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A HIGHLY SENSITIVE HPLC METHOD WITH NON-EXTRACTIVE SAMPLE PREPARATION AND UV DETECTION FOR TRACE DETERMINATION OF CINACALCET IN HUMAN PLASMA

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A highly sensitive HPLC method with non-extractive sample preparation and UV detection has been developed and validated for the trace determination of cinacalcet (CIN) in human plasma. Paracetamol (PCM) was used as the internal standard. CIN and PCM were isolated from plasma by protein precipitation with acetonitrile. Chromatographic separation was achieved in isocratic mode on a C18 column (150 mm × 4.6 mm, i.d. 5µm particle size) by a mobile phase consisted of acetonitrile and 50 mM phosphate buffer (50:50 v/v) adjusted to pH of 7.4, at a flow rate of 1.0 mL/min. The eluted compounds were monitored by UV detector at 235 nm. Under the optimum chromatographic conditions, a linear relationship with good correlation coefficient (0.9998) was obtained between the peak area ratio of CIN to that of PCM and the concentration of CIN in a range of 5-5000 ng/mL. The lowest limits of detection and quantitation of the proposed method were 2.5and 7.7 ng/mL, respectively. The intra- and inter-day precisions were satisfactory; the relative standard deviations did not exceed 4.12%.The accuracy of the method was proved; the recoveries of CIN from spiked human plasma were in the range of 95.19 - 99.47 ± 0.11-4.12%.The method has high throughput because of its simple sample preparation procedure and short run time (<6 min). The results demonstrated that the proposed method would have great value when applied for determination of CIN in human plasma for the purposes of pharmacokinetic and bioequivalence studies.

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Keywords: Cinacalcet; HPLC, UV; Plasma; Pharmacokinetic; Bioequivalence studies.

1. Introduction

Cinacalcet (CIN) is described chemically as N-[1-(R)-(−)-(1-naphthyl) ethyl]-3-[3-(trifluoromethyl) phenyl]-1-aminopropane, and its structural formula is given in Fig. 1. CIN is a selective calcimimetic agent, which acts on a calcium-sensing receptor of the parathyroid gland. This principal negative regulator of parathyroid hormone release increases its selectivity to activation by extracellular calcium, thus decreasing parathyroid hormone levels [1,2]. CIN is effective in Clinical setting and it has been approved for the treatment of secondary hyperthyroidism in patients with chronic kidney disease placed on dialysis [3], and for the treatment of elevated calcium levels in patients with parathyroid carcinoma [4].

The effective and safe therapy with CIN is basically depending on the quality of its pharmaceutical dosage forms, and assessing its concentrations in patient's plasma for the purposes of pharmacokinetic studies. Nevertheless, the therapeutic profile of CIN is anticipated to encourage the development of new pharmaceutical generics for CIN. As a consequence, there is an increasing demand for proper analytical technologies for determination of CIN in determining its pharmacokinetic parameters and in bioequivalence studies.

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To the best of our knowledge, based on an extensive literature survey, only four methods exist for analysis of CIN [5-8]. The first two methods were thin-layer [5] and HPLC [5,6]; these methods were employed for the qualitative enantiomeric separation of CIN enantiomers in laboratory-made racemic mixtures. The third method was HPLC with UV detection for detection of impurities in presence of CIN in bulk drug manufacturing [7].The fourth method was liquid chromatography-coupled with tandem mass spectrometric detector (LC-MS-MS) [8]. This method offered the adequate sensitivity for determination of CIN in plasma. However, this method employed the tandem mass detector that is expensive and not available in most clinical laboratories. As well, this method involved laborious solid-phase extraction procedures for the sample. These extraction procedures are usually negatively affect the convenience of the method, and decrease the accuracy of the results. For these reasons, the development of a new alternative simple method with adequate sensitivity for the determination of CIN in plasma samples was very essential.

