

DEVELOPMENT AND VALIDATION OF A RAPID ISOCRATIC RP-HPLC METHOD FOR THE QUANTIFICATION OF SALMON CALCITONIN

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Abstract

In this study, we have developed and validated a simple and rapid isocratic reverse-phase high performance liquid chromatographic method with UV detection (220 nm) for quantification of salmon calcitonin. Chromatography was performed using an Agilent Eclipse XDB-C₁₈ column (150x4.6 mm i.d., 3.5 µm particle size) with a mobile phase (water:acetonitrile 65:35, v/v), containing 0.1% v/v trifluoroacetic acid at a flow rate of 1 mL/min. Each analysis requires less than 7 min including triamcinolone acetonide as an internal standard. The method has been validated for accuracy, precision, linearity, specificity, and sensitivity. The method has proved to be specific, rapid, accurate, precise and reproducible according to ICH standards. The calibration curve is linear in the concentration range 2.5-50 µg/mL; the correlation coefficient was 0.999. The method has showed good recoveries (average 100.64%) and the relative standard deviations intra- and inter-day were < 2.0%. The method has been successfully applied for the rapid and accurate quantification of salmon calcitonin in raw materials and commercial formulations.

Key words: HPLC, Salmon calcitonin, Validation, Isocratic, Triamcinolone acetonide

Salmon Kalsitoninin Miktar Tayininde Hızlı ve İzokratik Ters Faz Yüksek Basıncılı Sıvı Kromatografisi Yöntemi Geliştirilmesi ve Validasyonu

Bu çalışmada salmon kalsitoninin miktar tayini için ters faz sıvı kromatografisi kullanarak UV dedektör ile (220 nm) basit ve hızlı bir izokratik yöntem geliştirilmiş ve valide edilmiştir. Kromatografi için Agilent XDB-C₁₈ marka kolon (150x4.6 mm i.d., 3.5 µm partikül boyutlu) kullanılmıştır. Hareketli faz % 0.1 h/h trifloroasetik asit içeren su:asetonitril 65:35, h/h karışımı olup akış hızı 1 mL/dak olarak belirlenmiştir. Her bir analiz iç standart olarak kullanılan triamsinolon asetonitle birlikte 7 dk'dan kısa sürmüştür. Yöntem ICH standartlarına göre özgül, hızlı, doğru, kesin ve tekrarlanabilir bulunmuştur. Kalibrasyon eğrisi 2.5-50 µg/mL aralığında doğrusallık göstermiştir (R²=0.999). Yöntemin geri kazanımı ortalama % 100.64'dür. Gün içi ve günler arası yüzde bağıl standart sapma değerleri < % 2.0 dir. Yöntem salmon kalsitoninin ham madde ve ticari preparatlarda miktar tayininde başarı ile hızlı ve doğru bir şekilde uygulanmıştır.

Anahtar kelimeler: Yüksek Basıncılı Sıvı Kromatografisi, Salmon kalsitonin, Validasyon, İzokratik, Triamsinolon asetonit

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INTRODUCTION

Calcitonin (CT) is a water-soluble peptide hormone, discovered by Copp and colleagues in 1961 (1). It is produced by the parafollicular cells of the thyroid gland in mammals and the ultimobranchial gland of birds and fish (2). Synthetic or recombinant CTs from different species (human, salmon, porcine and eel) have been used for medical purposes. Salmon calcitonin (sCT) is by far the most widely used preparation in clinical practice due to its 40-50 times higher intrinsic potency when compared to human calcitonin, and its improved analgesic properties (3). The amino acid sequence of CT varies between species, but all CTs consist of 32 amino acids in a single chain, with a disulphide linkage between cysteine residues in positions 1 and 7, forming a loop at the N-terminus, and with a proline amide at the C-terminus (Figure 1) (4,5).

sCT has been available as a therapeutic agent for metabolic bone diseases for more than 30 years. It is approved for the treatment of postmenopausal osteoporosis in more than 90 countries. It decreases blood calcium levels and inhibits bone resorption by directly affecting osteoclast activity, and it is suggested to have an anabolic osteoblastic effect on bone. Other approved indications include Paget's disease, bone-associated pain conditions, and hypercalcemia (emergency, for injectable sCT). Nevertheless, approved indications vary from country to country (6,7). sCT is commercially available as an injectable formulation for intravenous, intramuscular or subcutaneous use, and as a nasal spray. An oral sCT preparation is currently under clinical development. In this new oral formulation of sCT, the 5-CNAC-disodium salt functions as a carrier which provides bioavailability for the sCT peptide. The oral calcitonin formulations have been developed and enabled using Emisphere's Eligen[®] technology (7).

