Acta Pharm. 52 (2002) 243–250 Original research paper

Simultaneous quantification of cefaclor and cephalexine in blood plasma using high-performance liquid chromatography with UV detection

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Concentration of cefaclor and cephalexine were measured simultaneously in blood plasma after protein precipitation, using a newly developed RP high-perfomance liquid chromatographic method on C_8 colum. The method was found to be reproducible with a relative standard deviation less than 6.0% over the concentration range 0.2–30.0 mg mL^{-1} for cefaclor and 0.5– 50.0 mg mL^{-1} for cephalexine in blood plasma samples. The limits of quantification were 0.1 and 0.25 mg mL^{-1}, respectively.

Keywords: cefaclor, cephalexine, high-performance liquid chromatography

Received September 27, 2002
Accepted November 11, 2002

Cefaclor [6R-[6α,7β(R*)]]-7-[(aminophenylacetyl)amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate, and cephalexine [6R-[6α,7β-R*)]]-7-[(aminophenylacetyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2--carboxylic acid, are cephalosporin antibiotics (Fig. 1). Cephalexine is a first-generation cephalosporin antibiotic (1). It is administered by mouth for the treatment of infections of the biliary, respiratory, and urinary tracts and of the skin. Cefaclor is an antibiotic administered by mouth similarly to cephalexine in the treatment of infections of the upper and lower respiratory tract, skin infections, and urinary-tract infections. Some classify cefaclor as a second-generation cephalosporin and its stronger activity against *Haemophilus influenzae* makes it more suitable than cephalexine for the treatment of infections such as *otitis media* (1).

In order to monitor blood plasma levels of cefaclor and cephalexine in patients and to carry out pharmacokinetic studies, a rapid, sensitive and reproducible method for their

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determination is required. Various high-performance liquid chromatographic (HPLC) methods (2, 3) have been reported for the determination of cefaclor and cephalexine in pharmaceutical preparations. Agbaba *et al*. (4) reported a high-performance thin-layer chromatographic (HPTLC) method for simultaneous determination of cefaclor and cephalexine in pharmaceuticals. Huang *et al.* (5) investigated the effects of column, ion--pairing reagents and mobile phase pH on the retention times of five amphoteric beta--lactam antibiotics using reversed-phase high-performance chromatography.

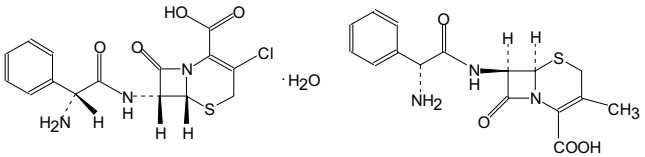

Fig. 1. Structural formulae of cefaclor (a) and cephalexin (b).

There are several methods for the determination of cefaclor and cephalexine in biological fluids by high-performance liquid chromatography (6–12). Nakagawa *et al.* (6) investigated the retention properties of the internal-surface reversed-phase silica packing and recovery of drugs from human blood plasma. Many authors have performed determinations of cefaclor or cephalexine in blood plasma or serum after protein precipitation using a mixture of cold methanol-sodium acetate (7) or acetonitrile (9). These methods yield poor separation of cefaclor and cephalexine from blood plasma and serum endogenous interferences. Other investigators have performed separation of cefaclor or cephalexine from blood plasma with the solid-phase extraction technique by means of C_{18} solid-phase cartridges (11, 12).

Some of the previously published methods require an evaporation step and they are time consuming. Therefore, in this paper, we report a simple, sensitive, and easy to operate HPLC method using UV detection for a simultaneous determination of cefaclor and cephalexine in human blood plasma.

EXPERIMENTAL

Materials

All chemicals and reagents used were of HPLC grade or analytical grade. Cefaclor was kindly supplied by Lupin Limited (India). Cephalexine was purchased from SUN Pharmaceuticals Ltd. (India). Other chemicals of reagent grade and solvents of analytical and HPLC grade were purchased from Merck (Germany).