The present study describes, for the first time, the development of a highly sensitive and simple HPLC method with UV detection for the determination of CIN in human plasma. The method involved a very simple non-extractive isolation of CIN from plasma samples using protein precipitation with acetonitrile followed by direct injection of the supernatant into the HPLC system. The method was successfully applied to determination of CIN in spiked human plasma samples.

2. Experimental

2.1. Chromatographic System

Chromatographic analysis was performed on Acme 9000 HPLC system (Young Lin Instruments Co., Ltd., Anyang, Korea) consisted of a SP930D0 gradient pump, Rheodyne-7725i (SUS) manual injection valve with variable loop volume of 20 µL, column oven, and UV730D UV-Visible detector. The system was controlled and data was acquisitioned by Autochro-3000 software provided with the system. The chromatographic separations were performed on Mediterraneasea C18 column (150 mm × 4.6 mm i.d., particle size of 5 µm) manufactured by Teknokroma (Barcelona, Spain). The column temperature was kept constant at 25 ± 2 °C. Separations were performed in the isocratic mode with a mobile phase consisting of aqueous phosphate buffer (50 mM):acetonitrile (50:50, v/v) adjusted to pH of 7.4 at a flow rate of 1.0mL/min. The mobile phase was filtered by a Millipore vacuum filter system equipped with a 0.22 µm filter, and degassed by ultrasonic bath. The sample injection volume was 20 µL. The UV detector was set at 235 nm. The system control and data acquisition were performed by Autochro-3000 software. The ratio of peak area of CIN to that of the internal standard (paracetamol; PCM) was used for the quantitation of CIN.

2.2. Materials

Cinacalcet hydrochloride was obtained from Amgen Inc. (Thousand Oaks, CA, USA) and used as received; its purity was > 99%. Paracetamol was obtained from Sigma Chemical Co. (St. Louis, USA). Human plasma samples were collected from normal healthy volunteer at King Khalid University Hospital (Riyadh, Saudi Arabia), and they were stored in deep-freezer at –20 ºC until analysis. All solvents were of HPLC grade (Merck, Darmstadt, Germany). All other materials were of analytical grade.

2.3. Preparation of solutions

2.3.1. Standard solutions of CIN and PCM. An accurately weighed amount (50 mg) of each of CIN and PCM was quantitatively transferred into a 25-mL calibrated flask, dissolved in 20 mL distilled water, completed to volume with the same solvent to obtain a stock solution of 2

mg/mL. The stock solutions were found to be stable for at least two weeks when kept in a refrigerator. The stock solutions were further diluted with water to obtain working solutions in the range of 5-5000 ng/mL for CIN and a fixed concentration of 500 ng/mL for PCM.

2.3.2. Phosphate buffer solution. An accurately weighed amount (3.0 g) of disodium hydrogen phosphate was dissolved in 500 mL. The pH of the solution was adjusted to 7.4 with 0.1 M NaOH using a calibrated pH meter.

2.3.3. Plasma samples. Aliquots of 100 µL of drug-free plasma were dispensed into eppendorf tube and were spiked with 100 µL of CIN standard solution (20─1000 ng/mL) and 50 µL of paracetamol solution (10 µg/mL). To each solution, 750 µL of acetonitrile was added and the mixture was vortexed for 3 min, and centrifuged at 6000 rpm for 30 min. The precipitated protein was removed by centrifugation and the clear supernatant was used for analysis; 20 µL was injected into the HPLC system.

3. Results and discussion

3.1. Strategy for method development

HPLC is one of the most efficient and widely used analytical techniques in both quality control and clinical laboratories. The wide applicability of HPLC is attributed to its high sensitivity, accuracy, and more importantly, its inherent high selectivity. HPLC methods with UV detection, as far as they offer the required sensitivity for their particular tasks, are more convenient and less complex than HPLC-MS. Since CIN is a chromophoric molecule, the present study was devoted to the development of UV-based HPLC method with adequate sensitivity for the determination of CIN plasma samples. Darwish *et al.* [9,10] has successfully developed simple and convenient non-extractive procedures for HPLC analysis of some pharmaceuticals in plasma. Therefore, this non-extractive procedure was employed in the development of the HPLC method described herein. The selection of PCM as IS in the present study was based on its UV absorption at those of CIN, and preliminary experiments that indicated its possible resolution from CIN under the same chromatographic conditions.

