derived fibroblasts and is a biosafe substance in wound healing. The results revealed that the wound cross-section had the highest healing rate in the group receiving fibroblasts and collagen simultaneously. In addition, over time, the thickness of the wound shell in this group was greater than that in other groups, and the wound

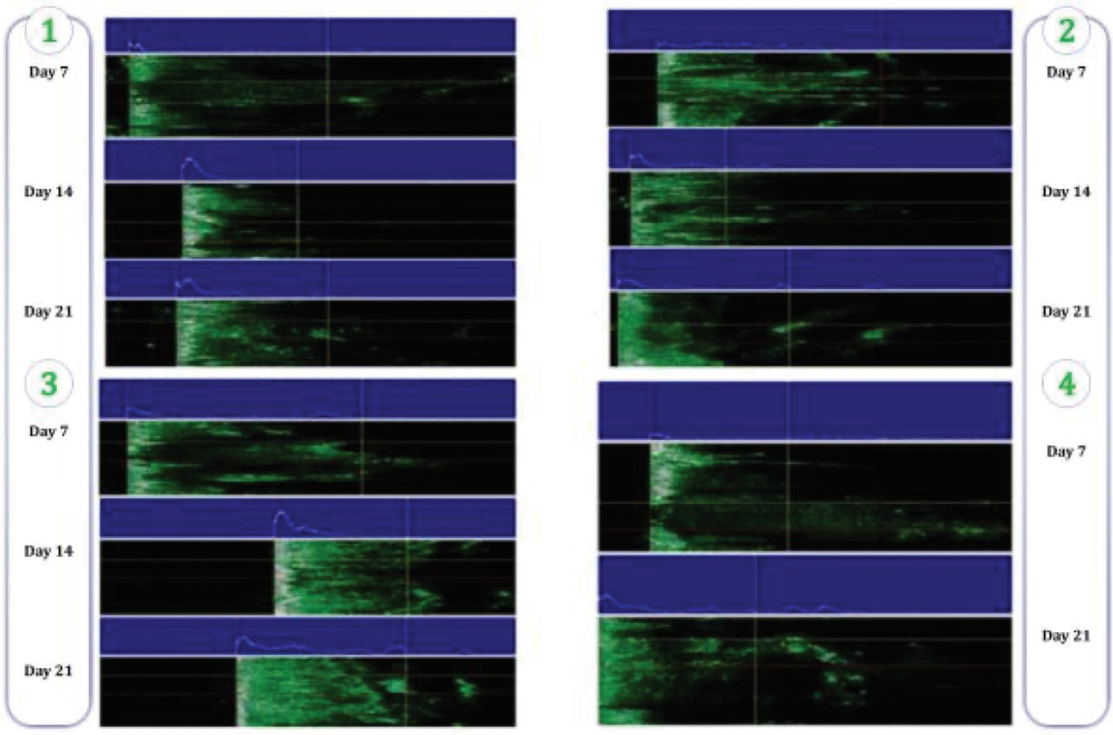

Figure 10. Ultrasound imaging for measurement of the density and thickness of the wound area skin using a 75 MHz ultrasound probe on the days 7, 14, and 21 after the treatment

diameter decreased more and the wound healing process was faster. Histological studies with two types of H&E and Mason Tricom showed that the number of macrophages decreased on the day 21 in all 4 groups. This amount was significantly lower in the cellular hydrogel group than in the other three groups. Epithelial formation was observed on the 7th day and increased until the 14th day. On days 7, 14, and 21, the percentage of epithelial formation in the cellular hydrogel group was always significantly higher than that in the other three groups. On day 7, angiogenesis in the cellular hydrogel group was significantly lower than that in the other three groups. According to these results, Bai et al.[24] reported that diabetic wounds treated with bone marrow stem cells (BM-MSCs) cultured in chitosan hydrogel compared with the control group completely healed the wound 15 days after treatment. Shen et al.[25] displayed that the wounds of diabetic rats in the collagen-treated group accelerated wound healing compared with the control group and achieved complete healing on the 18th day after the treatment. In a study, Shi et al.[1] examined the effects of fat-derived stem cells on skin ulcers in male diabetic rats and reported that stem cell transplantation significantly reduced ulcers on day 15. In another study, Pomatto et al.[6] examined the effects of fat-derived mesenchymal cells on skin ulcers in diabetic rats and showed that fat-derived mesenchymal cells repair wounds by increasing angiogenesis. In another study of the effects of fat-derived stem cells on skin wounds using flow cytometry and Western blotting on DBW mice, Pak et al.[26] reported that fat-derived stem cells maximize signaling. Paracrine and angiogenesis *via* the PI3K/AKT pathway have synergistic effects on wound healing.[26] Nuschke[27] examined the effects of MSCs using MTT and immunohistochemistry. The results of this study showed that MSCs through tissue collagen deposition can reduce tissue inflammation and induce angiogenesis and wound healing. In another study, Kaisang et al.[28] examined the effects of collagen hydrogels cultured with fat-derived stem cells on wounds using histological methods in diabetic rats. Their findings showed that collagen hydrogels cultured with fat-derived stem cells improve and optimize stem cell function to increase diabetic wound healing.[27] In addition, they investigated the effects of hydrogels cultured with bone marrow MSCs (BMSCs) on the wounds of diabetic rats using histological and immunohistochemical methods. They reported that this treatment involved granulation, angiogenesis, and extracellular matrix secretion and showed that rapid epithelialization helps heal diabetic skin ulcers.[28] In a study, Branco da Cunha et al.[29] examined the effects of collagen and alginate hydrogels cultured on fibroblast cells by flow cytometry and reverse transcription-polymerase chain reaction. Immunohistochemical results confirmed that cultures of encapsulated skin fibroblasts enhance the morphology of various cells and that this biomaterial can regulate wound healing progress.[29] In another study, Yu et al.[9] examined the effects of fat-derived stem cells (ASC) on immunohistochemical methods in mouse skin wounds, and their results showed that stem cells could reduce macrophage uptake and increase paracrine by collagen deposition to heal wounds. Uysal et al.[30] studied the effects of MSCs, bone marrow-derived stem cells, and ASCs on histological and immunohistochemical methods on skin lesions in male Fisher mice, and their results

showed that the fastest wound healing rate was observed by MSCs while increasing the rate of epithelialization through angiogenesis. In a study, Rodriguez et al.[31] examined the effects of ASC on immunohistochemical methods on skin wounds in nude mice, and their results showed that stem cells could repair wounds through blood perfusion. The results displayed that the concomitant recipient group of fibroblasts and collagen cells with amazing ability increased the thickness and percentage of skin density in the wound area. In addition, only the fibroblast and collagen cell receiving group significantly increased the gross skin elasticity (R2) in the wound area. Regarding the results of the study of net skin elasticity (R5) and recovery process (R7), all the studied groups, except for the collagen-receiving group alone, revealed significant incremental changes. According to these findings, Kittana et al.[32] showed that different concentrations of carbon nanotubes increase the thickness of the epidermis in the wound area. Luna et al.[33] examined the effects of bone marrow mesenchymal cells on skin wounds using histological and Western blotting methods in diabetic rats. The results indicated that bone marrow mesenchymal cells inhibit wound healing and are a treatment option for wounds in patients with diabetes.[33] Huang et al.[7] examined the effects of collagen hydrogel cultured with umbilical cord stem cells on diabetic wounds using histological methods in diabetic rats and found that collagen hydrogel is a desirable scaffold. Hydrogel-loaded stem cell factor as a dressing is a promising treatment for diabetic tissue regeneration.[34] In a study, Williams et al.[34] examined the effects of collagen hydrogels on wounds using histological and immunohistochemical methods in non-diabetic rats. The results showed that collagen hydrogel-treated wounds showed significant improvement compared with controls.[34] In contrast, Qiu et al.[35] studied the effects of fibroblasts on wound collagen production in diabetic rats and found that transplanted fibroblasts at the wound site did not significantly increase collagen production. In terms of the mechanism of action of fibroblasts and collagen hydrogels on the healing of diabetic wounds, it seems that these cells increase the number of fibroblasts and consequently increase their secretion. Increased secretion leads to an increase in collagen and interstitial matrix, followed by epithelial cells that have a high ability to migrate to the granular tissue and can block the wound opening in less time and cause the wound to heal.[36]

CONCLUSION

The results of the present study indicated that the bonding of collagen hydrogels with fibroblasts can increase the thickness, density, and elasticity of the skin in the wound area by increasing collagen synthesis. This dressing also increases angiogenesis, stimulate macrophages, and accelerates epithelial formation. The present study has limitations in measuring growth factors in wound tissue as well as the expression of regenerative genes involved in skin repair in skin cells and the epidermis. The results of this study can be used to clarify the association between collagen hydrogel and fibroblasts and diabetic ulcers, as well as to improve the management and prevention of diabetic skin ulcers. However, more research is needed to examine the process of wound healing through other types of hydrogels on other stem cells and to determine how the bonding of collagen hydrogels to fibroblasts affects wound healing.

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Ethics

Ethics Committee Approval: All applicable international, national, or institutional guidelines for the care and use of animals were followed. All the study procedures were carried out according to the local guidelines for the care of laboratory animals of the Faculty of Pharmacy and Pharmaceutical Sciences, Tehran Medical Sciences, the Islamic Azad University, Iran (IR.IAU.PS.REC.1399.148).

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ORIGINAL ARTICLE

A Cross-Sectional Survey of Knowledge, Attitude, and Practices Regarding Influenza Vaccination Among Jordanians Aged 18-64 Years with Chronic Diseases

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ABSTRACT

Objectives: Influenza is a frequent infectious disease that can be prevented and is associated with significant mortality and morbidity. The most economical way to prevent influenza is through vaccination, although this method is not widely used. This study aimed to assess the seasonal influenza vaccination rates and the knowledge and attitudes of Jordanian adults with chronic illnesses toward the influenza vaccine.

Materials and Methods: A cross-sectional design was employed. A 26-item online survey was used to gather data about patients' knowledge of and attitudes toward the influenza vaccine as well as their status as influenza vaccine recipients.

Results: A total of 19% of the 564 study participants received an influenza vaccination. Most (81%) of individuals reported inconsistent vaccination uptake. The most important factor affecting vaccination is the belief that the flu is not a threat (39%) and that their doctors did not advise them about the vaccination (32%). Participants with no health insurance and those with public insurance had a lower level of vaccination than those with private insurance (p= 0.008).

Conclusion: The adult population of Jordan with chronic diseases has subpar immunization rates. What is also revealed is a blatant misunderstanding about the value of routine influenza vaccination. These findings emphasize how urgently the public needs to be made aware of the effectiveness of the influenza vaccine.

Key words: Influenza, vaccination, chronic disease, Jordan

INTRODUCTION

Influenza is a contagious viral respiratory infection; up to 5 million people worldwide suffer from the severe influenza-related illness each year, and 645,000 people die as a result.[1,2] The seasonal influenza vaccine is the most efficient method of preventing infection and lowering influenza-related morbidity, death, and hospitalization, as it is with most infectious diseases.[3-5] For instance, it has been demonstrated that immunization in the elderly reduces the danger of death from influenza-related problems by 80%.[6]

To boost the proportion of individuals protected from the flu, the World Health Organization (WHO) and the United States Centers

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for Disease Control and Prevention (CDC) recommend that anyone aged 6 months and older obtain influenza vaccination. To reduce the risk of thoughtful consequences, those in high-risk groups must receive a once-a-year influenza vaccination.[1] High-risk patients include expectant mothers, children, the elderly, people with chronic heart or pulmonary diseases, and people with immunosuppressed situations.[1,7,8] Despite abundant evidence of the benefits of the influenza vaccine, only a minimal proportion of people follow the provided advice.[9,10] Based on data from several locally representative surveys, the CDC makes an educated projection of the yearly influenza vaccination exposure in the United States. Nearly half of the Americans had yet to receive the advised influenza vaccination as of the beginning of November for the 2020-2021 flu season.[11]

In December 2019, a novel virus that caused coronavirus disease-19 (COVID-19), is known as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2).[12,13] SARS-CoV-2, which spread quickly and was deemed a pandemic on March 11, 2020.[14] The COVID-19 pandemic is currently a significant global community health issue. More than 500,000 cases and more than 8,000 fatalities have occurred in Jordan.[15]

Seasonal influenza viruses and COVID-19 exhibit comparable illness symptoms.[16] Two viruses generate respiratory signs, which can indicate a variety of disorders ranging from asymptomatic to fatal. Acute respiratory distress syndrome, multi-organ failure, pneumonia, and even mortality are devastating illnesses that certain COVID-19 patients may get.[17] Elderly patients and those with comorbid conditions, particularly those with diabetes, cardiovascular diseases, and chronic pulmonary diseases, had higher death rates and a higher hazard to emerging series problems.[18,19]

A number of studies have evaluated the relationship between the coverage rate of seasonal vaccination against influenza and COVID-19 mortality.[20-22] The rationale for the significance of raising the exposure rate of influenza vaccine has appeared as a result of the resemblance in signs and symptoms of two infectious diseases and the strong evidence of reduction of COVID-19 disease mortality rate in influenza-vaccinated persons.[23] The burden that seasonal influenza infections place on medical institutions could be reduced by increasing the use of the influenza vaccine, which could also lower COVID-19 death rates.[23,24]

Most Jordanians' insurance policies do not cover influenza vaccination, and the country's mandatory immunization program excludes it.[25,26] Prior calculations of the influenza vaccination rate in Jordan revealed that the percentage rate of immunization is much below ideal (9.9% to 27.5%).[25]

It is always important to have up-to-date information about Jordanians' awareness of, attitudes toward, and use of the influenza vaccine. Therefore, determining vaccination exposure rates and comprehending the views of Jordanians with chronic illnesses on the influenza vaccine is a crucial first step in developing methods to increase uptake. Because patients with chronic illnesses are more likely to develop influenza complications, the goal of this study was to assess the seasonal influenza vaccination uptake among Jordanians with chronic diseases and identify the factors influencing such behavior. In addition, it assesses the same patients' knowledge about and attitudes toward the seasonal flu vaccine.

MATERIALS AND METHODS

Study design

A cross-sectional plan was established to study the rate of influenza vaccination among the Jordanian population who suffer from chronic diseases aged 18-64 years, whose attitudes and knowledge regarding the influenza vaccine were evaluated. The survey was conducted among all target patients at the beginning of the influenza season.

Sample

The participants for this study were the patients with chronic diseases. The sample was recruited using a non-probability convenience sampling procedure through an electronic survey. Patients were considered suitable for this study, if they had chronic diseases, were 18-65 years old, could read and comprehend survey questions in Arabic, and agreed to participate. The sample size was determined using power analysis, yielding approximately 400 participants. To be more conservative and compensate for the non-response rate, the authors collected 564 respondents.

Ethical considerations

Ethical approval was obtained from Al-Balqa Applied University's Institutional Review (approval no: 26/3/2/213). To protect the persons' anonymity, no personal data were collected. All participants knew that the participation was completely voluntary, and they had the option to withdraw at any time without facing potential risks. All patient details were saved in electronic versions on the corresponding computer.

Instrument

Based on the current literature, a 26-point questionnaire was developed. The questionnaire was used to document influenza vaccination history and medical status. Knowledge and attitudes concerning the vaccine were also gathered among Jordanians aged 18-64 who suffer from chronic diseases. The questionnaire consisted of six sections: socio-demographics (gender, age, educational level, health insurance, smoking, and physical activity), medical history (frequency of medical visits, medical conditions, and the number of medicinal drugs taken daily), vaccination status (received or did not receive the influenza vaccine), and general knowledge about the influenza vaccine. As binary outcomes, awareness variables were evaluated. Willingness to be vaccinated, perceived hurdles to vaccination, and vaccine information sources (physician, pharmacist, family and friends, media, organizational foundations) were also described. A test study on 20 candidates was conducted to examine the clarity of the survey questions. Because of the volunteers' feedback, minor changes were made to the survey questions' language and design.

Statistical Analysis

To achieve the objectives of the study, data were entered and analyzed using the SPSS statistical program, and descriptive statistics were used to estimate arithmetic averages, frequencies, and percentages of the demographic variables of the sample, such as age, gender, and education level, as well as for the variables describing the social habits of the sample, such as smoking, exercise, and statistical comparisons chi-square tests: to detect the statistical significance of the correlations, the Pearson correlation coefficient and a multivariate logistic regression were used to predict the variables associated with the regular use of influenza vaccine among the study sample as dependent variables with independent variables. The study dealt with it, and the statistical significance value (α: 0.05) or less was adopted so that the correlations could be considered statistically significant.