Chromatography

A series of parameters, including composition and pH of the mobile phase, column packing, flow rate and detection wavelength, were tested for the location and shape of the peaks of cefaclor and cephalexine in the respective chromatograms. The final choice of the stationary phase giving satisfaction resolution and run time was the Hibar Lichrospher 100 RP 8 (250 ×4 mm) with 5 μm particle size, protected by a guard column Lichrospher 100 RP 8 (4 ×4 mm, 5 μm, Merck). The composition of the mobile phase was methanol – 0.025 mol L^{-1} KH_2PO_4 (pH 2.2) (25:75, *V/V*) delivered by a pump Perkin-Elmer LC series 200 (Perkin-Elmer, USA). pH of the buffer solution was adjusted by adding a very small amount of concentrated *ortho*-phosphoric acid. The mobile phase was filtered and degassed with helium. The flow-rate of the mobile phase was 1.2 mL min^{-1}. The column was thermostated at 25 °C. An ultraviolet diode array detector (Perkin Elmer LC 235 C) was used for detection and 255 nm was chosen as the optimal wavelength for simultaneous determination of cefaclor and cephalexine. The samples were introduced into the column using an autosampler Perkin Elmer LC ISS Series 200 and the injection volume was 120 μL. The chromatographic system was controlled by the software package Turbochrom Version 4.1. plus and UV-spectrometric data were produced by TurboScan Version 2.0.

Standards

Stock solution of cefaclor was prepared in water in a concentration of 1000 μg mL^{-1}. Stock solution of cephalexine was prepared in methanol in a concentration of 1000 μg mL^{-1}. These solutions were prepared monthly and stored at 4 °C. No change in stability over a period of 1 month was observed. Working solutions were prepared by diluting appropriate aliquots of these solutions with distilled water.

Samples

Human plasma samples were prepared by centrifuging (at 1000×*g*) the heparinized whole blood samples collected from healthy volunteers, who later participated in a bioequivalence study of cefaclor and cephalexine. This study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Sts. Cyril and Methodius University, Skopje, Macedonia.

Prepared plasma samples were stored at –20 °C. Before the analysis, the samples were thawed at 20 °C for about 10 minutes. A 0.25-mL volume of the sample was transferred into a vial and vortexed with 0.25 mL of 6% trichloroacetic acid for 30 s. After protein precipitation and centrifugation (5 min, 4000 rpm), 120 μL of supernatant was injected onto the HPLC column.

Calibration

Typical calibration curves were constructed with six blank blood plasma samples spiked with appropriate aliquots of the standard solutions. The calibration range was 0.2–30.0 μg cefaclor and 0.5–50 μg cephalexine per 1 ml of blood plasma. The standard samples were prepared according to the procedure as unknown samples. Peak heights were recorded.

RESULTS AND DISCUSSION

A series of studies were conducted in our laboratory in order to develop a convenient and easy-to-use method for quantitative analysis of cefaclor and cephalexine in biological fluids. The effects of several HPLC variables on the separation of cefaclor and cephalexine from the matrix were investigated. In our extensive preliminary experiments, a series of aqueous mobile phases, containing buffer solutions with different pH values in combination with different modifiers such as acetonitrile, methanol and triethylamine with different volume fractions, were tested. The best results were obtained with the mobile phase made up of 0.025 mol L^{-1} KH_2PO_4 (pH 2.2) and methanol in volume fractions 75:25; the best location and resolution of the peaks of cefaclor and cephalexine from the interfering peaks was achieved at 1.2 mL min^{-1} rate. A set of column packings including C_8, C_{18} and RP-select B with different lengths and particle sizes were tested and the C_8 packing showed best separation. A typical chromatogram of standard solutions of cefaclor (10 μg mL^{-1}) and cephalexine (10 μg mL^{-1}) produced by the developed HPLC method is shown in Fig. 2b. Retention time of cefaclor and cephalexine is 8.3 min and 9.3 min, respectively.

Typical chromatograms of blank blood plasma in comparison with spiked samples are shown in Fig. 2a, c. Under the chromatographic conditions described, cefaclor and cephalexine peaks were well resolved. Endogenous blood plasma components gave no interfering peaks.