3.2. Optimization of the chromatographic conditions

The chromatographic conditions were optimized to achieve an efficient separation, adequate response with sharp peaks, and a short run time for separation of CIN and PCM. These conditions were the composition of the mobile phase and the type of column. Preliminary separation experiments were initiated using isocratic elution mode on a reversed-phase C18 column. Mobile phases consisting of different ratios of methanol-water and acetonitrile-water were examined. These mobile phases produced quite satisfactory separation, however the response of CIN was not adequate for its determination in plasma at its therapeutic concentration range (10–70 ng/mL). Previous studies have shown that the addition of an appropriate ratio of buffer solution in the mobile phase may improve the chromatographic response [11-13]. Different ratios of acetonitrile and phosphate buffer solution were studied. The best resolution was achieved when the ratio of acetonitrile:phosphate buffer was 50:50 (v/v). Since both CIN and PCM are pH sensitive compounds, the effect of pH of the mobile phase was investigated. Mobile phases of varying final pH values (from 5.5 to 9.0) were studied. Good resolution was achieved at pH 8 and 9, however the retention times of both CIN and PCM were short; the peak of PCM overlapped with those of the eluted plasma components. The mobile phase with final pH value of 7.4 gave the best results in terms of resolution and run time. Different concentrations of phosphate buffer solution (10, 25, and 50 mM) in the mobile phase were tested. The results showed that the lowest concentration of buffer solution cause broadening and overlapping of the peaks. The best resolution was obtained when the buffer concentration was 50 mM. Employing the mobile phase which consisted of

acetonitrile:50 mM phosphate buffer (50:50, v/v), at a flow rate of 1 mL/min gave the best results in terms of good resolution, adequate response, and better peak shape. Under these chromatographic conditions, the retention times were 4.1 ± 0.12 and 5.2 ± 0.14 min for PCM and CIN, respectively. A representative chromatogram for CIN and PCM, along with their chemical structures is given in Fig. 1, and the chromatographic parameters are given in Table 1.

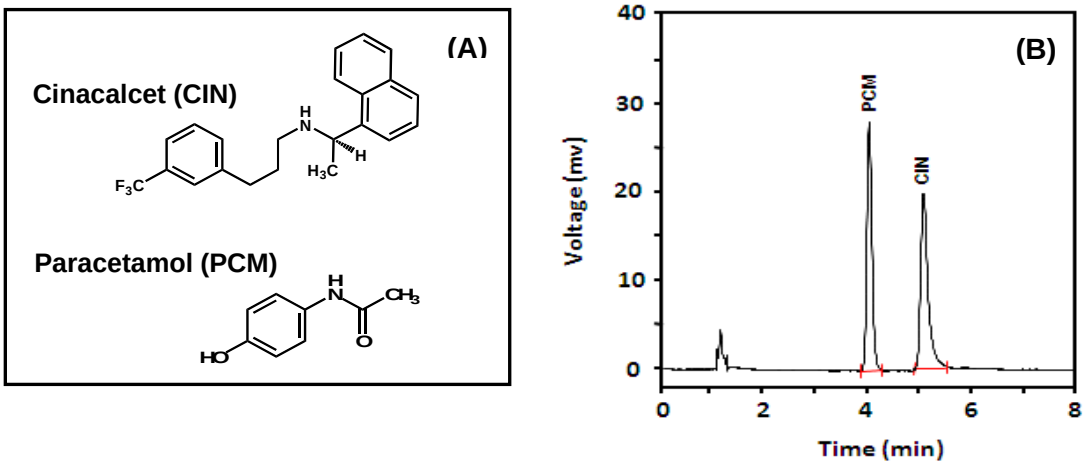

Figure 1. Chemical structures of CIN and PCM (A), and the chromatogram of their standard solution (B). The concentrations of CIN and PCM were 250 and 500 ng/mL, respectively.