RESULTS

Sample

Five hundred forty six respondents completed the questionnaire. Among the 564 study participants, (35%, n: 199) were males vs. females (65%, n: 365). Almost all participants had university-grade education (46.3%, n: 261), and 31.6% (n: 178) had a diploma degree, while 125 (22.2%) had low levels of education. More than two-thirds of the participants were not smokers (64.2%, n: 362), and the remaining were either not smokers (28.9, n: 163) or previously smokers (6.9%, n: 39). In Table 1, the socio-demographic information of the participants is described.

Rates of influenza vaccine uptake

In this survey, the total 2018-2019 influenza vaccination rate for 2018-2019 was 19%. Most participants (81%) reported irregular uptake of the vaccine. Most of the respondents did not report getting immunizations against influenza. For example, 86% of patients with cardiovascular diseases, 85% with diabetes, 71% with respiratory diseases, and 76% of patients on chronic steroid management did not receive the annual influenza vaccine.

Association of patients' socio-demographic, lifestyle features, and annual influenza vaccine uptakes

The correlations that associate socio-demographic and lifestyle features and regular uptake of influenza vaccine are summarized in Table 2. There is no significant effect of socio-demographic and lifestyle characteristics on the vaccination rate. However, patients without health insurance and those with public health insurance had a lower level of vaccination compared with private ones (p= 0.008). Table 2 displays the socio-demographic and lifestyle features and influenza vaccination.

Relationship between chronic disease type and influenza vaccination

When detecting the rates of vaccination uptake among participants with definite comorbidities, a significant association was observed between chronic disease conditions and influenza vaccination. Subjects who suffer from respiratory diseases were found to have nearly a two-fold rate of periodic influenza vaccination compared to people without this situation (50% *vs.* 26.3%, p= 0.001), as shown in Table 3.

Knowledge, attitude and practice of influenza vaccination

Table 4 shows associations between knowledge, attitude, and practice of influenza vaccination. Nine of ten factors are significantly associated with vaccination. Better knowledge about the risk of influenza is linked with a higher regular vaccination rate (p= 0.01). When evaluating the reason for refusing to take the vaccine, we found that most unvaccinated participants did not feel a risk (39%) or were not recommended by their physician (32%). When evaluating the vaccine source of information, we found that the physicians and the pharmacists are accompanied by higher regular vaccination rates. The government was shown to play the lowest role in publicizing information on the influenza vaccine. On the other hand, the majority of participants who were not vaccinated did not have information about the vaccine (23%). Table 4 presents the knowledge, attitude, and practice of influenza vaccination.

Multivariate analysis and correlations of regular influenza vaccination

The findings presented in Table 5 displayed the variance values expressed by (R) and the explanatory variance expressed by (R square) in the predicted dependent variable (the use of influenza vaccine), note that all[17] variables were entered. The regression

Table 1. Demographic and clinical characteristics of respondents (n: 564)

Variable	Frequency (n)	Percentage (%)
Gender		
Male	199	35%
Female	365	65%
Age (years)		
18-30	182	32.3%
31-40	98	17.4%
41-50	120	21.3%
51-64	164	29.1%
Educational level		
High school or less	125	22.2%
Diploma	178	31.6%
University level	180	31.9%
Postgraduate	81	14.4%
Smoking status		
Non-smoker	362	64.2%
Smoker	163	28.9%
Previously smoker	39	6.9%
Exercise		
Never	335	59.4%
Less than twice/week	139	24.6%
2-3 times/week	44	7.8%
More than 3 times/week	46	8.2%

Table 2. Sociodemographic, lifestyle characteristics, and influenza vaccination

	Characteristic	Regular influenza virus vaccination n: 108 (19%)	Irregular influenza virus vaccination n: 456 (81%)	*p* value
Gender	Male	42 (39%)	157 (34%)	0.223
	Female	66 (61%)	299 (66%)	
Age	18-30 years	41 (38%)	141 (31%)	0.328
	31-40 years	20 (19%)	78 (17%)	
	41-50 years	17 (16%)	103 (23%)	
	51-64 years	30 (28%)	134 (29%)	
Education level	High school or less	26 (24%)	99 (22%)	0.365
	Diploma	28 (26%)	150 (33%)	
	University level	34 (31%)	146 (32%)	
	Postgraduate	20 (19%)	61 (13%)	
Smoking status	Non-smoker	69 (64%)	293 (64%)	0.121
	Smoker	36 (33%)	127 (28%)	
	Previously smoker	3 (3%)	36 (8%)	
Exercise	Never	67 (62%)	268 (59%)	0.296
	Less than twice/week	29 (27%)	110 (24%)	
	2-3 times/week	8 (7%)	36 (8%)	
	More than 3 times/week	4 (4%)	42 (9%)	
Medical visit	Routinely	34 (31%)	122 (27%)	0.378
	When needed	64 (60%)	302 (66%)	
	Once/year	10 (9%)	32 (7%)	

Table 3. Chronic disease conditions and influenza vaccination

Characteristic		Regular influenza virus vaccination n: 108 (19%)	Irregular influenza virus vaccination n: 456 (81%)	*p* value
Heart diseases	Yes	18 (14%)	113 (86%)	0.01*
	No	90 (21%)	343 (77%)	
Respiratory disease	Yes	30 (29%)	74 (71%)	0.03*
	No	78 (17%)	382 (83%)	
Gastrointestinal disease	Yes	2 (11%)	16 (89%)	0.00*
	No	106 (19%)	440 (81%)	
Diabetes mellitus	Yes	25 (15%)	144 (85%)	0.01*
	No	83 (21%)	312 (79%)	
Disease requiring steroids treatment	Yes	8 (24%)	25 (76%)	0.03*
	No	100 (19%)	431 (81%)	

*Significant at ≤0.05

analysis model and the (Stepwise) method of analysis were chosen, which is the most appropriate method to determine the factors that explain the most significant possible amount of variation in the dependent variable. (Doctor's advice), this factor alone explained (0.161) of the variance, (belief that the vaccine is safe) with the previous factor (doctor's advice) explained

Table 4. Knowledge, attitude, and practice of influenza vaccination

Characteristic		Regular influenza virus vaccination n: 108 (19%)	Irregular influenza virus vaccination n: 456 (81%)	*p* value
What is the reason to refuse taking the vaccine?	The physician is not recommended	37 (34%)	148 (32%)	0.00*
	I don't feel I have a risk	26 (24%)	180 (39%)	
	Vaccine is not effective	11 (10%)	25 (5%)	
	Vaccine is expensive	19 (18%)	21 (5%)	
	Vaccine is not safe	15 (14%)	82 (18%)	
The vaccine should be taken at a specific time	Yes	71 (66%)	173 (38%)	0.00*
	No	5 (5%)	42 (9%)	
	I don't know	32 (30%)	241 (53%)	
Have medical insurance	Yes	94 (87%)	350 (77%)	0.01*
	No	14 (13%)	106 (23%)	
Your medical insurance covers the price of the influenza vaccine	Yes	37 (34%)	104 (23%)	0.01*
	No	48 (44%)	196 (43%)	
	I don't know	23 (21%)	156 (34%)	
Aware of vaccination needs/ availability	Yes	81 (75%)	200 (44%)	0.00*
	No	14 (13%)	85 (19%)	
	I don't know	13 (12%)	171 (38%)	
Do you think, presence of chronic disease increases the risk of influenza?	Yes	76 (70%)	268 (59%)	0.01*
	No	17 (16%)	61 (13%)	
	I don't know	15 (14%)	127 (28%)	
Your physician advises you about vaccine	Yes	70 (65%)	87 (19%)	0.00*
	No	38 (35%)	369 (81%)	
Source of information about influenza vaccine	Don't have information	10 (9%)	104 (23%)	0.00*
	Family	13 (12%)	56 (12%)	
	Pharmacist	19 (18%)	69 (15%)	
	Physician	42 (39%)	63 (14%)	
	Government	10 (9%)	59 (13%)	
	Media	14 (13%)	105 (23%)	
Are you ready to take vaccine if we provide you?	Yes	96 (89%)	227 (50%)	0.00*
	No	12 (11%)	229 (50%)	
What are the reasons to refused to take vaccine in case its available?	Not safe	25 (23%)	76 (17%)	0.15
	I think I don't need it	46 (43%)	237 (52%)	
	Not recommended by physician	37 (34%)	143 (31%)	

*Significant at ≤0.05

(0.200) of the variance (readiness to take the vaccine if you are provided with it in the next times). It was explained with the previous two factors (doctor's advice and belief that the vaccine is safe) (0.221) from the variance in the dependent variable. The remaining variables were excluded from the regression equation because they were not good predictors and did not explain significant values of the variance.

Table 5. Multivariate analysis: correlates of regular influenza vaccination

The predictor	R	R square	Adjusted R square	Standard error of prediction	*p* value
Physician advise	0.402	0.161	0.160	0.361	0.00*
+ Physician advise Thinks the vaccine is safe	0.448	0.200	0.198	0.353	0.00*
Physician advise + thinks the vaccine is safe + readiness to take vaccine if we supplied you next time	0.470	0.221	0.217	0.348	0.00*

*Significant at ≤0.05

DISCUSSION

Diverse opinions and attitudes are present among Jordanian adults about influenza vaccination. Our study aimed to measure the knowledge, attitude, and practice of adults in Jordan who suffer from chronic diseases toward influenza vaccine and the readiness of adults to take influenza vaccine. Results of this analysis are created for a specific sample of the Jordanian population based on the sample size determined to be satisfactory by statistical analysis. The findings of this study may help with broad immunization tactics in the future.

Rates of influenza vaccine uptake

Our findings indicated a 19% overall vaccination rate. Because our participants completed the questionnaire on their own, the reported prevalence of vaccination among Jordanian adult subjects may be overstated. A major problem for worldwide community health is low immunization rates. Continuous research is being conducted across nations to project and apply useful treatments for raising the rates of influenza vaccinations. Each strategy needed to be more creative to overcome this problem, from patient reminders and memory systems to posters in doctor clinics tracking vaccination success.[24] Indeed, according to the CDC-sponsored National Internet Flu Survey, 48.4% of adults in the United States will be immunized against the flu during the 2019-2020 influenza season.[11] The recommended targets for influenza vaccination in European nations are likewise difficult to meet.[27] In high-risk groups, low vaccination coverage is especially alarming. Patients with chronic diseases, *e.g.* cardiovascular disease, cancer, or diabetes, or immunosuppressed states had inadequate immunization rates in our study, which ranged from 14% to 29%. According to the European CDC, immunization rates for older adults and people with chronic conditions should reach a target of 75% coverage by the 2014-15 winter season.[27]

Factors affecting the acceptance of seasonal influenza vaccines

Future immunization campaigns must focus especially on populations with low vaccine acceptance. Therefore, it is crucial to identify people, who refuse to receive the vaccine or are against it. The availability of vaccinations is a significant contributing factor. In fact, the majority of unvaccinated patients believed the vaccination was pricey and did not have medical insurance for the flu shot.

Analysis of patients with chronic illnesses revealed that a statistically significant fraction of them were immunized against the influenza virus, although a significant number of patients were still unvaccinated. Given that influenza vaccination has been shown to decrease hospitalization, morbidity, and death in both the elderly and chronically ill patients, these studies emphasize the need to boost immunization energies in the individual, who suffers from chronic disease.[3-5] Again, immunization has been demonstrated to reduce mortality from influenza-related complications and pneumococcal illnesses in the elderly by 50% and 80%, respectively.[6] Additionally, it has been demonstrated that routine vaccinations given to diabetic children and adults reduce hospital admissions due to diabetes by about 79%.[28]

Knowledge and attitude toward vaccination

Despite a high level of education among the responders, most did not receive an influenza vaccination. Vaccinations were administered to 19% of the subjects. These results are consistent with those of a previous study conducted in Jordan, which showed that 20.4% of the subjects had ever received the vaccine.[29] The study by Zalloum et al.[30] revealed that the COVID-19 pandemic did not boost Jordanians' acceptance of vaccination.

According to the study's findings, 39% of participants do not think influenza is a serious problem, while the remaining participants have concerns about the vaccine's safety. These results are consistent with a recent survey conducted in Jordan, which discovered that 22%, 19.4%, and 18.9% of respondents, respectively, did not consider influenza to be a threat, had doubts about the vaccine's effectiveness, and had misgivings about the vaccine's safety.[29] Similar hurdles have been seen in many places throughout the world; for instance, in the United States, one study found that worries about the safety and effectiveness of the influenza vaccine were the most often cited reasons for vaccination refusal.[31] Additional research revealed justifications for vaccine refusal, including concerns about vaccine-related infections, doubts about its effectiveness, and conviction that the vaccine is unnecessary. In the UK, where safety concerns and fear of side effects were the biggest obstacles to getting the vaccine among competent nurses, similar results were also attained in an earlier study.[32]

Surprisingly, most individuals do not perceive influenza as a threat. This belief is risky because there is a greater chance of infection, especially from aggressive influenza viruses.[33,34] Although most participants were not yet ready for vaccination, they generally had respectable opinions regarding the value

of becoming vaccinated against influenza. This is a key notion since it would be conceivable to encourage them to receive the influenza vaccine to raise Jordan's coverage rate. Because there is a correlation between influenza vaccination rates and influenza mortality rates, encouraging these individuals to get the vaccine could help reduce influenza mortality in high-risk patients.[10,22,23,35] In Italy, a study demonstrated that general practitioners are the most reliable source of information on the yearly influenza vaccination. The current study results display that pharmacists and doctors play a poor role in providing patients with vaccination information. As a result, campaigns should encourage pharmacists and doctors to play an important part in spreading the word about the value of influenza vaccination, particularly considering the COVID-19 pandemic. In addition, the government should make a concerted effort to direct citizens to sources of information about influenza vaccines and emphasize the importance of doctors and pharmacists in educating the public about the vaccine. Campaigns on media to provide the public with information related to the importance of the influenza vaccine are crucial.

Awareness of the influenza vaccine is not sufficient; 18% of unvaccinated participants think that the vaccine is unsafe. Consequently, there should be a harder work to be done to improve awareness and clarify the picture about the safety and efficacy of influenza vaccine. Social media is a platform where portion of people acquire vaccine information. Owing to the inadequate roles played by pharmacists and doctors, this is a genuine reason why some patients experience anxiety after receiving vaccinations. In addition, health care professionals play a very small part in informing the public about the advantages of receiving an influenza vaccination. Physicians in Italy are the most reliable source of information regarding influenza vaccination, in contrast to the situation in Jordan.

The significance of pharmacists' and doctors' roles in educating the public about the value of the influenza vaccine should be emphasized. Furthermore, because Jordan has no national influenza vaccination programs, it is advised to create a financed government program for influenza vaccines.

Source of vaccine information

The primary sources of vaccination information for all study participants were their doctors and pharmacies. This observation emphasizes the importance of healthcare workers in encouraging and reinforcing vaccination faithfulness. Other studies found that healthcare practitioners' instructions for routine immunization were favorable predictors of adherence.[29,36] As a result, physicians, pharmacists, and other healthcare workers are being asked to establish their understanding of the present immunization procedures and recommendations. Surprisingly, the government's function as a foundation of information was discovered to be minor in society.

Jordan's health ministry should be urged to teach the people the value of vaccination and to remove obstacles to vaccine access. Accepting the influenza vaccine as part of the countrywide immunization program, creating awareness operations, and assigning general finances to support it are critical stages.

Study limitations

Our study has interesting results; however, it may have certain limitations. Contributors self-reported their data as in all patient-directed surveys, and vaccination statuses and awareness criteria were scored using binary outcomes. The time of the study may have also influenced the outcomes, as subjects may have been more aware of the importance of the influenza vaccine following a coronavirus attack and during the winter. The study sample was drawn from social media, which may have introduced bias into excluding persons, who have difficulty using electronic devices. Despite the limitations highlighted, the most essential strength of our study is that it was oriented to general adult populations suffering from chronic diseases throughout the kingdom and reported on their periodic vaccination knowledge, attitudes, and practices.

CONCLUSION

To the best of our knowledge, the current survey is the first to document immunization rates between a specified population of Jordanian adults with chronic illnesses. The immunization rates for seasonal flu were relatively low. Chronic disease patients receive subpar vaccinations. The vaccine was considered unnecessary, which is the main obstacle to vaccination abstinence. The recent findings thus highlight the urgent need to increase public awareness of the effectiveness and advantages of seasonal influenza vaccination in Jordan. Enhancing immunization possibilities requires regular interaction with medical professionals. In addition, it is advised that governmental entities and healthcare professionals work together to enhance immunization practices through professional and public awareness initiatives.