A number of assays have been reported for the quantification of sCT. Reported analytical methods of sCT include liquid chromatography-mass spectroscopy (LC-MS) (8-10). Some of the analytical methods of sCT typically utilize biological or radioimmunoassay techniques which have high variability and low accuracy (11,12). These limitations can be overcome by use of chromatographic techniques. High performance liquid chromatography (HPLC) is one of the most widely used and rapidly expanding techniques for the separation of polypeptides and peptide hormones (13). Many of these assays include precolumn derivatization with fluorescence labeling agents (14,15). However, this includes one additional step before injection and variability is possible. There are assays reported for fractionation of glycopeptides also (16). Gradient reverse-phase HPLC (RP-HPLC) separations of sCT were reported previously (17-22), but this approach can take a long time (from 12 min to 38 min) to identify and quantify sCT, which is not only time consuming but also consumes additional organic solvents. Isocratic HPLC separations of sCT were also reported previously, but details about the chromatographic efficiency were not given (23-25).

The aim of this work was to develop and validate a simple, rapid and isocratic RP-HPLC method for quantification of sCT in raw materials and pharmaceutical dosage forms.

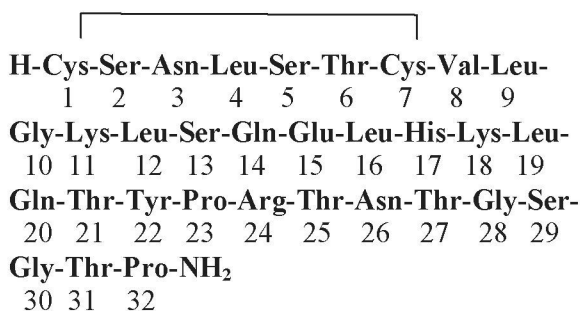


Figure 1. Graphic formula of sCT

EXPERIMENTAL

Chemicals and reagents

Certified reference material of sCT was purchased from Bachem Bioscience (King of Prussia, PA, USA). Pharmaceutical formulations containing sCT (Injectable and nasal spray formulations) were purchased commercially. Acetonitrile HPLC grade (EMD Chemical Inc., Gibbstown, NJ, USA), trifluoroacetic acid (TFA), (Fischer Scientific, Fair Lawn, NJ, USA) and deionized water was used.

Apparatus and chromatographic conditions

The HPLC system is Agilent 1200 Series (Germany) Quaternary LC system consisting of G1311A quaternary pump, G1329A ALS autosampler, G1314B VWD operating at 220 nm and a data station with HP Chemstation.

Chromatographic separation was performed using a reverse-phase Agilent Eclipse XDB-C18 column (150 x 4.6 mm i.d., 3.5 μ m). The mobile phase consisted of acetonitrile and water (35:65 v/v) containing 0.1% trifluoroacetic acid (TFA) degassed prior to use. The column temperature was 65 $^{\circ}$ C and the flow rate was set at 1 mL/min. Triamcinolone acetonide (TA) was used as an internal standard (IS).

Preparation of standard solution

Stock solutions of sCT (1000 μ g/mL) were prepared by dissolution in 10 mM citrate buffer pH 3.5 and this solution was stored at 4 $^{\circ}$ C until use. Further dilution of the stock solution for preparation of the calibration standards of sCT (2.5, 5, 10, 25 and 50 μ g/mL) was prepared using mobile phase.

Method Validation

Linearity

The calibration curves were constructed with five concentrations including the lower limit of quantification (LLOQ) ranging from 2.5 to 50 μ g/mL. The peak area ratio of the drug to the IS was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method (26).

Limits of detection (LOD) and Limit of quantification (LOQ)

Detection limit is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified under the stated experimental conditions. Detection limit was determined by preparing a solution that is expected to produce a response that is \sim 3 to 10 times baseline noise. The solution was injected three times, and the signal and the noise for each injection was recorded. Each signal-to-noise ratio (S/N) was then calculated, and averaged. The concentration of the solution was used to determine the detection limit if the average S/N ratio is between 3 and 10. If outside this range, the solution concentration was modified as necessary and the experiment was repeated. The limit of detection (LOD) may be expressed as:

$$\text{LOD} = 3.3\sigma / S$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve.

Limit of quantification (LOQ) can be determined in the same manner by using the formula:

$$\text{LOQ} = 10\sigma / S \text{ (26).}$$

Precision

Precision (26) is determined in terms of instrumental precision, intra-assay precision, and inter-assay precision. The instrumental precision was studied by repetitive injections (n=10) of standard solutions of sCT (5, 10 and 25 µg/mL).