Table 1. Chromatographic parameters for CIN and PCM by the proposed HPLC method

Parameter	Value
Retention time of CIN (min)	5.2 ± 0.14
Retention time of PCM (min)	4.1 ± 0.12
Capacity factor	1.26
Separation factor	1.97
Resolution factor	2.34
Peak asymmetry factor	1.08
Theoretical plate number	3136
Limit of detection (ng/mL)	2.5
Limit of quantitation (ng/mL)	7.7

3.3. Validation of the method

3.3.1. Linearity and sensitivity. Under the above optimum conditions, linear relationship with good correlation coefficient (r = 0.9998, n = 3) was found between the peak area ratio of CIN to PCM (Y) versus CIN concentration (X) in the range of 5–5000 ng/mL. The mean regression equation of the calibration curve obtained from nine points was: $Y = 0.2267 + 0.0026\,X$. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to the ICH guidelines for validation of analytical procedure based on the standard deviation of the response and the slope of the calibration curve [14]. The LOD and LOQ were found to be 2.5 and 7.7 ng/mL, respectively.

3.3.2. Precision and accuracy. The precision of the method was evaluated in terms of repeatability (intra-day) and intermediate precision (inter-day) [14]. Intra-day precision was studied at six concentration levels (Table 2) of CIN. Three aliquots were prepared from each

concentration level, and injected into the HPLC system. Inter-day precision was carried out on five different days at the same concentration levels of CIN samples freshly prepared daily. The relative standard deviation (RSD) values were used as a measure for the precision. The method gave satisfactory precise results as the RSD values did not exceed 4.12% (Table 2).

Table 2. Precision and accuracy data for analysis of CIN by the proposed HPLC method in spiked human plasma.

Nominal CIN (ng/mL)	Measured CIN (ng/mL)	Recovery (%)	RSD (%)
Intra-day			
20	19.70	98.59	0.99
100	95.52	95.52	3.72
200	193.80	96.90	1.22
500	475.95	95.19	4.12
1000	961.50	96.15	0.12
3000	2984.10	99.47	0.11
Inter-day			
20	19.04	95.20	1.91
100	96. 93	96. 93	2.17
200	192.02	96.01	1.68
500	475.30	95.06	3.50
1000	960.50	96.05	0.52
3000	2970.90	99.03	0.47

Accuracy was determined using the data that have generated from precision assessment, and calculating the recovery percentages, which was the ratio of the measured concentration to that of the nominal ones, expressed as percentages. The proposed method gave satisfactory results as the recovery values were 95.06 – 99.47% (Table 2).

3.3.3. Selectivity. The typical chromatogram of standard solution of CIN and PCM is given in Fig. 1B. It is obvious that both CIN and PCM were well separated under the applied HPLC conditions. Retention times were 4.1 ± 0.12 and 5.2 ± 0.14 min for PCM and CIN, respectively. This good separation indicated the appropriate selectivity of the proposed HPLC method.

Since the proposed method is devoted to the analysis of CIN in plasma, it was necessary to assess the selectivity of the method using plasma samples. Experiments were carried using blank human plasma to identify the peaks due to the plasma components, and check their resolution from the peaks of CIN and PCM. Typical chromatograms obtained from blank plasma, plasma spiked with PCM, and plasma spiked with both CIN and PCM are shown in Fig. 2. The chromatograms showed complete separation of CIN and PCM from the endogenous plasma constituents. This indicated the selectivity of the proposed method for determination of CIN in plasma samples.