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Ethics

Ethics Committee Approval: Ethical approval was obtained from Al-Balqa Applied University's Institutional Review (approval date: 26/3/2/213).

Informed Consent: Participants provided electronic informed consent to participate in the study.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: O.A.B., Design: O.A.B, I.A.B., Data Collection or Processing: O.A.B., E.G., O.A-F., M.A., F.A-T, R.B., Analysis or Interpretation: O.A.B, I.A.B., R.B, F.A-T, M.A., Literature Search: O.A.B, I.A.B., E.G., Writing: O.A.B., I.B, O.A-F.

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ORIGINAL ARTICLE

The Development and Full Validation of a Novel Liquid Chromatography Electrochemical Detection Method for Simultaneous Determination of Nine Catecholamines in Rat Brain

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ABSTRACT

Objectives: Chemical neurotransmission, managed by neurotransmitters, has a crucial role in brain processes such as fear, memory, learning, and pain, or neuropathology such as schizophrenia, epilepsy, anxiety/depression, and Parkinson's disease. The measurement of these compounds is used to elucidate the disease mechanisms and evaluate the outcomes of therapeutic interventions. However, this can be quite difficult because of various matrix effects and the problems of chromatographic separation of analysts. In the current study; for the first time, an optimized and fully validated high-performance liquid chromatography-electrochemical detection (HPLC-EC) method according to Food and Drug Administration and European Medicines Agency Bioanalytical Validation Guidance was developed for the simultaneous analysis of nine neurotransmitter compounds, namely dopamine, homovanilic acid, vanilmandelic acid, serotonin (SER), 5-hydroxyindole-3-acetic acid, 4-hydroxy-3-methoxyphenylglycol, norepinephrine, 3,4 dihydroxyphenylacetic acid, and 3-methoxytyramine and simultaneously determined in rat brain samples.

Materials and Methods: Separation was achieved with 150 mm x 4.6 mm, 2.6 µm Kinetex F5 (Phenomenex, USA) column isocratically, and analysis was carried out by HPLC equipped with a DECADE II EC detector.

Results: The method exhibited good selectivity, and the correlation coefficient values for each analyte's calibration curves were › 0.99. The detection and quantification limits ranged from 0.01 to 0.03 ng/mL and 3.04 to 9.13 ng/mL, respectively. The stability of the analyses and method robustness were also examined in detail in the study, and the obtained results are presented statistically.

Conclusion: The developed and fully validated method has been successfully applied to actual rat brain samples, and important results have been obtained. In the rat brain sample analysis, the lowest number of SER and the highest amount of noradrenaline were found.

Key words: Catecholamine, electrochemical detection, high-performance liquid chromatography, neurotransmitter, rat brain

INTRODUCTION

It is an essential group of biogenic amines that contain monoamines, dopamine (DA), noradrenaline (NA), epinephrine (EP), serotonin (SER), and their metabolites in the central nervous system (CNS).[1,2] Their primary function is neurotransmission with hormonal or neuronal signals during various physiological events. In addition, the amount of monoamines and dysfunction are closely related to various CNS diseases.[3] These monoamines are principally obtained from two amino acids, tryptophan and tyrosine.[4] EP and DA are primary catecholamines, which include metanephrine (ME), vanilmandelic acid (VA), and homovanilic acid (HVA) as emerging metabolites. NA metabolites are HVA and VA. The last pathway is HVA, when transforming into 3-methoxytyramine (3-MT) and 3,4-dihydroxyphenylacetic acid

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(DOPAC). SER synthesized from tryptophan amino acid turns into 5-hydroxyindole-3-acetic acid (5-HIAA).

Chemical neurotransmission is considered as neurotransmitters and affects brain processes such as memory, pain, learning, and fear or neuropathology such as epilepsy, schizophrenia, Parkinson's disease, and anxiety/depression. Therefore, changes in decreasing or increasing regions are the precursors of many mood disorders, diseases, or irreversible events such as tissue deformation.[5,6] Electrochemical detection (EC) reactions are often the basis of these deformations. Because these compounds have a small and straightforward structure, they contain only non-rigid electroactive functional groups.[6] In addition, these properties range from the dominance of EC detectors in their analysis.

In clinical and experimental neuroscience, neurotransmitters are widely studied in different biological samples, such as blood, plasma, urine, dialysates, and supernatants from tissue homogenates. In general, two main methods are used in tissue analysis of neurotransmitters. The samples were collected *in vivo*, and their determinations were made by a selected analytical method. Frequently preferred methods in sample preparation are microdialysis or push-pull perfusion techniques. Their determination is mostly achieved using high-performance liquid chromatography (HPLC). However, most of these compounds do not contain any chromophore groups; therefore, the ultraviolet-visible region or photodiode array detector cannot be used. Instead, two types of detectors are used, mostly mass spectrometry (MS) and electrochemical. EC detector equipment is cheaper and easier to use than MS. In addition, it does not require sample derivatization because of the high electroactivity of catecholamines and indolamine.

Moreover, EC detectors are specific and significantly rarely affected by other analysts' matrix or signal interference. Coulometry or amperometry can be used for EC detection. The main advantage of coulometry is the complete oxidation of the compounds entering the detection cell, resulting in a low limit of detection (LOD). Nevertheless, the technology for miniaturization of cells is currently limited. In contrast, the amperometric cell size can be easily reduced, and when connected to the microhole and capillary columns, amperometry can detect low compound levels despite a weak EC yield (~10%).

One of the works that need to be emphasized is that in 2011, Parrot et al.[7] developed a method for the analysis of NA, DA, and three metabolites using DOPAC, HVA, 3-MT, SER, and its metabolite 5-HIAA, using ultra-high performance liquid chromatography (UPLC)-ECD. For the method, validation studies were performed by selecting the working range of all compounds 10^{-9}–10^{-6} M, and they were applied to rat, cat, and mouse brain tissue.[7] Furthermore, in 2014, benzoyl chloride was used for derivatization for the analysis of cerebrospinal fluids from rat models such as SER, 5-HIAA, HVA, NA, EP, DA, glutamic acid, GABA, and DOPAC by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method.[8] Especially since 2018, the LC-MS/MS method has been developed to investigate the potential therapeutic effect of various diseases and many neurotransmitters, including DA, NA, 5-hydroxytryptamine, acetylcholine, *l*-tryptophan, γ-aminobutyric acid, glutamic acid, and aspartic acid.[9-13] Simultaneous determination in various tissues, such as the mouse brain was accelerated. A simple protein precipitation method is generally used for sample pretreatment. Brain samples were separated by the polar functional group attached column and detected in the LC-MS/MS system equipped with a positive ion in the mass detector and a turbo ion spray source in multiple reaction tracking modes.

The other efficient work is that in 2018, seven compounds, including monoamines and their metabolites, were analyzed by HPLC-ECD.[3] The method was validated to obtain a working range at the ng level and applied to three rat brain regions (posterior-anterior cortex, hippocampus, and striatum). In addition, voltammetry techniques are widely examined in the studies performed until today. However, because of selectivity problems, the analysis was carried out with electrodes modified using various techniques, and very successful results were obtained.[14-16] For example, in the 2014 study, a covalently bound electrode was designed by the condensation reaction of graphene oxide and 5,15-pentafluorophenyl-10,20-paminophenylporpyrin.[17] The modified electrode was used for DA and SER analyses at µM levels.

As seen above, much progress has been made, and studies have been published on analyzing neurotransmitters. The mass detector is particularly promising. However, this detector exhibits Achilles tendon matrix interference in biological fluid and tissue analysis.[18] Sensor work is still in its infancy. In brief, more efficient methods should be produced by developing chromatographic techniques. The purpose of the current study is to perform more compound analysis of neurotransmitters in brain tissue in a shorter time. For this reason, an efficient and promising HPLC-ECD method was developed for the routine analysis of DA, HVA, VA, SER, 5-HIAA, MHPG, NA, DOPAC, and 3-MT in brain samples. All method optimization parameters were examined in detail, and full bioanalytical validation parameters were performed according to international guidelines.[13,19,20] Thanks to the current method, 10 eluents, 9 analyses, and an internal standard (IS) were successfully separated in the required chromatographic efficiency and applied to the rat brain samples.

MATERIALS AND METHODS

Chemicals

The hydrochloride salts; DA, SER, and 3-MT, HVA, VA, 5-HIAA, MHPG, NA, DOPAC, 3,4-dihydroxybenzylamine hydrobromide (DHBA), triethylamine (TEA), ethylene-diamine-tetra-acetic acid (EDTA) disodium salt, 1-octanesulfonic acid (OSA), potassium dihydrogen phosphate, potassium chloride, citric acid, methanol sodium metabisulfite, and perchloric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced using a Milli-Q system (Millipore, Bedford, MA, USA). Whatman cellulose acetate membranes were purchased from Sigma-Aldrich (St. Louis, MO, USA), and PTFE syringe filters were purchased from IsoLab (Wertheim, Germany).

Preparation of the stability solution

The stability solution was used to prepare, store standard and brain solutions, and homogenize brain tissue to ensure adequate neurotransmitter substance stability. The prepared stability solution consisted of 0.1 M perchloric acid and 0.1 mM sodium metabisulfite of water.[21] For this purpose, 4.8 mg of sodium metabisulfite and 2.156 µL of perchloric acid were placed in a 250 mL volumetric flask with distilled water, and the volume was then completed with water.

Preparation of the standard solutions

Each standard was weighed at approximately 1 mg and placed in a volumetric flask (10 mL), and the completed volumes were with the stability solution. Subsequently, they were stored at 4 °C for optimization and validation studies by making necessary dilutions. We examined whether the solutions made any degradation under the storage conditions and how long they remained in the analysis. As a result, the compounds could be safely stored in the freezer and thawed, and they remained intact for a minimum of 60 h during the analysis period.

Preparation of the mobile phase solution

The mobile phase composition for liquid chromatography was prepared in water with 0.07 M KH_2PO_4, 20 mM citric acid, 5.3 mM OSA, 100 mM EDTA, 3.1 mM TEA, 8 mM KCl, and 11% (*v/v*) methanol. The mobile phase was filtered through a 0.22 µM cellulose acetate filter before use. Under these conditions, the mobile phase's measured pH value was optimized as 3.2 ± 0.1 isocratically.

Removal of brain tissue from rats

Approximately 9-10-week-old male Sprague-Dawley rats were used for the experiments. Rats were fed a standard rodent diet and tap water. The animals sacrificed without any experimental procedure to obtain tissue were anesthetized with pentobarbital (60 mg/kg) and decapitated. All animal procedures were performed according to protocols approved by the national ethical requirements for animal research and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU).[22] After each animal was sacrificed immediately, all brain tissue was removed and placed in tubes containing prepared stability solutions. The tissues were quickly frozen with liquid nitrogen and stored in a -40 ºC freezer for EC analysis. The experimental protocols of this study were approved by the Local Ethical Committee on Animal Experimentation of Anadolu University, Eskişehir, Türkiye (Decision no: 2022-12, date: 06.04.2022).

Preparation of the brain tissues

The brain tissue was removed from the freezer and placed in a sonicator bath, allowing it to dissolve quickly. The effect of the sonicator bath on the tissues' dopaminergic substances was tested by applying various parallel sample preparation procedures, and it was found that it did not affect them. The defrosted brain samples were gently removed from the stability solution, accurately weighed, and placed in the homogenization vessel. The brain tissue samples were homogenized in an ice-cold stability solution for approximately 25 min by adding the IS DHBA at a concentration of 61.6 ng/mL concentration. The amount of solvent added *per* milligram of brain tissue is 7.5 µL.[23] All homogenization processes were carried out with Potter S (B. Braun Biotech International, Berlin, Germany) model homogenizer. The homogenates were then sonicated for 10 min, centrifuged at 11,000 rpm at 5 °C for 40 min, and filtered through a 0.22 µm PTFE filter. This process was repeated again, and the filtrate was placed in injection vials and analyzed.

Analysis parameters

The analysis system is equipped with UPLC from Shimadzu (Kyoto, Japan) and a DECADE II EC detector (Antec-Leyden, Netherlands) for chromatographic separation and detection. The system comprises two LC-30AD parallel piston pumps, a SIL-30AC refrigerated autosampler, a CBM-20A system control unit, and a DGU-A5R inline degassing unit. A Decade II EC detector (cell volume 80 nL, Antec, Leyden, Netherlands) equipped with a 2 mm glassy carbon working electrode, an *in situ* Ag-AgCl (ISAAC) reference electrode, a platinum auxiliary electrode, and a 25 µm spacer at 35 °C.

Separation was performed at 35 °C (oven-controlled) using 150 mm x 4.6 mm, 2.6 µm Kinetex F_5 (Phenomenex, USA). The mobile phase is pumped at a flow rate of 0.35 mL/min, and the injection volume is 1 µL. EC transformations at + 800 mV oxidation potential, the signal range is 2 nA, and the filter 0.005 Hz cell temperature was set to 35 ± 0.1 °C as the column temperature.

The evaluation of the results

Peak normalization (PN) techniques were used to investigate the results. The PN method could be used to minimize errors in the analysis. PN values are obtained by dividing the area of the relevant peak by the t_R value. In response to each concentration, the values obtained by dividing the PN values of the analyst and IS, DHBA, were accepted as reasonable responses. Based on the PN values, the concentrations of solutions were applied to method validation and optimization applications.

Method validation

The validation studies were conducted according to the bioanalytical method validation guidelines of the Food and Drug Administration and the European Medicines Agency.[19,20] The method validity practices were applied to the method development, and it was checked that the method provided analytically acceptable data such as specificity, precision, accuracy, linearity, LOD, limit of quantitation (LOQ), and system suitability tests (SST).[24] All statistical calculations were performed with the GraphPad Prism v6.0b (trial version) program.

The SST parameters showing the LC instrument's performance and the column used during the analysis are fundamental in the method development step. Parameters such as resolution (Rs), tailing factor (T_f) and asymmetry factor (As), theoretical plate number (N), capacity factor (k'), and selectivity factor (α) constitute the system suitability parameters.[25] During method development, each of these parameters was calculated using

the Shimadzu LCsolution v1.11 SP1 data analysis program, according to the method of the United States Pharmacopeia.

Selectivity

The presence of any matrix component that could interfere with each analyst signal in the detector was investigated. Each analyte's selectivity studies were conducted at three concentration levels on rat brain sample homogenates (80%, 100%, 120%).

Linearity

For the linearity studies; all analyses were prepared at six concentration levels (20%, 50%, 80%, 100%, 120%, and 150%). Linearity was investigated between intra-day and inter-day slopes, and correlation coefficients and intercepts at 95% confidence levels were calculated using linear regression analysis.

Accuracy

For accuracy studies, low, medium, and high concentrations (80%, 100%, and 120%) of neurotransmitter standard substance were added to the brain tissue solution before analysis. Three sets were prepared and analyzed for each concentration level, and the recovery values were calculated.

Precision

Precision studies include intra-day and inter-day measurements. The analyst solutions used in the linearity studies (80%, 100%, and 120%) were analyzed three times within the same day and for three consecutive days. In the statistical evaluation of the results, the mean, standard error of the mean, standard deviation (SD), and relative SD are given at 95% confidence level.

LOD and LOQ

According to the ICH recommendations for LOD and LOQ values, the ratio obtained in the calculations using the signal/noise ratio is accepted as 3 and 10, respectively. On the other hand, the LOD was calculated using the given in equation 1 for this study. This is recommended in the user manual of detector.[26]

$$C_{LOD} = \frac{3\sigma_{noise}}{signal} C_A \quad (1)$$

σ_{noise} is the length of the 0.2 x noise peak, and C_A is the analyte concentration injected.

Stability

The stability of the standard solutions was examined for one month. For this purpose, the standard solution mixture was analyzed with the developed method for one week, three weeks, and one month.