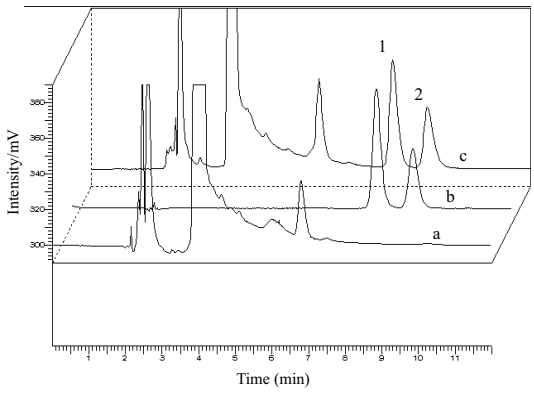

Fig. 2. Chromatograms of a) blank blood plasma, b) standard mixture of cefaclor and cephalexine, and c) spiked blood plasma sample containing 10 μg mL^{-1} cefaclor (1) and 10 μg mL^{-1} cephalexine (2).

The proposed method is of sufficient sensitivity and reproducibility to permit pharmacokinetic studies. The developed HPLC method can be used for analyses of blood plasma samples from healthy volunteers after oral administration of cefaclor and cephalexine. Typical chromatograms of blood plasma samples of patients before and after administration of 500 mg cefaclor and 1000 mg cephalexine are shown in Fig. 3. Chromatograms show no interfering peak at the cefaclor and cephalexine peak position.

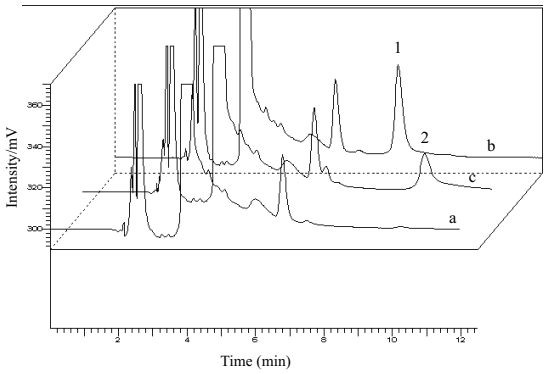

Fig. 3. Chromatograms of blood plasma samples from healthy volunteers: a) before, b) 1 h after administration of cefaclor (1) and c) 0.5 after administration of cephalexine (2).

Linearity, precision and accuracy

Calibration standards and spiked quality control samples of cefaclor and cephalexine were prepared by spiking blank blood plasma with known amounts of cefaclor and cephalexine. Linearity was tested on three different days at six concentration points ranging from 0.2 to 30.0 μg mL^{-1} of cefaclor and 0.5 to 50 μg mL^{-1} of cephalexine in blood plasma. The respective regression equations were: $y = 2538.6\,\gamma + 993.8$ for cefaclor and $y = 1668.1\,\gamma + 102.2$ for cephalexine. The correlation coefficients were 0.9993 and 0.9996, respectively.

Spiked samples from each concentration in one day and on three different days were prepared in triplicate and analyzed. The intra- and inter-day variations of the method throughout the linearity range are shown in Table I. For intra-day precision, the RSDs ranged from 0.9 to 5.7% for cefaclor and from 1.2 to 6.2% for cephalexine; for inter-day precision, RSDs ranged from 0.9 to 5.9% for cefaclor and from 1.9 to 6.0% for cephalexine. These data indicate a considerable degree of precision. Moreover, similar repeatability and intermediate precision were observed for both analytes.

Intra- and inter-day accuracy was determined by measuring the blood plasma quality control samples at low, middle and high concentration levels of cefaclor and cephalexine. Indication of accuracy was based on the calculation of the relative error of the mean observed concentration compared to the nominal value. Accuracy data are presented in Table II. Relative errors at all three concentrations studied for cefaclor and cephalexine are less than 6% and it is obvious that the method is remarkably accurate, which ensures reliable results.

Limit of quantification

The limit of quantification was defined as the signal to noise ratio 1:10 ($n = 5$). The limits of quantification were found to be 0.1 μg mL^{-1} for cefaclor and 0.25 μg mL^{-1} for cephalexine in blood plasma samples.