3.3.4. Robustness and ruggedness. Robustness was examined by evaluating the influence of small variation in the method variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in the method variables did not significantly affect the procedures; recovery values were 96.55 – 98.83% ±1.63 – 0.15 (Table 3). This indicated the reliability of the proposed method during its routine application for the determination of CIN in plasma.

Ruggedness was also tested by applying the proposed method to the assay of CIN using the same operational conditions but using two different instruments at two different laboratories

and different elapsed time. Results obtained from lab-to-lab and day-to-day variations were reproducible as the RSD did not exceed 2%.

3.4. Applications of the method

The applicability of the method to the determination of CIN in human plasma was investigated. The therapeutic mean plasma concentrations after treatment with 30 and 60 mg of CIN were reported to be 24.7 and 69 ng/mL, respectively [15], which are higher than the LOQ of the proposed method (7.7 ng/mL). Plasma samples were spiked with CIN in its reported therapeutic plasma levels (10–70 ng/mL), and the spiked samples were subjected to the analysis by the proposed method. The results were satisfactorily in terms of the accuracy and precision as the recovery values were 98.32–102.41 % with low RSD (Table 4). These data indicated the applicability of the proposed HPLC method for the accurate determination of CIN in plasma in bioequivalence and pharmacokinetic studies.

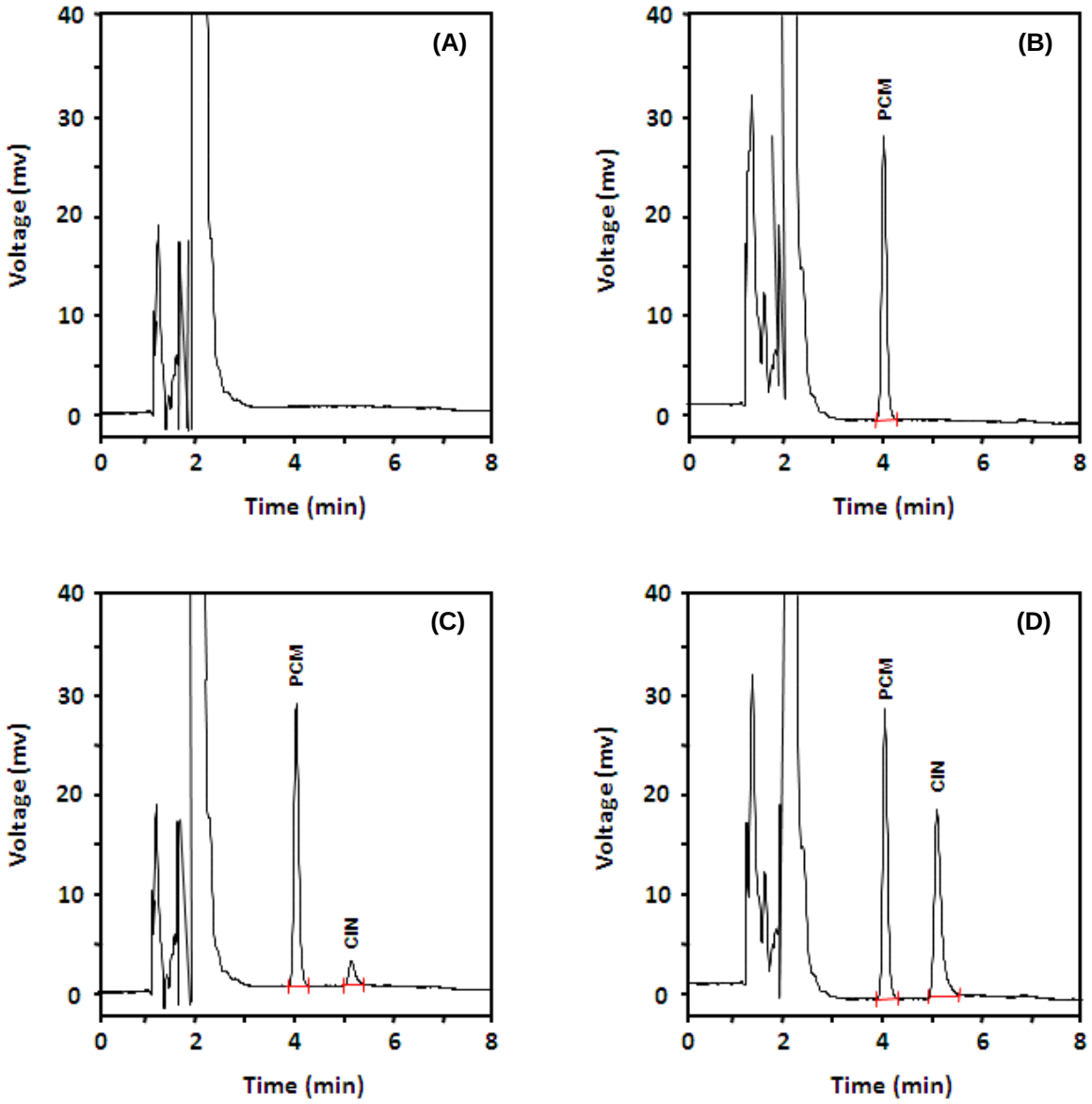

Figure 2. Representative chromatograms from (A) blank CIN-free human plasma, (B) plasma spiked with PCM (500 ng/mL), (C) plasma spiked with PCM (500 ng/mL) and CIN (7.7 ng/mL; LOQ), and (D) plasma spiked with CIN (250 ng/ml) and PCM (500 ng/mL). mV is the detector response in millivolts.

Table 3. Influence of small variations in the assay conditions on the analytical performance of the proposed HPLC method for determination of CIN.

Parameters	Recovery (% ± SD) [a]