RESULTS

Stability of the analyte solutions

The extraction of neurotransmitters from tissues and cells was performed by homogenization and sonication in stability solutions. The stability solution damaged the tissues, revealed the target compounds, and stabilized the neurotransmitter compounds. In previous studies, perchloric acid is the most active compound in the extraction of neurotransmitters.[27] While it provides efficient extraction by adsorption of perchlorate ions in the perchloric acid structure, it also contributes to stabilizing the compounds because of its acidic pH value. Because acidic pH is stable due to neurotransmitters' structure, and as the pH increases, they undergo oxidation effortlessly.[28] Another component of the stability solution is a preservative with antioxidant activity. In other studies, ascorbic acid could be an antioxidant component but not sufficient alone, and bisulfite ion or EDTA was added.[29] Sodium metabisulfite was preferred in the current analysis because its preservative feature alone is sufficient. Moreover, the stability solution had to be selected to minimize analyte loss by preventing tissue damage and should not lead to any loss or increase of the detector signal. This mixture was found by using the literature because of various stability trials.

The effect of the stability solution on brain tissue was also studied in detail. For this purpose, fresh brain tissue taken from the rat immediately after decapitation was divided into two symmetrical parts. Brain tissue was immediately homogenized and analyzed. The other brain tissue, subjected to the same experimental treatment, was immediately placed in the stability solution and frozen for analysis later with liquid nitrogen. Then, this tissue was prepared and analyzed according to the procedure mentioned above, and it was calculated whether there was a significant difference between the analysis of fresh brain tissue. Because of the analyses, it was seen that there was no significant difference.

Chromatographic separation

The separations were first made in the reverse phase of the Ascentis Express (St Louis, MO, USA) brand 10 cm x 4.6 mm with a C_{18} functional group produced with core-shell silica technology with a 2.7 µm particle diameter operating at high efficiency. However, due to the increasing number of analyses after a while, the column had poor separation, and method optimization was continued with Kinetex F5 (Phenomenex, USA) brand 15 cm x 4.6 mm, 2.6 µm column filled with core-shell silica particles with F_5 functional group. Both columns' features are compared, particularly in Table 1. The chromatographic separation studies presented that column functional group change did not have much effect on the retention time of the compounds, whereas the separation efficiency significantly increased. In this effect, of course, the contribution of using a 15 cm Kinetex F5 core-shell column instead of the 10 cm long column of Ascentis Express cannot be ignored. Although it had no dominant effect on the peak morphology, it showed excellent performance separating of structures close to each analyte. However, as the SER and 3-MT eluents, already quite late in the previous short column, had slightly higher retention times in the long column, an increase in tailing was observed in their peak morphology.

It is observed that the compounds with free carboxylic acid in their structure were eluted first and had short retention times.

The retention times began to increase as the free amine group existed. In particular, SER, which does not contain any methyl or carboxylic acid in the structure, was the last to leave the column, while 3-MT with a free methyl group remained in the column for approximately 9 min. The mixture standard solution chromatogram obtained is given in Figure 1. We also checked whether the stability solution gave any peaks by injecting, as shown in Figure 2. In addition, SST is applied to show the performance of the LC device and method. In these tests, parameters such as N, R_s, T_f, A_s, α, and k' were calculated and are given in Table 2.

Method development

The purpose of this study was to develop a method for the separation and determination of DA, HVA, VA, SER, 5-HIAA, MHPG, NA, DOPAC, 3-MT, and DHBA for the presence of many interfering compounds in the rat brain. Nine neurotransmitter compounds were analyzed by EC detection reverse-phase ion-pair chromatography, DHBA was used as an IS, and thus, the unpredictable factors internal or external were eliminated. In experiments with matrix effects, such as biological analyses, the use of an IS is highly recommended. Today, with the development of analytical instruments, lower detection and quantitation limits can be obtained, and the effect of various attempts from interference components becomes more critical. DHBA is the most preferred IS in the analysis of neurotransmitters. Structural similarity and acting similar to catecholamines in chromatographic separation are the main reasons for preference. While calculating the analytical data, the error was minimized using the PN method, which considers both the retention time of the analyst and IS and their peak areas.

Table 1. Features of Ascentis Express C_{18} and Kinetex F5 columns

Properties	Ascentis® Express	Kinetex F5
Particle size (µm)	2.7	2.6
Functional group	C_{18}	F_5
Macropor/mesopor size (µm)	10	0.35
Core size (µm)	2.7	1.9
Surface area (m^2/g)	450	200
Carbon load (%)	25	9
Total permeability (m^2) (k_0)	-	5.81×10^{-11}
Pore volume/diameter (Å)	90	100
Range of pH	2-9	1.5-8.5
Surface coating (µmol/m^{-2})	3.7	2.8

The effect of each of the mobile phase components on separation is examined in detail using various mobile phases in the literature.[7] According to the results obtained, EDTA reduces the noise by holding free metal ions and provides a lower level of LOD and LOQ values, while achieving a smoother baseline. However, no effect of increasing after a specific EDTA concentration was observed. The EDTA concentration was chosen at this breaking point. KCl was used in the analyses as the reference electrode. This system, called *in situ* Ag-AgCl (ISAAC), was designed as EC cell T-, and the KCl concentration was determined according to the potential to be analyzed. Such reference electrodes are very stable but have the disadvantage that the EC reaction media volume is relatively small. Although the diffusion increases, the mobile phase prepared must be renewed in 48 h at the latest.[30] KH_2PO_4 and citric acid were used to control the pH of the mobile phase. It was observed that the optimum pH value for buffering was approximately 3.2. This pH value is around the pK_a value of the analytes to be distinguished. Lower acidity values can provide more stable and better interaction but, at the same time, reduce the life of

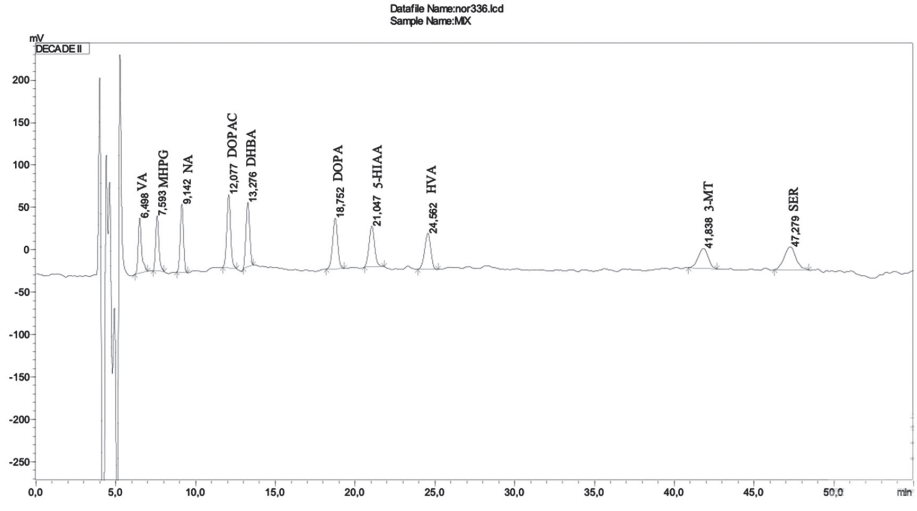

Figure 1. The chromatogram of the standard mixture (VA 89.60 ng/mL, MHPG 14.43 ng/mL, NA 148.0 ng/mL, DOPAC 35.30 ng/mL, DOPA 34.80 ng/mL, 5-HIAA 27.40 ng/mL, HVA 42.30 ng/mL, 3-MT 5.50 ng/mL and SER 0.186 ng/mL) in optimized conditions

VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, NA: Noradrenaline, DOPAC: 3,4 Dihydroxyphenylacetic acid, DOPA: 3,4-Dihydroxyphenylalanine, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin

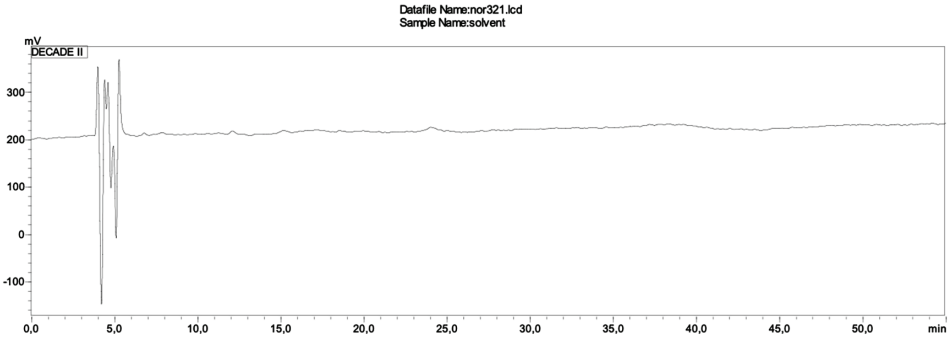

Figure 2. The chromatogram of the stability solution recorded under the optimized conditions.

Table 2. SST results obtained in the analysis of monoamines in the Kinetex F5 column

Parameter	VA	MHPG	NA	DOPAC	DHBA	DOPA	5-HIAA	HVA	3-MT	SER	Recommended
T_R	6.49	7.59	9.14	12.07	13.27	18.73	21.03	24.55	41.75	47.22	
SD of T_R	0.07	0.01	0.01	0.01	0.21	0.03	0.03	0.02	0.13	0.08	
N	35.504	40.818	48.736	61.713	73.442	109.916	100.321	98.880	106.521	91.375	> 2.000
HETP	28.166	24.499	20.519	16.204	13.616	9.098	9.968	10.113	9.388	10.944	-
T_f	1.410	1.255	1.046	1.184	1.206	0.921	1.023	1.007	1.891	1.063	< 2
A_s	1.137	1.105	1.18	1.161	1.050	1.13	1.052	0.991	1.200	1.030	0.95 < A_s <1.2
k'	0.235	0.443	0.737	1.295	1.521	2.560	2.998	3.667	6.927	7.973	2 < k < 10
α	1.887	1.663	1.757	1.175	1.683	1.171	1.223	1.889	1.151	1.530	> 1
R_s	3.683	2.946	3.791	6.313	2.364	10.04	3.637	4.712	16.136	3.753	> 2
USP width	0.356	0.388	0.427	0.501	0.505	0.583	0.685	0.806	1.318	1.611	-

SST: System suitability test, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, T_R: Retention time, SD: Standard deviation, N: Number of theoretical plate, T_f: Tailing factor, HETP: Height equivalent of theoretical plate, As: Asymmetry factor, k': Capacity factor, α: Selectivity factor, R_s: Resolution, USP: United States Pharmacopeia, NA: Noradrenaline

the stationary phase. It should be kept in mind that the optimum working values of silica-based columns are in the acidity range of 3-7. In addition, citric acid acted as an ion-pairing reagent with OSA, which was observed as the lead ion-pair reagent in optimization studies. It regulated the peak morphology and increased the eluent column interactions. As the OSA concentration increased, it provided more sharp peaks, and because of its micelle feature, it also increased the viscosity of the mobile phase.

Another antitailing additive component is TEA. In the mobile phase, two different types of anti-tailing components were used because the dominant functional group of some neurotransmitters was carboxylic acid and some were amines. Analytes with amine groups interact with TEA, and those with carboxyl groups interact with OSA. Another critical point was the effect of the anti-tailing mobile phase additive on the mobile phase's pH. TEA is a strong-basic substance, its pK_a 10.2, and while the concentrations of TEA were increased, the mobile phase pH was tried to stabilize at 3.2. For the organic phase of the mobile phase, methanol and acetonitrile were examined for optimum separation. Acetonitrile rapidly eluted all compounds from the column, and its separation efficiency was inferior. On the other hand, methanol provided a slower elution rate, increased the interaction of the stationary phase with analysts, and optimum separation efficiency was obtained.

An EC detector detected signals with the flow cell VT-03. The cell's characteristics, which have a three-electrode configuration, are as follows: glassy carbon working electrode (3.00 mm in diameter and 0.18 µL internal volume with 25 µm spacer seal), platinum auxiliary electrode, and *in situ* Ag-AgCl (ISAAC) comparison electrode.

Method validation

Linearity/calibration curve

One of the most valuable experiences we gained from our studies with the EC detector is that it is not difficult to achieve excellent linearity, when optimum conditions are provided. This study evaluated the calibration curve with the linear regression value at the ng/mL level. The correlation coefficient (R^2) of linearity greater than 0.99 was consistently good for all determinations in the respective calibration ranges (Table 3).

Table 3. Linearity studies (20-150%)

	VA	MHPG	NA	DOPAC	DOPA	5-HIAA	HVA	3-MT	SER
Linearity range (ng/mL)	17.9-134.4	2.9-21.65	29.6-222	7.1-52.9	6.9-134.4	5.5-41.1	8.5-63.5	1.1-8.25	0.037-0.279
Slope ± SD (intraday, n: 6, ratio)	0.0143 ± 0.0004	0.0954 ± 0.0043	0.00821 ± 0.0001	0.02943 ± 0.0008	0.01889 ± 0.0004	0.0192 ± 0.0006	0.01137 ± 0.0004	0.04591 ± 0.003	1.4474 ± 0.067
Intercept ± SD (intraday, n: 6, mA)	0.1508 ± 0.034	0.10182 ± 0.061	0.01756 ± 0.016	0.15497 ± 0.029	-0.00157 ± 0.014	-0.01537 ± 0.016	-0.01474 ± 0.017	0.00158 ± 0.0157	0.00716 ± 0.012
Regression coefficient (intraday, n: 6)	0.9970	0.9917	0.9992	0.9968	0.9982	0.9959	0.9947	0.9937	0.9915
LOD (ng/mL)	2.15	0.02	3.04	0.71	1.02	1.01	1.64	0.36	0.01
LOQ (ng/mL)	6.45	0.06	9.13	2.12	3.06	3.03	4.93	1.08	0.03

SD: Standard deviation, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, LOD: Limit of detection, LOQ: Limit of quantitation, NA: Noradrenaline

Table 4. Recovery results (n: 3)

Compound	Concentration (ng/mL)	% recovery (mean ± SD)	% difference
VA	71.70	98.15 ± 1.43	-1.85
	89.60	99.03 ± 0.15	-0.97
	107.5	98.83 ± 0.78	-1.17
MHPG	11.50	99.25 ± 1.01	-0.75
	14.43	99.13 ± 0.84	-0.87
	17.30	98.46 ± 0.56	-1.54
NA	118.4	99.75 ± 0.77	-0.25
	148.0	98.89 ± 1.07	-1.11
	177.6	97.99 ± 0.06	-2.01
DOPAC	28.20	99.01 ± 0.28	-0.99
	35.30	98.53 ± 0.72	-1.47
	42.40	98.78 ± 1.14	-1.22
DOPA	27.80	101.1 ± 0.52	+1.10
	34.80	99.56 ± 0.45	-0.44
	41.80	100.8 ± 0.34	+0.80
5-HIAA	21.90	98.82 ± 0.67	-1.18
	27.40	99.14 ± 0.43	-0.86
	32.90	100.3 ± 1.00	+0.30
HVA	33.80	98.56 ± 1.09	-1.44
	42.30	99.20 ± 0.89	-0.80
	50.80	101.2 ± 0.63	+1.20
3-MT	4.40	99.76 ± 0.47	-0.24
	5.50	98.44 ± 1.07	-1.56
	6.60	97.14 ± 0.35	-2.86
SER	0.149	96.58 ± 1.20	-3.42
	0.186	97.15 ± 0.64	-2.85
	0.223	99.26 ± 1.32	-0.74

SD: Standard deviation, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, NA: Noradrenaline

Quantitative and detection limits

LOD and LOQ were calculated for all analyses according to the method section. The obtained results are given in Table 3. The lowest quantitation limit belongs to NA, and SER is the highest.

Recovery

The precision and accuracy results of the three calculated concentrations are given in Table 4. Both precision and accuracy results were within acceptable limits according to the validation parameters. For the three concentrations, SD was lower than 1.5 for each compound. The accuracy of all concentration levels

for all analyses tested was 96-100%. The average absolute recovery for each analyst measured in triplicate for all three concentration was consistently above 95%.

Stability

The stability results of the standard solution and three rat brain tissue samples are shown in Table 5. When all monoamines and their metabolites were injected at 24 and 48 h, they were stored at autosampler 5 °C. As seen in the results, there was no significant change in any analyst signal for the standard solution and brain samples.