The intra-assay precision was performed by analysis of replicate injections of sample solutions of three different concentrations on the same day. The intermediate precision was evaluated by replicate analysis of sample solutions of three different concentrations on three different days. The values of percent relative standard deviation of peak areas of sCT for instrumental, intra-assay, and intermediate precision were determined.

Accuracy

The accuracy was evaluated by the recovery of known amounts of the reference substance added to a sample solution (containing 20 µg/mL of sCT and excipients) to obtain solutions with final concentrations of 32, 40 and 48 µg/mL, equivalent to 80, 100, and 120% of the nominal analytical values, respectively. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix (26,27).

Specificity

Specificity (27,28) was investigated to check the absence of interference due to the excipients of the pharmaceutical formulation. It was verified by comparing sample solution, standard solution, and placebo chromatograms. A placebo was prepared by weighing and mixing the excipients of commercial formulations of sCT and was processed in a similar way to the sample.

Robustness

Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters during normal usage. Parameters that were investigated are percent organic content in the mobile phase (± 2 %); pH of buffer in mobile phase (up to ± 0.5 pH units); column temperature (± 1 to 5 °C); flow rate (± 0.2 mL/min) and different HPLC columns (lots and/or suppliers). These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment (26, 29).

In the present study, robustness was evaluated by variation in the percentage of acetonitrile in the mobile phase, flow rate of mobile phase and temperature of the column.

System suitability

The system suitability was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed using six replicate analyses of the drug at a concentration of 10 µg/mL. The parameters evaluated were resolution factor, tailing factor, selectivity, capacity factor and relative standard deviation (RSD%) of retention time (27, 28).

Assay of sCT in pharmaceutical formulations

Dilutions of commercial formulation of sCT (Injectable and nasal spray formulations) were made with mobile phase and final concentration was adjusted to 25 µg/mL and filtered through a membrane filter having a pore diameter of 0.45 µm.

RESULTS AND DISCUSSION

Development and optimization of the analytical methodology

Drug analysis is undertaken during various phases of pharmaceutical development, such as formulation, stability studies, cleaning validation, quality control, and pharmacological testing in animals and humans. All these require reliable analytical methods to measure drugs in pharmaceutical formulations and biofluids (30).

The aim of the present study was to develop a simple and rapid isocratic HPLC assay for the quantification of sCT in raw materials and pharmaceutical formulations.

A stock solution of sCT was prepared in 10 mM citrate buffer, because this buffer system provides the best stability conditions for sCT (31).

To obtain the best chromatographic conditions, the wavelength for detection, the column, and the mobile phase composition were adequately selected. The use of acetonitrile and water (35:65, v/v) containing TFA at a ratio of 0.1 % resulted in a better peak symmetry (1.17) and in a relatively short retention time (2.32), which allowed rapid determination of the drug. The column temperature was kept constant at 65 °C.

An ion-pairing reagent, TFA, was selected for use in the mobile phase to obtain a good peak shape. sCT has a hydrophilic structure, so the mobile phase is often adjusted to low pH values (below 3) to decrease the polarity of the peptide and to increase interaction with the nonpolar stationary phase (32). Most of the carboxylic groups of the amino acid residues are in undissociated form at this pH value. But some of the amine residues (lysine, arginine and histidine) are charged at this pH value. So, we must use an appropriate reagent that can pair with these ions to produce a molecular complex that possesses sufficient hydrophobicity to interact with the stationary phase. TFA is a good candidate as an ion pairing reagent because of its desirable properties like volatility, protein solubilization and adsorption characteristics. Another positive approach for TFA is that it does not disrupt the native protein confirmation. On the other hand, TFA is a powerful partition reagent and has strong surface-active properties. Therefore, it can be used to mask the remaining silanol groups on the support surface (4, 33).

The use of an appropriate IS can enhance the precision and accuracy of the method by means of correcting fluctuations in the detector response. Often, sample preparation steps that include filtration, reaction, extraction and so on result in sample losses. If a suitable IS can be added prior to sample preparation, sample losses in the preparation steps can be corrected (34). A different compound from the analyte, triamcinolone acetonide (TA), was used as the IS in this study. Although the chemical structure of TA is not similar to sCT, it shows similar absorptivity and chromatographic behavior pattern to sCT. On the other hand, maximum UV absorbance of both substances seems very near to each other (35). Indeed, our chromatograms showed that TA presented a shorter retention time with a symmetrical peak having sharp and better resolution (Figure 2).