The recommended condition [b]	97.29 ± 1.95
Ratio of acetonitrile:phosphate buffer	
45:55	96.55 ± 1.63
55:45	98.26 ± 0.64
pH of the mobile phase	
7.2	96.89 ± 2.19
7.6	97.03 ± 2.09
Flow rate (mL/min)	
0.8	98.83 ± 0.15
1.2	96.88 ± 1.56

[a] Values are mean of 3 determinations.
[b] The recommended conditions were given in the Experimental Section.

Table 4. Application of the proposed HPLC method in the determination of CIN in spiked human plasma.

Spiked concentration (ng/mL)	Measured concentration (ng/mL)	Recovery (% ± RSD) [a]
10	9.83	98.32 ± 2.82
20	19.81	99.05 ± 0.98
30	30.57	101.89 ± 2.37
40	39.92	99.81 ± 0.22
50	50.27	100.55 ± 1.25
60	59.55	99.25 ± 0.33
70	71.69	102.41 ± 0.23

[b] Values are mean of three determinations.

4. Conclusions

The sample preparation procedure was very simple and robust as it did not involve liquid-liquid or solid-phase extraction of CIN from plasma. It was based on only protein precipitation with acetonitrile followed by injecting the supernatant into the HPLC system. The chromatographic separation was based on a reversed phase mechanism carried out under isocratic elution mode for short run time (< 6 min). The method employed UV detector for detecting the eluted compounds, which is the basic component and most commonly used HPLC detector. The analytical results demonstrated that the proposed method is suitable for the accurate determination of CIN in plasma at concentrations as low as 7.7 ng/mL, and has a wide linear range. Compared to the previously reported LC-MS-MS method [8], the proposed method provided more simple procedure and less instrumentation cost and complexity. The simple procedure and the short run-time added the property of a higher throughput to the method. Although real CIN plasma samples

were not analyzed by the proposed method, however, the performance of the method makes it valuable for the combined pharmacokinetic studies and bioavailability evaluation of CIN in human subjects after oral administration of low therapeutic CIN dose.

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