Table 5. Stability studies of catecholamines (n: 3)

% stability of compounds (mean ± SD)			
		24 h	48 h
Standard solution	VA	99.89 ± 1.46	99.85 ± 1.86
	MHPG	99.81 ± 0.57	99.73 ± 1.35
	NA	99.09 ± 1.66	98.68 ± 1.90
	DOPAC	99.03 ± 1.89	98.85 ± 2.18
	DOPA	99.32 ± 1.16	99.18 ± 1.65
	5-HIAA	99.34 ± 1.28	99.26 ± 1.76
	HVA	99.05 ± 1.05	98.86 ± 0.63
	3-MT	100.52 ± 0.76	99.72 ± 0.83
	SER	99.13 ± 0.56	99.72 ± 0.84
Rat brain tissue	VA	99.78 ± 0.08	97.48 ± 1.14
	MHPG	99.11 ± 0.77	99.11 ± 0.34
	NA	98.21 ± 0.29	98.23 ± 2.39
	DOPAC	97.45 ± 0.58	99.89 ± 1.02
	DOPA	99.75 ± 1.81	98.01 ± 0.27
	5-HIAA	99.59 ± 0.26	98.98 ± 0.45
	HVA	96.23 ± 2.39	93.46 ± 3.57
	3-MT	97.62 ± 0.36	99.20 ± 0.16
	SER	98.23 ± 0.07	97.86 ± 1.28

SD: Standard deviation, VA: Vanilmandelic acid, MHPG: 4-Hydroxy-3-methoxyphenylglycol, DOPA: 3,4-Dihydroxyphenylalanine, DOPAC: 3,4-Dihydroxyphenylacetic acid, DHBA: 3,4-Dihydroxybenzylamine hydrobromide, HIAA: Hydroxyindole-3-acetic acid, HVA: Homovanilic acid, MT: Methoxytyramine, SER: Serotonin, NA: Noradrenaline

Sample applications

The optimized and validated method was applied to three rat brain tissues. Brain samples from three rats were collected, prepared, and analyzed according to the above-mentioned procedure. IS was used to calculate each analyte's concentration in brain tissue samples.[31] The chromatogram of brain tissue is shown in Figure 3. According to available data, in rat brain tissue, VA was 28.20 ng, MHPG was 5.59 ng, NA 147.15 ng, DOPAC 11.07 ng, DOPA 81.95 ng, 5-HIAA 48.89 ng, HVA 10.68 ng, 3-MT 1.71 ng, and SER 0.30 ng *per* µg tissue was found. The results obtained are also compatible with the literature data.[32,33]

CONCLUSION

For the first time, in this study, an optimized and fully validated method was developed for the simultaneous analysis of nine neurotransmitter compounds, DA, HVA, VA, SER, 5-HIAA, MHPG, NA, DOPAC, and 3-MT, in the rat brain The Kinetex F5 column separated successfully, and the DECADE II EC detector determined the signal of the target compounds. DHBA was used as an IS. The most important advantages of this study are; fast and efficient, does not require sophisticated sample pre-treatment procedures, can provide the stability of analysts, and is analytically evaluated. In the study, all parameters affecting the separation of neurotransmitters were examined in detail. In addition, the applicability of the method was demonstrated by the analysis of three rat brains.

The study's weaknesses could be that the separation efficiency of stationary phases with different structures cannot be examined, and more brain sample applications cannot be performed. Although it is possible to reduce the analysis time by using stationary phases with a shorter length and smaller particles called UPLC columns, it will be useful to consider that such columns in ECD systems are short-lived, and the routine analysis cost increases significantly.

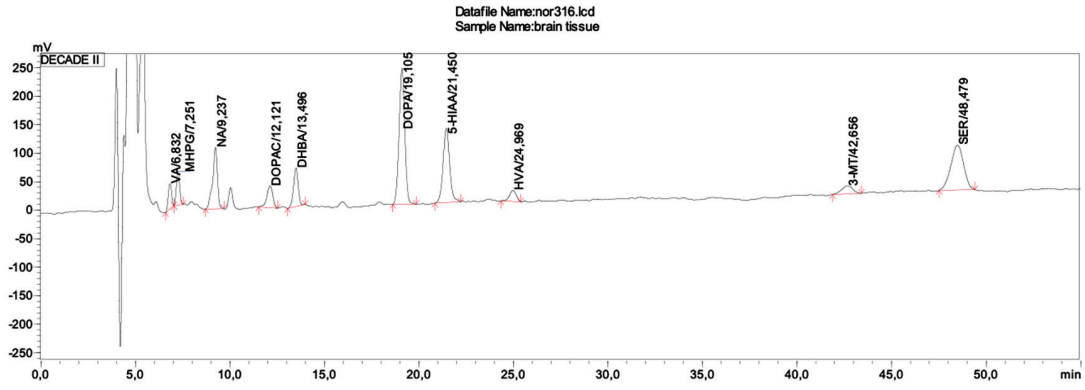

Figure 3. Brain tissue chromatogram obtained under optimized analysis conditions

Ethics

Ethics Committee Approval: The experimental protocols of this study were approved by the Local Ethical Committee on Animal Experimentation of Anadolu University, Eskişehir, Türkiye (decision no: 2022-12, date: 06.04.2022).

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.Ö., Concept: S.Ö., N.Ö.C., Design: S.Ö., N.Ö.C., Data Collection or Processing: S.Ö., A.G., M.K., Analysis or Interpretation: S.Ö., A.G., Literature Search: S.Ö., M.K., Writing: S.Ö.

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

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ORIGINAL ARTICLE

Ultrasound- and Vortex-Assisted Dispersive Liquid-Liquid Microextraction of Parabens from Personal Care Products and Urine, Followed by High-Performance Liquid Chromatography

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ABSTRACT

Objectives: Parabens, which are *p*-hydroxybenzoic acid esters, are used as preservatives in personal care products, pharmaceuticals, and food because of their antimicrobial activity. However, they are also classified as suspected endocrine disruptors and carcinogens. In the present study, we aimed to optimize an ultrasound and vortex-assisted dispersive liquid-liquid microextraction (DLLME) procedure for the simultaneous extraction of methyl, ethyl, isopropyl, propyl, isobutyl, and butyl parabens from personal care products and urine.

Materials and Methods: The extraction solvent type, extraction solvent volume, disperser solvent volume, sodium chloride concentration, ultrasonication time, and vortex application time were evaluated to obtain optimum recoveries by ultrasound and vortex-assisted DLLME. Parabens were detected using a validated high performanc-liquid chromatography (HPLC) method with fluorescence detection. Method validation was performed by examining linearity, the limit of detection, limit of quantification, accuracy, and precision.

Results: The limits of detection and quantification of the HPLC method were between 0.09-0.18 µg/mL and 0.28-0.54 µg/mL, respectively. Precision was examined as the relative standard deviation, which was 0.22-1.81% and 1.12-2.03% for intra- and interday studies. Recovery percentages were higher than 96.00%. Samples of two paraben-free personal care products and synthetic urine were spiked with the analyses at 0.02 µg/mL and were successfully analyzed using the developed procedure with recovery values higher than 82.00%.

Conclusion: The proposed procedure provided quantification of selected parabens at 20 ng/mL in analyzed personal care products and urine matrices with good precision and accuracy.

Key words: High-performance liquid chromatography, liquid-liquid microextraction, paraben, personal care product, urine

INTRODUCTION

Parabens, which are *p*-hydroxybenzoic acid esters, are widely used in various types of food, pharmaceuticals, and personal care products as preservatives because of their antimicrobial activity within a wide pH range, high stability, water solubility, and low cost. Among them, methylparaben (MP), ethylparaben (EP), propylparaben (PP), and butylparaben (BP) are mostly used individually or as mixtures.[1,2] However, recent studies have shown the affinity of parabens for binding to estrogen.[3,4] Their estrogenic effect was assumed to be able to cause breast cancer.[5,6] In addition, some negative impacts on the male reproductive system were reported.[7] Regarding the related research, parabens are classified as suspected endocrine disruptors and carcinogens.

One of the ways of exposure to parabens is through personal care products containing various types of parabens as preservatives because parabens are absorbed through the skin.[8] The maximum permitted level for PP and BP is 0.14%, when

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used individually or together with other esters in cosmetics by the European Commission. The use of isopropylparaben (IPP), isobutylparaben (IBP), phenylparaben, benzylparaben, and pentylparaben has been restricted.[9]

The reliable analysis of parabens has become an issue of great scientific interest because of their suspected damage to human health. Various types of pre-treatment techniques have been developed for the pre-concentration or extraction of parabens, considering low concentrations and complex sample matrices.[8,10,11] Among them, dispersive liquid-liquid microextraction (DLLME) is a common technique with advantages, including the requirement of a lower extraction solvent volume, lower sample amount, and less time-consumption compared with traditional procedures. The extraction solvent is immiscible with the sample solution, and the disperser solvent is used to obtain better contact between them.[12] In a previous study, MP, EP, PP, and BP were successfully extracted by DLLME from breast milk.[8] de Oliveira et al.[13] also determined 17 potential endocrine-disrupting chemicals, including MP, EP, PP, BP, and benzylparaben, by DLLME coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) in human saliva. In another study, DLLME of MP, EP, PP, and BP from pharmaceuticals and personal care products was performed.[14]

Parabens are generally extracted from various matrices using chlorinated solvents, which may negatively affect human health and are not environmentally friendly.[13,14] A special technique of DLLME, called ultrasound and vortex-assisted DLLME (USVADLLME), was developed by which the required volume of hazardous extraction solvents was reduced. In the USVADLLME procedure, ultrasonication provides better dispersion, and vortexing prevents biphasic system formation.[15]

High-performance liquid chromatography (HPLC)[16,17] and gas chromatography (GC)[18,19] are two common methods for the detection of parabens in different types of sample matrices such as food products,[20] biological fluids,[16,17,21] environmental samples,[22,23] pharmaceuticals,[24,25] and personal care products.[26,27] Among them, GC methods may require steps of derivatization or preconcentration. HPLC with ultraviolet (UV) or diode array detection has disadvantages, such as interference of other ingredients and high detection limits. LC-MS or LC-MS/MS may avoid all of these drawbacks, but these systems are unavailable in many laboratories because of their high costs. On the other hand, HPLC with fluorescence detection (FD) may also be used because it has higher selectivity than UV detection and is more available than MS systems. An HPLC-FD method was developed, validated, and applied successfully for four types of parabens, namely MP, EP, PP, and BP, in cosmetic products in a recent work.[28] In addition, Yılmaz and Tokat[29] developed a method for MP, EP, PP, IBP, and benzyl paraben (BzP) using HPLC-FD in cosmetics.

In the present study, we aimed to optimize a USVADLLME procedure for extracting six parabens (MP, EP, IPP, PP, IBP, and BP) (Figure 1) from personal care products and synthetic urine. For the quantification of the extracted parabens, an HPLC-FD method was developed and validated according to the following parameters: linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and the advantages of FD mentioned above. The proposed USVADLLME technique has never been used for extracting selected analysts. To the best of our knowledge, the proposed work is the first USVADLLME coupled with the HPLC-FD method for the detection of parabens in personal care products and urine.

MATERIALS AND METHODS

Reagents and chemicals

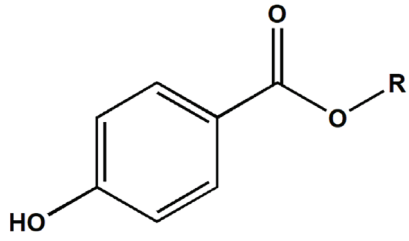
Standard materials of MP, EP, IPP, PP, IBP, BP, and synthetic urine (Surine™ Negative Urine Control) were purchased from Sigma (Darmstadt, Germany). HPLC grade methanol (MeOH), *o*-phosphoric acid, sodium chloride (NaCl), dichloromethane (CH_2Cl_2), and chloroform ($CHCl_3$) were obtained from Merck (Darmstadt, Germany). The chemicals were of analytical grade. A stock solution at 100.00 µg/mL for each analyte was prepared with MeOH (HPLC grade) and stored at 4 °C. The stock solution was diluted daily with the mobile phase to obtain standard paraben solutions at the desired concentrations. The paraben-free tonic sample (T) and the paraben-free micellar water sample (MW) were obtained from a commercial supplier in İstanbul, Türkiye (2018).

HPLC conditions

The analysis of the parabens was performed by an HPLC system (LC20AT, Shimadzu, Kyoto, Japan) with FD (RF20A). Analytes were separated using a C18 analytical column (4.6 x 250 mm, 5.0 µm) (Intersil ODS-3, GL Sciences Inc., Tokyo, Japan). The mobile phase system consisted of 50% phosphate buffer (0.1 M, pH 7) and 50% MeOH. Isocratic elution was applied at 1.0 mL/min. The column temperature was 40 °C. The excitation and emission wavelengths were 254 and 310 nm, respectively. The injection volume was set to 20 µL. Data analyses were performed using LabSolutions software (version 1.25).

USVADLLME procedure

A total of 150 µL of $CHCl_3$ and 50 µL of MeOH were transferred into a conical-bottom glass test tube with a screw cap containing 5 mL of sample solution. NaCl was then added (2.0 g/L). The



R: CH_3, methyl paraben
R: C_2H_5, ethyl paraben
R: C_3H_7, propyl and isopropyl paraben
R: C_4H_9, butyl and isobutyl paraben

Figure 1. Chemical structures of the analyzed parabens

solution was vortexed (VTX-3000L, Harmony, Tokyo, Japan) for 4 min and ultrasonicated for 90 s (Elma Hans Schmidbauer GmbH & Co. KG, Siegen, Germany). Following ultrasonication, the solution was centrifuged for 3 min at 4,000 rpm (VWR Compactstar CS4, VWR International Ltd, Leicestershire, UK). A microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used to separate the $CHCl_3$ phase, which was then evaporated under N_2 flow. The residue was dissolved in 100 µL of the mobile phase before HPLC analysis. The extraction procedure was performed in triplicate for all analyzed samples.

RESULTS AND DISCUSSION

The selection of HPLC conditions

Various mobile phase types were examined using MeOH, acetonitrile, acetic acid, formic acid, and phosphate buffers for the suitable separation of the parabens. A mobile phase system consisting of 50% phosphate buffer (0.1 M, pH 7) and 50% MeOH with isocratic elution was selected, considering the parameters of baseline drift, retention time, and resolution. An excitation wavelength of 254 nm and an emission wavelength of 310 nm were selected to obtain optimum signals for all analytes.

Method validation

Validation of the proposed HPLC-FD method was evaluated using the parameters of linearity, LOD, LOQ, precision, and accuracy. A representative chromatogram of the analytes (2.50 µg/mL) is shown in Figure 2.

A 6-point calibration curve was prepared for each paraben (0.50-10.00 µg/mL). The linearity was examined using regression results. Suitable linearities were obtained for all analytes ($r > 0.99$) (Table 1).

LODs were calculated as 3.3 times, whereas LOQs were determined as 10 times the standard deviation/slope ratio of the calibration curve. The LODs were in the range of 0.09-0.18 µg/mL, and LOQs were between 0.28-0.54 µg/mL. The analytical figures of merit for parabens are given in Table 1.

Precision was examined by intra- and interday studies at 0.50, 2.50, and 10.00 µg/mL. The standard solutions at three concentration levels were analyzed in triplicate consecutively on first day and in triplicate on three different days (Table 2). The results were calculated as the percentage of relative standard deviation (RSD %). The accuracy was determined as the recovery percentage (%) (Table 2). All %RSD values were lower than ≤ 2.03, and the recoveries were higher than 96.00.

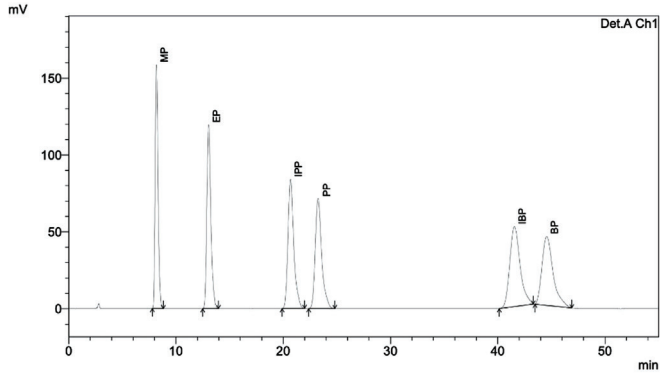


Figure 2. Representative HPLC-FD chromatogram of the analyzed parabens (at 2.50 µg/mL)

HPLC: High performance liquid chromatography, FD: Fluorescence detection

Optimization of the USVADLLME procedure

To optimize the USVADLLME procedure, extraction solvent type, extraction solvent volume, disperser solvent volume, NaCl concentration, ultrasonication, and vortex times were examined. All trials were performed in triplicate. The extraction recovery values were evaluated to determine the optimal extraction conditions.