Table I. Intra- and inter-day precision data

Nominal concentration ($\mu g\ mL^{-1}$)	Intra-day		Inter-day	
	Mean observed concentration ($\mu g\ mL^{-1}$)[a]	RSD (%)	Mean observed concentration ($\mu g\ mL^{-1}$)[b]	RSD (%)
Cefaclor				
0.2	0.22	4.5	0.23	4.4
1.0	1.05	5.7	1.01	5.9
5.0	5.12	2.7	5.05	2.6
10.0	9.88	1.8	9.95	1.7
15.0	14.99	1.1	15.04	0.9
30.0	29.83	0.9	29.78	1.2
Cephalexine				
0.5	0.54	5.6	0.54	5.6
1.0	0.97	6.2	1.00	6.0
5.0	4.97	2.8	4.97	2.6
10.0	9.92	2.2	9.93	2.2
30.0	29.65	2.8	30.13	2.8
50.0	50.34	1.2	50.36	1.9

Number of independent analyses: [a] $n = 3$, [b] $n = 9$.

Table II. Intra- and inter-day accuracy data

Nominal concentration ($\mu g\ mL^{-1}$)	Intra-day		Inter-day	
	Mean observed concentration ($\mu g\ mL^{-1}$)[a]	Relative error (%)	Mean observed concentration ($\mu g\ mL^{-1}$)[b]	Relative error (%)
Cefaclor				
0.5	0.52	4.0	0.53	6.0
8.0	7.97	–0.4	7.94	–0.8
20.0	19.54	–2.3	19.41	–3.0
Cephalexine				
0.8	0.83	3.8	0.83	3.8
8.0	7.92	–1.0	7.96	–0.5
40.0	39.66	–0.9	40.10	0.3

Number of independent analyses: [a] $n = 3$, [b] $n = 9$.

Stability of cefaclor and cephalexine in blood plasma samples

Stability of cefaclor and cephalexine in blood plasma was investigated using spiked samples at two different concentration levels, prepared in duplicate. Spiked samples were analyzed after different storage conditions: immediately, after standing in an autosampler for 2, 12 and 24 hours at room temperature, after one and two freeze/thaw cycles and after 1 month storage at –20 °C. The investigation results show that cephalexine added to blood plasma samples is stable under different storage conditions. Also, stability studies show that cefaclor added to blood plasma samples is stable except in the case of 1 month storage at –20 °C.

Ruggedness

Ruggedness was tested using a new clean analytical column of the same type, by determining the linearity, precision and accuracy. Linearity was checked at six points within the same concentration range as described above for both analytes. The regression equations obtained were: for cefaclor $y = 2735.7\,\gamma + 550.9$ ($r = 0.9997$) and for cephalexine $y = 1691.7\,\gamma + 375.6$ ($r = 0.9991$). Intra-day precision and accuracy were determined by measuring three series of blood plasma quality control samples. Relative stan- dard deviation at all three concentrations studied for cefaclor and cephalexine was less than 5.5%. Relative errors ranged from 0.5 to 3.8% of the nominal concentrations of investigated drugs. Ruggedness expressed as RSD for the data obtained by two C_8 columns was found to be between 3.5 and 4.8%. Similarity of the results indicates that this HPLC method for simultaneous determination of cefaclor and cephalexine in spiked human blood plasma samples is rugged enough.

CONCLUSIONS

A sensitive and rapid HPLC method has been developed for simultaneous determination of cefaclor and cephalexine in blood plasma samples. The typical assay time is about 12 min. No internal standard is required and this simplifies the method. The proposed method is advantageous compared to the known methods for the determination of cefaclor and cephalexine in blood plasma because of its simplicity, short time of analysis, efficient clean-up of the complex biological matrix and a high recovery of cefaclor and cephalexine. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. Also, the method can be used to monitor cefaclor and cephalexine levels in clinical samples.

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S A Ž E T A K

Simultano određivanje cefaklora i cefaleksina u krvnoj plazmi pomoću tekućinske kromatografije visoke učinkovitosti uz UV detekciju

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Razvijena je jednostavna, inverzno-fazna tekućinska kromatografija visoke učinkovitosti na C_8 koloni za određivanje cefaklora i cefaleksina u krvnoj plazmi nakon precipitacije proteina. Metoda je reproducibilna, s relativnom standardnom devijacijom manjom od 6,0%, u koncentracijskom rasponu 0,2–30,0 μg mL^{-1} za cefaklor i 0,5–50,0 μg mL^{-1} za cefaleksin. Granice određivanja su 0,1, odnosno 0,25 μg mL^{-1}.

Ključne riječi: cefaklor, cefaleksin, tekućinska kromatografija visoke učinkovitosti

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