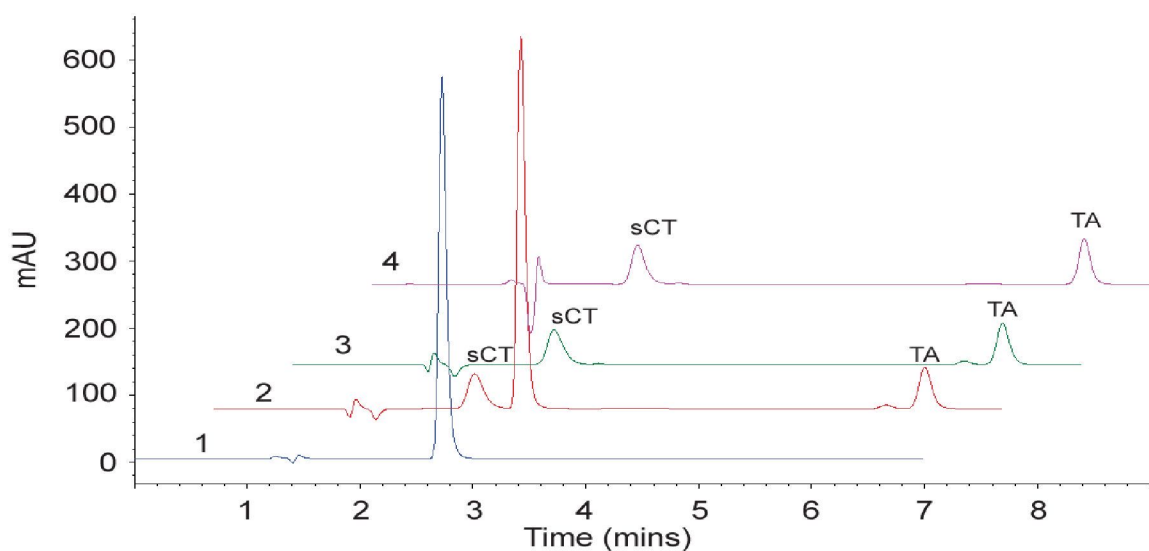


Figure 2. Chromatogram overlay of placebo (1), commercial injectable formulation (2), commercial nasal spray formulation (3) and standard (4).

The detection wavelength was defined from the absorption spectrum of sCT in the UV-vis range. The wavelengths of 210, 215 and 220 nm were initially tested, and the third one proved to be most sensitive to quantification of the drug.

The isocratic RP-HPLC conditions described allowed the separation of sCT and TA within a run time of 7 min. sCT was eluted at 2.32 min whereas TA was eluted at 6.27 minutes. No interfering peaks were observed in the chromatograms. Figure 2 represents a typical chromatogram, obtained in the chromatographic conditions defined for the method.

Validation of the Method

Linearity

The linearity of the method is a measure of how well a calibration plot of response versus concentration approximates a straight line, or how well the data fit to the linear equation. Linearity and range of the method were determined by analyzing different solutions containing 2.5-50 µg/mL of sCT under the chromatographic conditions mentioned previously (n=5). Then, 50 µL solutions were injected into the column and the chromatogram was recorded. Peak area ratios ($A_{\text{sample}} / A_{\text{IS}}$) were plotted against corresponding concentrations in the different concentration ranges of the compounds. Linear regression parameters of the peak area ratios versus concentrations of the compounds are presented in Table 1. The results showed highly reproducible calibration curves with correlation coefficients of >0.999.

Table 1. Analytical parameters of calibration curve of sCT.

Parameters	sCT
Linearity range	2.5-50 µg/mL
Correlation coefficient	0.999
Slope ± S.D.	0.0393 ± 0.0003
Intercept ± S.D.	0.0528 ± 0.0011

Limits of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated as given in the Methods section. LOD and LOQ of sCT were 1 µg/mL and 2.5 µg/mL, respectively.

Precision

Data obtained from analysis of the samples on the same day (n=3) and on consecutive days (n=3) are given in Table 2. As evident, the %RSD values of the data obtained were well below 2% (i.e., in the range of 0.99-1.51% and 1.33-1.73% for intra- and inter-day, respectively). The % RSD values indicate that the method was sufficiently precise.

Table 2. Instrumental, intra-day and interday precision of the method.

Added (µg/mL)	Instrumental Precision		Intra-day		Inter-day	
	Injection Repeability RSD%		Measured (µg/mL)	RSD%	Measured (µg/mL)	RSD%
5	0.94		5.07±0.05	0.99	5.21±0.09	1.73
10	0.59		9.91±0.11	1.11	9.79±0.13	1.33
25	0.37		25.23±0.38	1.51	24.32±0.33	1.36

Accuracy

Percentage recovery was calculated from differences between the peak areas obtained for fortified and unfortified solutions. As shown from the data in Table 3, recoveries close to 100% were made at each added concentration, confirming that the method was accurate.