Optimization of extraction solvent type and volume

A literature survey revealed that chlorinated organic solvents were effective for the extraction of parabens from various sample matrices.[13,14] The extraction capabilities of CH_2Cl_2 and $CHCl_3$ were examined. The selected solvents match the criteria for liquid-liquid extraction because they have higher density than the sample solutions, are poorly soluble in the sample solutions, and are volatile enough to be easily separated. The extraction trials were performed with the standard solution containing each analyte at a concentration 0.02 µg/mL. $CHCl_3$ provided better extraction recoveries for all the analytes (Figure 3). Different volumes of $CHCl_3$ as 100, 150, and 200 µL were used to determine the optimum extraction solvent volume and 150 µL provided almost the same extraction performance with 200 µL (Figure 4).

Optimization of the disperser solvent volume

A cloudy solution of the sample and the extraction solvent is formed using the disperser solvent, which determines the degree of dispersion. The disperser solvent was MeOH because of its good dispersing ability in mixtures of water and $CHCl_3$. The extraction trials were performed with 25, 50, and 100 µL of MeOH, and 50 µL of MeOH was suitable for complete dispersion with similar recovery results obtained with higher volumes (Figure 5).

Optimization of NaCl concentration

The presence of NaCl lowers the solubility of the parabens in the aqueous phase by the salting-out effect. Trials were performed without the addition of NaCl and with the addition of NaCl at concentrations of 2 g NaCl/L and 3 g NaCl/L. The optimum concentration was 2 g NaCl/L (Figure 6).

Optimization of ultrasonication and vortex times

The time ranges of 30, 60, and 90 seconds (sec) were examined to determine the optimum ultrasonication time for high recovery of the parabens, whereas the effect vortex time was examined at 2, 4, and 6 min. Recoveries higher than 80.00% were achieved with an ultrasonication time of 90 sec and vortex time of 4 min (Figures 7, 8).

Real sample analysis

The developed procedure was used for the extraction and determination of six parabens simultaneously in a cosmetic T,

Table 1. Analytical figures of merit for parabens

Analyte	t_R (min)[a]	Calibration range (µg/mL)	Linear equation	r	LOD (µg/mL)	LOQ (µg/mL)	Tailing factor (t)	Resolution (R_s)
MP	8.168 ± 0.004	0.50-10.00	y: 1114911 x 105530	0.9972	0.10 ± 0.01	0.30 ± 0.02	1.250 ± 0.009	-
EP	13.031 ± 0.010	0.50-10.00	y: 1209791 x 320800	0.9956	0.10 ± 0.02	0.30 ± 0.03	1.293 ± 0.010	8.459 ± 0.013
IPP	20.660 ± 0.013	0.50-10.00	y: 1205857 x 129613	0.9973	0.10 ± 0.02	0.30 ± 0.02	1.325 ± 0.017	9.506 ± 0.032
PP	23.229 ± 0.017	0.50-10.00	y: 1134769 x 123760	0.9973	0.09 ± 0.01	0.28 ± 0.01	1.356 ± 0.012	2.595 ± 0.011
IBP	41.523 ± 0.037	0.50-10.00	y: 1329085 x 158082	0.9970	0.12 ± 0.03	0.36 ± 0.03	1.273 ± 0.017	13.343 ± 0.099
BP	44.521 ± 0.042	0.50-10.00	y: 1248312 x 164808	0.9976	0.18 ± 0.03	0.54 ± 0.03	1.447 ± 0.024	1.709 ± 0.007

[a]Mean ± SD, n: 6, SD: Standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben, LOD: Limit of detection, LOQ: Limit of quantification

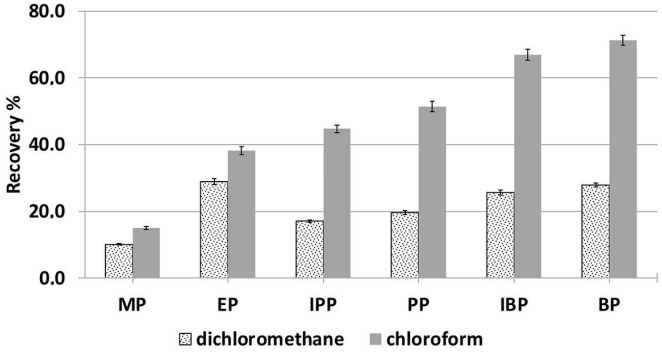


Figure 3. Optimization of the extraction solvent type. Disperser solvent (MeOH) volume, 50 µL; extraction solvent volume, 100 µL; ultrasonication time, 30 sec; vortex time: 4 min. n: 3, RSD values were in the range of 2.12-3.05%

MeOH: Methanol, RSD: Relative standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben

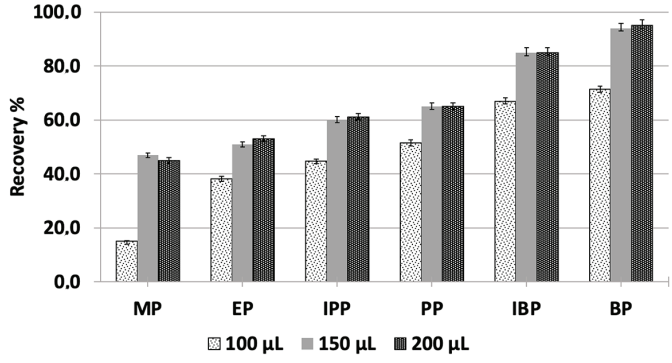


Figure 4. Optimization of the extraction solvent volume. Extraction solvent, $CHCl_3$; disperser solvent (MeOH) volume, 50 µL; ultrasonication time, 30 sec; vortex time: 4 min. n: 3, RSD values were in the range of 1.59-2.25%

$CHCl_3$: Chloroform, MeOH: Methanol, RSD: Relative standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben

MW, and synthetic urine sample. The conditions were optimized as: extraction solvent volume, 150 µL; dispersing solvent volume, 50 µL, NaCl concentration, 2 g/L; ultrasonication time, 90 sec; vortex time, 4 min. Because the samples were paraben-free, they were spiked at 0.02 µg/mL before the extraction procedure. The extraction procedure and HPLC-FD analyses

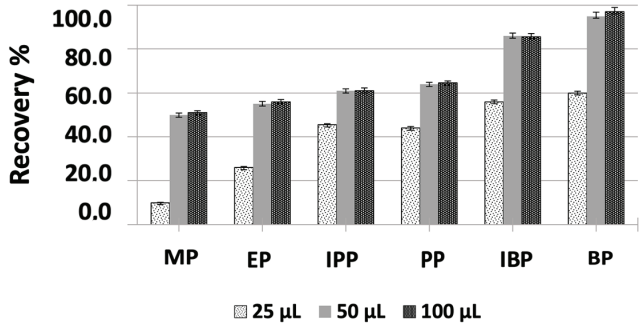


Figure 5. Optimization of the disperser solvent volume. Extraction solvent ($CHCl_3$) volume, 150 µL; disperser solvent, MeOH; ultrasonication time, 30 sec; vortex time: 4 min. n: 3, RSD values were in the range of 1.00-2.10%

$CHCl_3$: Chloroform, MeOH: Methanol, RSD: Relative standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben

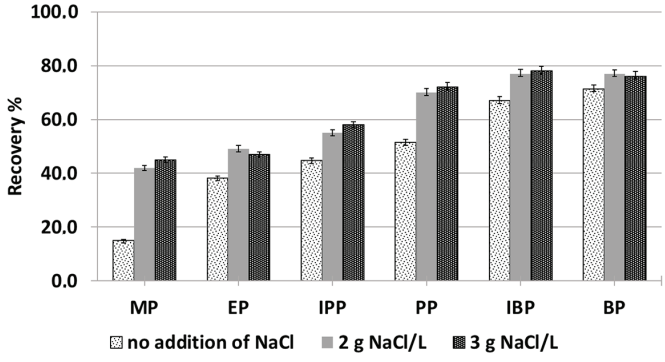


Figure 6. Optimization of the NaCl concentration. Extraction solvent ($CHCl_3$) volume, 100 µL; disperser solvent (MeOH) volume, 50 µL; ultrasonication time, 30 sec; vortex time: 4 min. n: 3, RSD values were in the range of 2.08-2.79%

NaCl: Sodium chloride, $CHCl_3$: Chloroform, MeOH: Methanol, RSD: Relative standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben

were performed in triplicate. Recoveries were higher than 82.00, and the enrichment factors were in the range of 41.07-49.05 (Table 3).

The optimized USVADLLME procedure provided the determination of parabens in different matrices at 20 ng/mL.

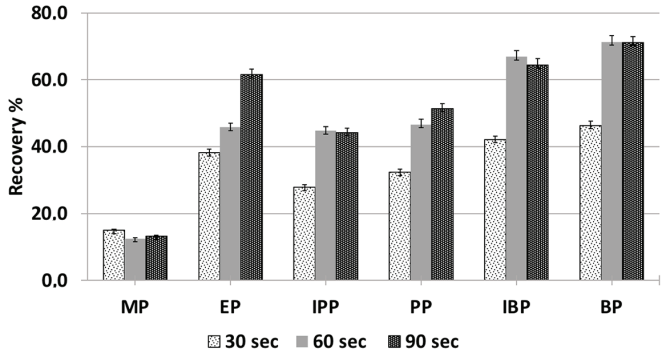


Figure 7. Optimization of ultrasonication time. Extraction solvent ($CHCl_3$) volume, 100 µL; disperser solvent (MeOH) volume, 50 µL; vortex time: 4 min. n: 3, RSD values were in the range of 2.51-3.12%

$CHCl_3$: Chloroform, MeOH: Methanol, RSD: Relative standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben

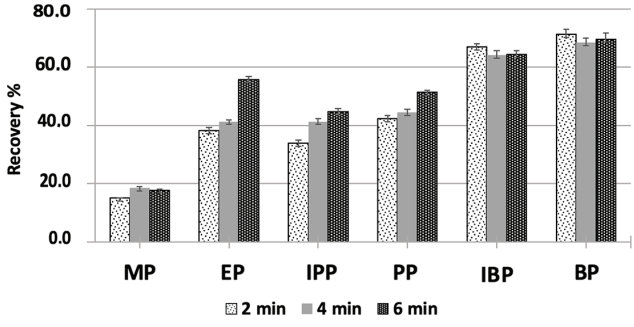


Figure 8. Optimization of the vortex time. Extraction solvent ($CHCl_3$) volume, 100 µL; disperser solvent (MeOH) volume, 50 µL; ultrasonication time, 30 sec. n: 3, RSD values were in the range of 1.15-2.93%

$CHCl_3$: Chloroform, MeOH: Methanol, RSD: Relative standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben

Yılmaz and Tokat[29] also developed an HPLC-FD method for the determination of different parabens (MP, EP, PP, IBP, and BzP) in cosmetics. Distinctly, in that study, a preconcentration method was not applied. The LOQs were in the range of

Table 3. Analysis results of spiked (0.02 µg/mL) real samples[a]

Sample	Analyte	Recovery (%)[b]	RSD (%)	Enrichment factor
T	MP	82.13 ± 2.01	2.45	41.07
	EP	84.53 ± 2.44	2.89	42.27
	IPP	88.53 ± 3.03	3.42	44.27
	PP	92.80 ± 2.88	3.11	46.40
	IBP	94.93 ± 1.22	1.29	47.47
	BP	95.47 ± 2.44	2.56	47.73
MW	MP	83.85 ± 1.41	1.68	41.93
	EP	88.90 ± 1.64	1.84	44.45
	IPP	90.52 ± 0.55	0.61	45.26
	PP	94.99 ± 1.37	1.44	47.50
	IBP	95.53 ± 1.05	1.09	47.77
	BP	94.89 ± 1.82	1.91	47.45
Urine	MP	95.03 ± 2.01	2.45	47.52
	EP	94.51 ± 2.44	1.89	47.26
	IPP	98.09 ± 3.03	2.03	49.05
	PP	92.86 ± 2.88	2.10	46.43
	IBP	94.96 ± 1.22	1.85	47.48
	BP	95.45 ± 2.44	2.01	47.73

[b]Mean recovery % ± standard deviation SD: Standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben, MW: Micellar water, RSD: Relative standard deviation, T: Cosmetic tonic

Table 2. Precision and accuracy of the developed HPLC-FD method

		MP	BP	IPP	PP	IBP	BP
Intraday (n: 3)[a]	C[c] (µg/mL)						
	0.50	1.43	1.30	1.58	1.81	1.26	1.61
	2.50	0.61	0.52	0.56	0.50	0.62	0.74
	10.00	0.60	0.29	0.25	0.23	0.22	0.27
Interday (n: 3)	C (µg/mL)						
	0.50	1.59	1.77	1.32	1.12	1.28	1.42
	2.50	1.82	1.65	1.66	1.46	1.42	1.45
	10.00	1.80	1.94	2.01	2.03	1.54	1.58
Recovery (%) (n: 3)[b]	C (µg/mL)						
	0.50	98.41 ± 1.77	96.71 ± 1.18	101.34 ± 1.15	101.11 ± 1.26	100.74 ± 1.40	99.67 ± 1.67
	2.50	98.46 ± 0.61	98.50 ± 0.52	98.16 ± 0.55	98.28 ± 0.48	99.07 ± 0.61	98.75 ± 0.73
	10.00	98.55 ± 0.59	98.61 ± 0.29	98.68 ± 0.25	98.76 ± 0.22	99.78 ± 0.22	99.94 ± 0.27

[a]Relative standard deviation (%), Mean recovery % ± SD, [c]Concentration (µg/mL), SD: Standard deviation, MP: Methylparaben, EP: Ethylparaben, IPP: Isopropylparaben, PP: Propylparaben, IBP: Isobutylparaben, BP: Butylparaben, HPLC: High-performance liquid chromatography, FD: Fluorescence detection

0.88-0.97 µg/mL, and it was not possible to quantify the analytes at lower concentrations. This procedure can be used for much lower concentrations with good precision and accuracy, which is an important advantage, especially for biological samples. In addition, USVADLLME may be effective for separating various interferences in complex matrices. On the other hand, the sample preparation time is longer and a chlorinated solvent such as $CHCl_3$ is used for the extraction. However, LOQs are lower without requiring a more sophisticated instrument such as LC-MS or GC-MS.

CONCLUSION

To the best of our knowledge, the present report could be considered as the first research on the determination of the selected parabens simultaneously by USVADLLME-HPLC-FD. Reliable paraben analysis could be achieved by the developed and validated HPLC-FD method. The proposed extraction procedure provided quantification of parabens at 20 ng/mL level without using a more sophisticated instrument such as LC-MS or GC-MS, was easy to perform and could be used for different aqueous personal care products and urine matrices. In addition, the use of low volumes of the extraction and dispersing solvents lower the cost.

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: P.K.Y., U.K., Design: P.K.Y., U.K., Data Collection or Processing: P.K.Y., Analysis or Interpretation: P.K.Y., Literature Search: P.K.Y., Writing: P.K.Y.

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

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ORIGINAL ARTICLE

Deiodinase Type 3 Polymorphism (rs1190716) Affects Therapeutic Response to Levothyroxine

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ABSTRACT

Objectives: Levothyroxine (LT4) is a commonly used treatment for hypothyroidism. Deiodinase enzymes control the metabolism and homeostasis of thyroid hormones (THs). Deiodinase type 3 gene (*DIO3*) encodes deiodinase type 3 enzyme (D3), and the genetic polymorphisms of this gene could affect the levels of THs and the response to LT4 treatment. This study aimed to investigate the single-nucleotide polymorphism (SNP), rs1190716; C > T, of DIO3 as a candidate genetic variant that might affect the clinical response to LT4 treatment.

Materials and Methods: Two hundred Iraqi hypothyroid female patients aged 40 years were enrolled in this cross-sectional study. All of them were already on the LT4 treatment for at least 4 months. THs [thyroxin (T4), triiodothyronine (T3), reverse triiodothyronine (rT3), and diiodothyronine (T2)] were estimated. An allele-specific polymerase chain reaction technique was performed to detect the rs1190716; C > T SNP.

Results: The genotypes distribution of rs1190716; C > T SNP was 10 (4.5%) for the wild type (CC), 50 (22.7%) for the heterozygous mutant type (TC), and 160 (72.7%) for the homozygous mutant type (TT). The patients were divided into three groups according to their genotypes. Significant differences were found in the T4, T3, and T2 levels among the patients (p=0.019, p=0.039, p=0.032, respectively).

Conclusion: The rs1190716; C > T SNP could affect the activity of the D3 enzyme and the metabolic homeostasis of the THs; therefore rs1190716; C > T SNP could have an impact on the therapeutic response to LT4 in Iraqi female patients with primary hypothyroidism. Regarding *DIO3* gene, this is a novel finding; hence, further studies are needed to confirm it.

Key words: Primary hypothyroidism, tetra ARMS-PCR, thyroxin, triiodothyronine, deiodinases type 3 enzyme

INTRODUCTION

Hypothyroidism is a common illness caused by the deficiency of thyroid hormones (THs).[1] The prevalence of hypothyroidism varies according to geographic region, population, and whether it is overt or subclinical hypothyroidism. Although it is roughly 3.2% overt hypothyroidism and 14.1% subclinical hypothyroidism in the Iraqi population.[2] It has been reported that the incidence of hypothyroidism is higher in females than in males and in older people, or at least the elderly, who receive hypothyroidism treatment more frequently.[3]

Levothyroxine (LT4) is a synthetic thyroxin (T4) that is suggested for the treatment of hypothyroidism and is physiologically and biochemically identical to the endogenous hormone. Treatment lessens hypothyroidism symptoms and returns the thyroid stimulating hormone (TSH) and TH levels to a normal physiological range.[4] Initial dose requirements for LT4 therapy can range significantly from small doses (25-50 µg) that are given to mild or subclinical cases to greater doses (88-175 µg) that are given to people who barely have any thyroid gland activity.[5]

Deiodinase enzymes are responsible for the metabolism of LT4. THs are activated and inactivated by deiodinases by removing iodine from the outer or inner ring of the THs molecules. Deiodinases enzymes are classified into three different types: type 1 (D1), type 2 (D2), and type 3 (D3). While type 3 enzyme inactivates T4 to reverse triiodothyronine (rT3) and T3 to diiodothyronine (T2), the first two enzymes convert T4 to triiodothyronine (T3).[6] The primary deiodinase

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during embryonic development is D3, which also protects growing tissue from excessive TH synthesis at maturity. Under physiological conditions, it is mostly found in the skin and central nervous system and is absent or present in very small amounts in the other tissues.[7]

The deiodine type 3 gene (*DIO3*), which encodes D3 enzyme, is found in the imprinted region of the chromosomal locus 14q32.2. It is expressed from the human paternal allele preferentially during development.[8] DIO3 opposite strand upstream RNA (*DIO3OS*) gene, which overlaps with *DIO3* and is transcribed in the opposite direction, is located in same imprinting region but is dominantly expressed from a maternally inherited allele.[9] *DIO3OS* gene is a long non-coding RNA that regulates the expression of a gene during overlap with a target gene or promoter or by modification of the chromatin structure during epigenetic marks. The human *DIO3OS* gene is widely expressed in many tissues. It has been reported that DIO3OS has the potential to control the expression and activity of DIO3.[10] The *DIO3OS* gene contains six exons and two alternate polyadenylation sites. The genetic polymorphism rs1190716 is located in the intron region of this gene. Exonic sequence of DIO3OS overlaps with the DIO3 promoter and strongly regulates its expression.[11]

Many patients with hypothyroidism who received LT4 treatment complained that the disease symptoms persisted even after their serum TSH levels returned to normal.[12] This study is one of the studies series that investigated the impact of the deiodinases enzymes on the biochemical response to LT4 in Iraqi the effect of the rs1190716 variant of *DIO3* gene.

MATERIALS AND METHODS

Subjects and methodology

This cross-sectional study was approved by the Scientific and Ethical Committee of the College of Pharmacy at the University of Kerbala (reference number: 2021HU6).

Approximately 220 hypothyroid female patients were recruited from November, 2021 to March, 2022. Patients were recruited from outpatient clinics after they were diagnosed with primary hypothyroidism. The patients' age was 40 year old. They had already been on the treatment for at least 4 months. Patients that were not included in this study were male patients, pregnant patients, patients with an active neoplasm or a history of neoplasm, patients with a condition that may be the main cause of secondary hypothyroidism, patients who were taking drugs that may affect Th bioavailability, patients with autoimmune thyroid disease, and any patient who underwent thyroidectomy. All the participants were enrolled in this study after signing a written consent form that included a detailed explanation of the study purpose, and they were asked to fill out a specially designed questionnaire.

Blood (5 mL) was collected and divided into two parts, the first part (3 mL) was placed in an anticoagulant-free plain tube, and the second part (2 mL) was placed in an evacuated ethylenediaminetetraacetic acid (EDTA) tube. After 30 min of sampling, the blood sample in the plain tube was centrifuged, and the serum was separated and stored at -20 ˚C for the biochemical analysis. For the DNA extraction, samples in EDTA tubes were used.

Genetic analysis

The genomic DNA was isolated from the whole blood samples using a Prep genomic DNA Mini kit (China) based on the procedure recommended by the manufacturer. To detect the rs1190716 single-nucleotide polymorphism (SNP), the tetra primers amplification refractory mutation system-PCR (tetra ARMS-PCR) technique was used. The primers were designed using primer-BLAST software and were purchased as lyophilized products from Alpha DNA, Canada. Each primer was dissolved in specific volumes of nuclease-free water to obtain a stock solution with a concentration of 100 pmol/µL. Diluted work solutions were prepared by adding 90 µL of nuclease-free water to 10 µL of each stock solution of the outer forward and outer reverse primers. Water (95 µL) was mixed with 5 µL of the stock solution of each of the inner forward and inner reverse primers to make the work solution of each primer. The work solutions were kept at -20 ˚C until use. The sequences of primers are as outer forward primer 5`*ATCCCTGGGGCTAGAAAGAG3*`, the reverse primer 5`*TTTTCCCACAGTTAGTTTCAGAAA3*`, the inner forward 5` *CCACCCAGACCATACTTGCT3*`, the inner reverse g`*CGCATGTGCATGTGTTTG3*`. The PCR reaction mixture volume was total 25 µL, it contained 5 µL of Accu power PCR Pre Mix (Bioneer Company, Korea), 5 µL (100 ng/µL) of the extracted DNA, 1 µL (10 pmol/L) of each of the forward primers, 1 µL (5 pmol/µL) of each of the reverse primers and 11 µL of nuclease-free water. The program used for the amplification was as follows: initial denaturation for 3 min at 95 ˚C, 30 cycles of amplification (denaturation for 30 seconds at 95 ˚C, annealing for 30 seconds at 60 ˚C, extension for 1 min at 72 ˚C), and a final extension for 5 min at 72 ˚C. To confirm the amplification and detect the PCR products, electrophoresis using agarose gel [1.5% (*w/v*)] was performed.

Biochemical analysis

The competitive electrochemiluminescence immunoassay (ECLIA) was used to determine the levels of TSH, total T3 (tT3), free T3 (fT3), total T4 (tT4), and free T4 (fT4). Immunoassays were performed using laboratory kits (Snibe Diagnostics, China). A solid phase enzyme-linked immunosorbent assay (ELISA) was used to determine T2, rT3, and fasting serum insulin. T2 and rT3 analyzes were performed using research kits from BT LAB, China, while fasting serum insulin levels were estimated using a diagnostic kit from Mindray Company, China. A photometric assay method was used to determine the serum glucose concentration, which was estimated at a wavelength of 510 nm. This assay was performed using a diagnostic kit from Mindray Company, China.

Statistical analysis

To analyze the data, the statistical package for social sciences (SPSS) software (version 22, Chicago, USA) was used. The means of the three study groups were compared using one-way ANOVA. A *post-hoc* test was conducted to assess the

multiple comparisons between the means of the groups. The chi-squared goodness of fit test was performed to evaluate the allele distribution in accordance with Hardy-Weinberg equilibrium. The difference between the groups was considered significant when $p < 0.05$.

RESULTS

Two hundred twenty Iraqi females with primary hypothyroidism were enrolled in this study. Table 1 demonstrates the demographic characteristics of the studied patients.

To detect the rs1190716; C > T SNP, tetra-ARMS-PCR was performed. The PCR amplicon sizes differ according to the presence of C or T alleles. In case of the wild type (CC), two PCR bands with 253 and 121 bp appeared on the agarose gel. In mutant (CT), three PCR bands with 253 bp, 170 bp, and 121 bp appeared, whereas two PCR bands with 253 bp and 170 bp appeared in the case of the homozygous mutant type (TT), as demonstrated in Figure 1.

Table 2 demonstrates the allele and genotype distribution of the rs1190716; C > T SNP.

The age and body mass index did not show significant differences among the three groups of patients, but the duration of treatment was significantly different. The levels of TSH, fT4, fT3, and rT3 did not display significant differences among the groups of patients; however, there was a significant difference in tT3 level between CT and TT groups. A significant difference was also detected in T2 level between the same groups. The tT4 levels showed significant differences between CC and TT carriers (Table 3).

No significant differences were obtained in the blood pressure (systolic pressure, diastolic pressure, and the mean arterial pressure) among the groups of patients or in the glycemic indices (Table 3).

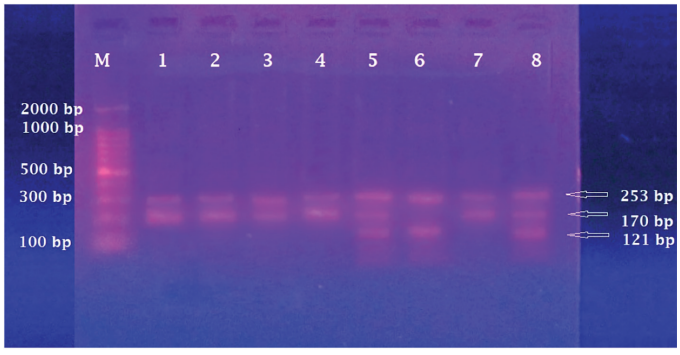

Figure 1. The agarose gel electrophoresis for identifying rs1190716; C > T SNP using tetra ARMS-PCR. M: DNA ladder, lanes 1, 2, 3, 4, and 7 represent the homozygous mutant type (TT), lanes 5 and 8 represent the heterozygous mutant type (CT), and lane 6 shows the wild type (CC)

Table 1. The characterization of the hypothyroidism patients

Parameters	Mean ± SD (n: 220)
Age (year)	49.15 ± 9.11
BMI (kg/m^2)	30.98 ± 5.83
Treatment duration (years)	4.47 ± 4.03

n: Number of studied subject, SD: Standard deviation, BMI: Body mass index

DISCUSSION

The rationale for LT4 replacement treatment is that two deioddinases, D1 and D2, convert T4 to T3 (the active form hormone), whereas D3 clears T4 to rT3 and T3 to T2. These three pathways restore the pool of T3 and the clinical euthyroidism.[13] After the observation of many patients, who were treated with the LT4, high serum T4/T3 levels were detected, and the symptoms of hypothyroidism have not resolved. However, the level of TSH was within the normal reference range. Thus, LT4 to restore TH levels came into question.[14] This could be the first study in Iraq and the Middle East that searches the impact of the rs1190716; C > T SNP on the clinical response to LT4 among patients with hypothyroidism. This response can be assessed by estimating the serum TSH and TH levels and the LT4 dose. We hypothesis is that the SNPs in *DIO3* gene, or other genes that overlap with it, such as DIO3OS, might impact on the activity of D3 enzyme. Accordingly, the conversion of T4 and T3 to rT3 and T2, respectively, might be affected, which affects the levels of T4 and T3 hormones.

This study indicated that there was no significant association between rs1190716; C > T SNP and TSH level, fT3, fT4, rT3, and LT4 dose. tT4, tT3, and T2 were significantly different among the three groups of patients. The patients with mutant allele (T) had significantly lower tT4 and greater tT3 levels; at the same time, they had slightly higher TSH levels and slightly higher LT4 dose (Table 3). The patient group carrying the wild-type allele had significantly higher T4 levels, slightly lower LT4 doses, and lower TSH levels. This could be due to the role of D2, which, as an enzyme inhibited by its substrate, D2 activity can be inhibited by the high T4 concentration. This results in decreasing T3 concentration, whereas serum TSH concentration could be within the normal range because of the slightly elevated T4 concentration.[15] According to a study conducted on rats, the hypothalamic tancytes and pituitary thyrotrophs absorb plasma T4 and locally convert it to T3 by

Table 2. The allele distribution of rs1190716; C > T SNP in the hypothyroidism patients

Genotype (n: 220)	Frequency (%)	Allele	Frequency	Chi-square	*p* value
CC (wild type)	10 (4.5)	C	0.16		
CT (heterozygous mutant type)	50 (22.7)	T	0.84	4.94	0.084
TT (homozygous mutant type)	160 (72.7)				

n: Numbers of the study subjects, SNP: Single-nucleotide polymorphism

Table 3. The demographic, the thyroid function, the blood pressure, and the glycemic analysis of the study subjects

Parameter	Genotypes (n: 220)			*p* value
	CC (n: 10)	CT (n: 50)	TT (n: 160)	
Age (year)	51.20 ± 2.96	47.98 ± 1.17	49.39 ± 0.73	0.48
BMI (kg/m^2)	29.55 ± 1.40	31.16 ± 0.65	31.02 ± 0.49	0.72
Duration of the treatment (years)	7.63 ± 1.83	4.38 ± 0.46	4.30 ± 0.32	0.04*[a,b]
tT3 (nmol/L)	1.49 ± 0.10	1.71 ± 0.07	1.52 ± 0.03	0.019*[c]
tT4 (nmol/L)	125.74 ± 9.52	108.81 ± 5.31	102.29 ± 2.22	0.039*[b]
fT3 (pmol/L)	6.40 ± 0.41	6.60 ± 0.28	6.56 ± 0.10	0.93
fT4 (pmol/L)	16.54 ± 1.47	15.65 ± 0.74	16.00 ± 0.58	0.91
TSH (µlU/mL)	3.03 ± 0.90	7.29 ± 2.20	5.94 ± 0.76	0.51
rT3 (nmol/L)	0.86 ± 0.10	1.01 ± 0.05	0.91 ± 0.02	0.21
T2 (nmol/L)	1.97 ± 0.30	2.38 ± 0.20	1.92 ± 0.07	0.032*[c]
tT3/tT4	1.22 ± 0.09	1.61 ± 0.07	1.56 ± 0.03	0.06
fT3/fT4	0.40 ± 0.03	0.44 ± 0.01	0.43 ± 0.009	0.57
T2/tT3	1.32 ± 0.18	1.54 ± 0.13	1.31 ± 0.05	0.54
rT3/T4	0.72 ± 0.10	1.01 ± 0.06	0.95 ± 0.03	0.19
T4 dose (µg)	67.50 ± 9.16	97.00 ± 5.23	93.34 ± 3.35	0.11
FBS (mg/dL)	123.14 ± 16.82	12149 ± 9.50	109.99 ± 3.23	0.27
Fasting insulin	24.24 ± 8.75	20.16 ± 2.93	15.82 ± 1.06	0.09
HOMA-IR	6.07 ± 1.61	6.18 ± 1.06	4.50 ± 0.37	0.13
Systolic BP (mmHg)	124.00 ± 3.05	126.60 ± 2.36	127.06 ± 1.21	0.82
Diastolic BP (mmHg)	80.00 ± 1.49	78.20 ± 2.17	78.12 ± 1.25	0.93
MAP (mmHg)	94.66 ± 1.87	94.33 ± 1.44	94.43 ± 0.82	0.99

Post-hoc test: a: CC *vs.* CT, b: CC *vs.* TT, c: CT *vs.* TT

The data is represented as mean ± standard error of the mean, *Significant at $p>0.05$. n: Numbers of the study subjects, T3: 3,3,5-triiodothyronine, T4: Thyroxin, T2: 3,5-Diiodothyronine, rT3: Reverse triiodothyronine, FBS: Fasting blood sugar, FSI: Fasting serum insulin, HOMA-IR: Homeostatic model assessment for insulin resistance, BP: Blood pressure, MAP: Mean arterial pressure

D2. TSH secretion is decreased because of the D2 action at these two locations.[14]

Although D3 enzyme is not implicated in the T4 to T3 conversion, *DIO3* gene is also not involved. It is worthy to mention that there are other candidate genes that could be potential effectors. Deiodinase type 1 (DIO1), deiodinase type 2 (DIO2), and Th transporters notably influence the response to LT4. Previous studies suggested that common genetic variations in these loci might have an impact on the changes in the levels of TH metabolites and, accordingly, the response to LT4 treatment.[16,17]

While normalizing serum TSH is the aim of LT4 therapy, a gradual increase in LT4 dose raises the circulating T3 levels while concurrently lowering TSH secretion (predominantly through D2).[18] Our patients, who had the mutant allele, had a higher LT4 dose but lower tT4 and higher TSH levels (Table 3). This indicated that LT4 treatment did not restore normal TSH levels, which could be explained by the carriers of the mutant allele (TC and TT) having lower tT4 levels. Thus, their TSH levels were above the reference range, so they needed a higher LT4 dose. This could be an indication that the rs1190716; C > T SNP increases D3 enzyme activity, which means increasing the conversion of T4 to rT3, thereby making the levels of tT4 lower in the mutant allele carriers. It was confirmed by the increased rT3 levels in the mutant allele carriers compared with the wild allele carriers, but not to a significant level (Table 3). As a result, the ratio of rT3/T4 was increased in the mutant types (CT and TT) groups but also in a non-significant manner (Table 3).