Table 3. Accuracy of method determined by recovery of sCT from commercial injectable solutions spiked with standard solution.

Sample	Level	Recovery %	Mean (3)	RSD %	Bias %
1	80	97.81	99.69	1.97	-2.22
2		101.73			1.72
3		99.54			-0.46
1	100	102.07	101.01	1.68	2.08
2		99.05			-0.95
3		101.92			1.93
1	120	100.31	101.20	0.78	0.31
2		101.49			1.51
3		101.81			1.81
Mean (9)		100.64			
RSD %		1.48			

Selectivity

Selectivity was assessed by a qualitative comparison between chromatograms obtained from sample, standard, and placebo solutions (Figure 2). It was observed that none of the peaks appear at the same retention time as sCT and TA peaks. As a result, we conclude that the method developed is selective in relation to the excipients of the final preparation.

System suitability

Evaluation of performance of both the analytical instrument and whole analytical method is a common and required analytical practice before its application. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results with acceptable accuracy and precision. System suitability tests are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. Based on United States Pharmacopeia (USP) 32, section 621 (32), system suitability tests were carried out on the chromatogram of freshly prepared standard solutions to check various parameters. These parameters included RSD% of retention times, tailing factor, resolution, capacity factor, selectivity factor and RSD% peak height or area for repetitive injections, and are shown in Table 4. Generally, it is sufficient to demonstrate system suitability tests if at least two of these criteria meet the requirement (27,29,34).

Table 4. Results of system suitability.

Parameters	Acceptance criteria	sCT
Retention time (min)	-	2.32
Resolution	>2.0	3.42
Tailing factor	<2.0	1.17
Theoretical plates	>2000	3984
Capacity factor	>2.0	4.69

We conclude that the developed method is the optimum according to the studied parameters. The capacity factor obtained is within the accepted values, above 2 for the sCT. The asymmetry to be controlled was within the limits established by these guidelines. Lastly, good resolution was obtained between two consecutive peaks in the developed method. Therefore, this method can be applied to its intended purpose with no problems, its suitability being demonstrated.

Robustness

In order to study the robustness of the proposed method, deliberate modifications in the percentage of acetonitrile in the mobile phase, flow rates of mobile phase and temperature of the column were made. The results are shown in Table 5. It can be seen that in every employed condition, the assay data remained acceptable. A change of $\pm 2\%$ on acetonitrile ratio of mobile phase, flow rate and column temperature had no significant impact on chromatographic performance and did not generate any extra peaks and distortions on peak morphologies.

Table 5. Influence of changes in experimental conditions on the performance of the HPLC system.

Factor	Level	R _T	Tailing Factor
Flow rate (mL/min)			
0.98	-1	2.34	1.43
1	0	2.32	1.36
1.02	1	2.29	1.25
Acetonitrile composition (%)			
34	-1	2.72	1.31
35	0	2.32	1.36
36	1	1.98	1.15
Column temperature			
63.5	-1	2.37	1.27
65	0	2.32	1.36
66.5	1	2.29	1.31

Assay of sCT in Pharmaceutical Formulations

The method was used to quantify sCT in commercial injectable and nasal spray formulations. As shown in Figure 2, chromatograms from sCT formulations and the sCT standard are no different, which indicates that other ingredients in the sCT formulations have no effect on the determination of sCT by this UV detection system.

Results from assay of the sCT injection and nasal spray are shown in Table 6; the values seem satisfactory and in agreement with the assumption that 1 IU sCT is approximately equivalent to 0.2 μg (36).

Table 6. Results from assay of sCT fom commercial formulations by isocratic HPLC method with UV detection (mean \pm S.D., n=6).

Formulation	Claim ($\mu\text{g}/\text{mL}$) (IU/mL)	Found ($\mu\text{g}/\text{mL}$)
Injectable	40 (200)	38.92 \pm 2.27
Nasal spray	440 (2200)	431.64 \pm 7.91

CONCLUSIONS

The proposed HPLC method has been evaluated over the accuracy, precision, linearity, specificity, and sensitivity and shown to be convenient and effective for the quality control of sCT in pharmaceutical dosage forms. The measured signal was shown to be precise, accurate, and linear over the concentration range tested (2.5 – 50 µg/mL) with a correlation coefficient 0.999. The main advantage of this new method is short analysis time, even with an IS. Moreover, isocratic elution makes this method applicable to any simple HPLC instrument. Solvent consumption is very low due to short analysis time (7.0 min) and this can reduce the cost of analysis in routine quality control of sCT in pharmaceutical preparations.

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