TSH level was lower (within the normal range) in the wild-type group than in both hetero and homo mutant type carriers, but not to a significant level (Table 3). This was due to the feedback inhibition of the elevated level of tT4 in this group. At the same time, this could indicate that the hypothyroidism patients, who were not carriers for the rs1190716; C > T SNP might have a better response to LT4 therapy and that D3 enzyme could have a role in TH regulation.

Our findings could be novel regarding rs1190716; C > T SNP and *DIO3* gene because previous studies demonstrated that this SNP and this gene had no impact on TH and TSH levels.[19-21]

The results also demonstrated that no significant differences in the glycemic parameters or blood pressure parameters were found among the three groups of patients. This role the rs1190716; C > T SNP from being involved as a risk factor for these parameters.

Study limitations

This study assessed the response to LT4 replacement therapy among the hypothyroidism patients by estimating serum Th levels. Deiodinase type 3 enzyme activity should be estimated in future studies to confirm the role of this enzyme.

CONCLUSION

The mutant allele (T) was the most frequent allele in our study subjects. The rs1190716; C > T SNP was significantly associated with the levels of tT3, tT4, and T2. Accordingly, SNP could affect the activity of the D3 enzyme and the metabolic homeostasis of the THs; therefore, rs1190716; C > T SNP could have an impact on the therapeutic response to LT4 in Iraqi female patients with primary hypothyroidism. This is a novel finding regarding *DIO3* gene; hence, further studies are needed to confirm it.

Ethics

Ethics Committee Approval: This cross-sectional study was approved by the Scientific and Ethical Committee of the College of Pharmacy at the University of Kerbala. The reference number for scientific and ethical approval is 2021HU6.

Informed Consent: Written permission was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.H.M., Concept: B.H., S.J., Design: B.H., S.J., Data Collection or Processing: A.H.M., B.H., S.J., Analysis or Interpretation: A.H.M., B.H., S.J., Literature Search: A.H.M., Writing: A.H.M., B.H., S.J.

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

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SHORT COMMUNICATION

Essential Oil Composition of *Capsella bursa-pastoris* (L.) Medik. Aerial Parts

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ABSTRACT

Objectives: *Capsella* Medik. belongs to Brassicaceae family and is represented by 4 species in Türkiye. Among them, *Capsella bursa-pastoris* (L.) Medik. is a cosmopolite species and grows naturally throughout Türkiye. There have been a few studies on the essential oil composition of different parts of the plant, and, as far as we are concerned, the essential oil composition of the plant growing in Türkiye has not been studied previously. Thus, in this study, we isolated and analyzed the essential oil composition of the aerial parts of *C. bursa-pastoris* growing naturally in Ankara, Türkiye.
Materials and Methods: Plant material was collected from Ankara University, Tandoğan Campus, and essential oil of the aerial parts was obtained from hydrodistillation using a Clevenger-type apparatus for 3 h and analyzed by gas chromatography (GC)-flame ionization detection and GC-mass spectrometry simultaneously.
Results: The essential oil yield was determined to be 0.2%, and 90.2% of the essential oil composition was identified, corresponding to 21 components. The major components of the oil were determined to be nonacosane (19.6%), phytol (19.3%), pentacosane (13.5%), heptacosane (9.9%), and hexadecanoic acid (9.9%).
Conclusion: Phytol was found to be the main component of the essential oil of leaves and aerial parts (16.34% and 13.14, respectively) in a literature study consistent with the results of our study. The essential oil content of other parts of the species, along with species collected from different localities, would be an important contribution to the species and the genus and should be performed in the future.
Keywords: *Capsella bursa-pastoris,* çobançantası, shepherd's purse, Brassicaceae, essential oil

INTRODUCTION

Capsella Medik. is a genus of the Brassicaceae family, and four species naturally grow in Türkiye: *Capsella grandiflora* (Fauché & Chaub.) Boiss, *C. lycia* Stapf., *C. rubella* Reut, and *C. bursa-pastoris* (L.) Medik. Among these species, *C. bursa-pastoris* is cosmopolitan and grows in Europe, Asia, America, Australasia, and Africa.[1]

The plant is mostly known as shepherd's purse in English [2,3] and is also called caseweed, lady's purse, rattle pouches, pickpocket, pepper-and-salt, and poor man's pharmacetti. It is known as "hirtentasche" in German; "bourse de Pasteur" in French, "madakat el raee" in Arabic,[3] and "çoban çantası, çıngıldaklı ot/çıngıldak otu" in Turkish.[4]

Morphological properties of the plant, such as size, fruits, and leaf shape, may vary according to locality; however, the species can be identified by its terminal racemose inflorescence bearing small white flowers and toothed rosette leaves.[1] The general appearance of the plant can be seen in Figure 1.

The plant is known to have various primary and secondary metabolites phytoalexins;[5] carotenoids, flavonoids, anthocyanins;[6] alkaloids[7] and is thus known as poor man's pharmacetti because it is also used in traditional medicine for various ailments and is also eaten as a salad or as cooked dishes.[8] Aerial parts of the plant are used against edema, enteritis, and nephritis in traditional Chinese medicine;[7] the plant is used as a uterine tonic and hemostatic;[9] and against ulcers, tumors, and uterine cancer.[10]

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Figure 1. General appearance of the plant (photo by. C.S. Kılıç)

Due to the rich composition of the plant, it is actually known to possess various biological activities such as inhibition of hepatocarcinogenesis[11] and inhibition of tumors;[12,13] antibacterial;[14] anticholinesterase;[15] anti-inflammatory;[16] antioxidant;[15-17] hepatoprotective[18] and anti-hemorrhagic,[19] antihemorrhoidal;[20] neuroprotective,[21] cholesterol lowering activities.[22] These biological activities are usually based on the traditional uses of the plant, which include its use against stomach cramps, bleeding (internal and external), wounds and burns, premenstrual syndrome, and malignant ulcers.[13]

In addition to studies performed on the composition, ethnobotanical usages, and biological activities of the species, studies related to the essential oil composition of different plant parts are scarce[17,23-26.] As far as we are concerned, essential oil obtained from the aerial parts of plants growing in Türkiye was not analyzed before. Therefore, in this study, we aimed to analyze the essential oil isolated from *C. bursa-pastoris* (aerial parts) that grows naturally in Türkiye; specifically in Ankara.

MATERIALS AND METHODS

Plant material

Aerial parts of the plant were collected on April 2021 from Tandoğan Campus of Ankara University (Türkiye). Herbarium specimens prepared from the collected species were placed in Herbarium of Ankara University Faculty of Pharmacy (AEF) with the number AEF 30718.

Essential oil isolation

Hydrodistillation was performed on air-dried plant material for a period of 3 h using a Clevenger-type apparatus as recommended by European Pharmacopoeia.[27] Isolated oil was dried with the help of sodium sulfate, anhydrous, and then stored at a temperature of +4 ˚C in a vial with the lid closed and in the dark until examination.

Essential oil analysis

Essential oil analysis was performed using gas chromatography-mass spectrometry (GC-MS) and GC according to the method used in a previous study.[28]

Determination of the volatile compounds

The of the essential oil was determined by comparing the relative retention times (RRI) of the components with the genuine samples or by comparing their RRI with *n*-alkane series. The obtained results were analyzed by computer-matching using the Wiley GC/MS Library and MassFinder 3 Library[29,30] and in-house "Başer Library of Essential Oil Constituents" formed by authentic compounds and the components from recognized oils.

RESULTS AND DISCUSSION

Essential oil yield was determined to be 0.2%, and 90.2% of this oil was identified, corresponding to 21 components, yielding major components as nonacosane, phytol, pentacosane, heptacosane, and hexadecanoic acid (19.6%, 19.3%, 13.5%, 9.9%, 9.9%, respectively). Table 1 lists the components of the oil.

RRI calculated against *n*-alkanes; %: calculated from FID data; IM: identification method, based on the RRI of genuine compounds on the HP innowax column; MS, determined by computer matching of the mass spectra with Wiley and MassFinder Libraries and comparing them with data found in the literature.

Some studies on the lipids of the aerial parts[31] and the fixed oil content of the seeds of the species can be found in the literature;[32-34,] however, studies analyzing the essential oil of different parts of the species are scarce.

In a study by Lee and Choi[24] performed on aerial parts, roots, and leaves of the species individually, phytol was stated to be the significant component of the essential oil of the leaves (16.34%) and aerial parts (13.14%). However, the percentages of nonacosane and pentacosane found in the aerial parts were lower (3.81% and 0.37%, respectively) than those in our study.

In another study by Miyazawa et al.,[23] camphor (20.2%) and α-phellandrene (7.8%) were determined to be the major components. In another work by Kamali et al.,[17] 1,1-dimethylcyclopentane, ethyl linoleate, palmitic acid, and phytane were found to be the major constituents.

There are also some other studies performed on different parts of the species[25,26] and one study performed on another *Capsella* species, namely *C. rubella*,[35] which has different main components.

To our knowledge, this is the first study performed on the essential of the aerial parts of the species growing naturally in Ankara city (Türkiye). Examination of the other parts of the

Table 1. List of essential oil components of the aerial parts

RRI	Compound	(%)	IM
1203	Limonene	0.3	RRI, MS
1294	1,2,4-Trimethylbenzene	0.1	MS
1398	2-Nonanone	0.5	MS
1571	*Trans*-*p*-menth-2-en-1-ol	0.3	MS
1604	2-Undecanone	1.6	MS
1706	α-Terpineol	0.4	RRI, MS
1933	Tetradecanal	0.5	MS
1958	(E)-β-Ionone	0.2	MS
2041	Pentadecanal	0.4	MS
2131	Hexahydrofarnesyl acetone	3.0	MS
2143	α-Cedrol	2.6	RRI, MS
2223	6,10,14-Trimethyl pentadecan-2-ol	0.2	MS
2226	Methyl hexadecanoate	0.4	RRI, MS
2300	Tricosane	5.5	RRI, MS
2500	Pentacosane	13.5	RRI, MS
2622	Phytol	19.3	MS
2700	Heptacosane	9.9	RRI, MS
2600	Hexacosane	1.9	RRI, MS
2700	Heptadecanal	0.1	MS
2931	Hexadecanoic acid	9.9	RRI, MS
2900	Nonacosane	19.6	RRI, MS
	Total	90.2	

RRI: Relative retention times, MS: Mass spectrometry, IM: Identification method

species along with species collected from different localities would be an important contribution to the literature on *Capsella* genus and more relevant studies should be performed in the future.

Ethics

Ethics Committee Approval: Not required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.F., D.K., B.D., C.S.K., Design: C.S.K., Data Collection or Processing: D.K., B.D., Analysis or Interpretation: S.F., D.K., B.D., C.S.K., Writing: S.F., D.K., B.D., C.S.K.

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

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LETTER TO EDITOR

Importance of Project-Based Learning for Pharmacy Education

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Key words: Education, pharmacy, project-based learning, students, PBL

Dear Editor,

Project-based learning (PBL) is defined as a question-based teaching approach that ensures the active participation of the learners in building knowledge by enabling them to perform meaningful projects and develop concrete products. PBL is generally regarded as an alternative to traditional teaching provided by teachers. It is recognized that PBL is a favorable approach that enhances student learning in higher education.[1] PBL was incorporated into educational practice in early 1980s. The history of PBL has its roots in the progressive tradition encouraged by John Dewey. He emphasized the concept of "learning through practice". John Dewey maintained that the lecture room should be a sort of society, and in the learning process, the focus should be on the students. PBL is a highly efficient technique that allows students; to express their views on topics that cover areas of their interest, raise their queries, use various tools, develop different concepts and theories, make use of skills attained through real and meaningful learning processes, and respond ingeniously to questions in and out of the classroom.[2]

The purpose of this letter is to focus on pharmacy education and the role of incorporating PBL into pharmacy training programs. The Doctor of Pharmacy is a 5-year professional degree that includes a multidisciplinary program. This program comprises theory sessions with hands-on demonstrations and experiments. With developing expertise in the area of therapeutics, the theoretical and training aspects of the pharmacy syllabus are progressively emphasized in the development of group work, collaborations, brainstorming, time management, and finding solutions for different problems. Over the past two decades, there has been a shift toward pharmacy practice. Currently, pharmacists are actively engaged in primary care services such as health promotion, patient education, drug utilization, disease surveillance, testing, and prevention, in addition to their traditional role of supplying medication products to patients. Because effective interdisciplinary collaboration is required to deliver these services, pharmacists need to develop competencies that promote interprofessional relationships. In the traditional education system, teaching is a common means of conveying basic knowledge and concepts, particularly to a wide audience. Despite the benefits of traditional education, many studies have shown that this mode of class style is incapable of introducing critical thinking skills essential for any professional degree, *i.e.*, doctor of pharmacy. Hence, there is an urgent need to transform teaching methods to ensure that pharmacy students have the necessary skills. One-Way to do this is to bring PBL into pharmacy teaching. PBL can be achieved through lab experiments, field studies, and work projects, leading to increased scientific knowledge and skills that can be implemented in many areas of pharmacy, such as analytical chemistry, biopharmaceutics, medicinal chemistry, pharmacognosy, pharmaceutical microbiology, industrial pharmacy, pharmacology, and clinical pharmacy.[3-5]

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PBL promotes a profound approach (*i.e.* not just shallow teaching) to learning and promotes autonomous and continuous learning skills. It motivates students to develop inner inspiration by delivering programmatic objectives that help them excel in collaboration and active learning. It provides a real-time solution to clinical challenges. In addition, it allows students to develop entrepreneurial skills to contribute their part to the country's economy by promoting decision-making capacities, finding opportunities, taking risks, communicating ideas, and building trust.[6] Therefore, it is recommended that instead of traditional tests and examinations that are done later, students' skills and attitudes should be evaluated on-site in classes while discussing problems and their solutions. However, assessing the progress of students in PBL tutorials continues to be a challenge because most traditional methods of evaluation are not harmonized and do not quickly evaluate what is learned in the PBL class. Peer assessment could be a way to gauge learning in PBL sessions. Peer review in higher education is a process in which students use predetermined criteria and norms to evaluate the work of their peers. Peer review allows students to receive input on their learning. Peer evaluation can be cumulative or formative and could include qualitative responses regarding the assessment criteria used instead of a quantitative focus on the actual score. Peer review may be conducted in the context of one-on-one or teamwork. Peer assessment in teamwork takes one of three forms, including intra-group, inter-group, and extra-group. As an intragroup, each member of a group assesses the performance or contribution of other individual members within the group regarding the shared product. In an intergroup context, one or more people in one group assess another team's performance or product. In an extra-group setting, individuals who are not part of the group judge the performance or product of a group. Through group work, peer review enhances student learning, builds self-confidence, encourages students to work collaboratively, improves their decision-making skills, and makes them aware of their own lives. Peer evaluation can also be beneficial for faculty members by reducing their workload, providing new perspectives on student learning processes, and encouraging staff to be more transparent about evaluation goals and scoring criteria. Since peer review and PBL focus on group collaboration and sharing key goals and philosophies, peer assessment appears to be an appropriate method for developing PBL tutorials. It is concluded that the traditional method of lectures is seen as a teacher-centered educational approach, in which knowledge is passed on by and from the instructor and passively acquired by the learners. PBL makes it possible to use contextual thinking and development that improves the richness of depictions in long-term memory and is identified as an effective cognitive approach for problem-solving. It is suggested that the implementation of PBL models in pharmacy education should be supported by well-designed pedagogical research.[3,7]

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Ethics

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