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Micromethod for determination of ceftriaxone in plasma and urine by high-performance liquid chromatography

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Abstract: A sensitive and rapid high-performance liquid chromatographic method for the determination of ceftriaxone in human plasma and urine is described. A C18 reversed-phase column is used; the mobile phase comprises water–methanol–triethylamine (750:250:4, v/v/v) adjusted to pH 3 with orthophosphoric acid. Quantitation is performed at 270 nm with cefazolin as the internal standard. This method involves precipitation of proteins from fluids with acetonitrile followed by extraction of endogenous compounds with chloroform and injection of the upper aqueous phase on to the chromatograph.

Relative standard deviations for between-day and within-day assays are ≤6.2%. The detection limit is 0.5 μg ml^{-1} in plasma and urine. Studies of drug stability during sample storage, sample pretreatment and chromatography showed no degradation of ceftriaxone or of the internal standard. The method is convenient for clinical monitoring and for pharmacokinetic studies.

Keywords: *Reversed-phase HPLC; cephalosporins; ceftriaxone.*

Introduction

Ceftriaxone (Fig. 1) is a third generation cephalosporin characterized by a broad antibacterial spectrum and a resistance to β-lactamase-producing organisms. In addition to its high antimicrobial activity, ceftriaxone exhibits a long elimination half-life, permitting less frequent administration. Also, the drug diffuses well into extravascular spaces, including cerebrospinal fluid [1, 2]. These characteristics are of considerable clinical interest.

Two types of methods for the determination of ceftriaxone in human body fluids have been described: microbiological assays and high-performance liquid chromatography

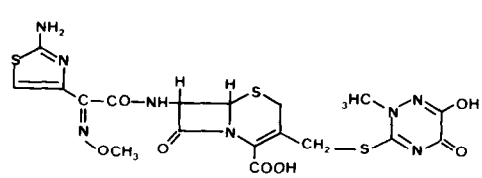

Figure 1
Structure of ceftriaxone.

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(HPLC) [3–9]. In comparison with microbiological assays, HPLC presents several advantages, such as rapidity and specificity, especially when the drug is analysed in the presence of active metabolites or other antibiotics. Among HPLC procedures reported for quantitation of ceftriaxone in biological fluids, ion-pair chromatography on a reversed-phase column is most commonly used. The use of R_4N^+ salts in the mobile phase was avoided in the present work, since these counter-ions can be destructive to packing materials thus causing the deterioration of column performance.

Generally a single protein precipitation by acetonitrile [3, 5, 6, 9] ethanol [8] or methanol [7] followed by injection of supernatant on to the column was used. Under the reversed-phase HPLC conditions in the present work, a single protein precipitation was not satisfactory since endogenous substances can prevent the determination of ceftriaxone. Thus the pretreatment includes another purification step: protein precipitation by acetonitrile is followed by removal of interfering endogenous compounds with an organic solvent (chloroform). A micromethod is described with the same chromatographic conditions and column for both serum and urine. The method can also be used for the determination of ceftriaxone in cerebrospinal fluid.

Cephalosporins are known to undergo remarkably facile cleavage of their β-lactam ring in aqueous solution [10–13]; therefore the stability of ceftriaxone has been studied during sample storage and analytical treatment.

Experimental

Chemicals

Ceftriaxone was supplied by Roche (Neuilly s/Seine, France) and cefazolin by Eli Lilly (Saint-Cloud, France). Acetonitrile was supplied by Rathburn (Walkerburn, Scotland, UK) and methanol by Prolabo (Paris, France). Water was deionized and doubly glass-distilled. All other chemicals (ammonium acetate, acetic acid, chloroform, orthophosphoric acid and triethylamine) were of analytical reagent grade (Merck, Darmstadt, FRG).

HPLC conditions

The analyses were carried out on a Waters Assoc. (Milford, MA, USA) chromatographic system: a Model 45 constant-flow pump, a Wisp Model 710 B automatic injector and a Lambda Max Model 480 ultraviolet detector operated at 270 nm. Compounds were chromatographed on a Radial Pak 10-μm Bondapak C18 (100 × 8 mm i.d.) (Waters) inserted in a radial compression Z module. The mobile phase was water–methanol–triethylamine (750:250:4, v/v/v), the pH being adjusted to 3.0 with orthophosphoric acid. Before use the mobile phase was filtered through a Durapore 0.22 μm filter (Millipore, Milford, MA, USA). The flow rate was 4 ml min^{-1} at ambient temperature. The detector was interfaced with a Model 730 data module (Waters). The chromatograms were recorded at a chart speed of 0.3 cm min^{-1} and peak-height ratios of ceftriaxone to cefazoline were measured. After use, the chromatographic system was flushed with water–methanol (750:250, v/v); when not in use, the analytical column was kept in methanol.

Standard solutions

Stock solutions of ceftriaxone and cefazolin (1 mg ml^{-1}) were prepared in doubly distilled water for ceftriaxone and in methanol for cefazolin. They were stored at −20°C

without degradation for 12 months. Appropriate dilutions of ceftriaxone stock solution were made in drug-free human plasma or urine to provide concentrations of 5–200 μg ml^{-1}. Urine samples were diluted 1:2 (v/v) with isotonic sodium chloride solution. The internal standard concentration was 80 μg ml^{-1} in 0.1 M ammonium acetate buffer (pH 5).

Sample preparation

In a 1.5-ml conical centrifuge tube, 100 μl of plasma or diluted urine and 100 μl of 0.1 M ammonium acetate buffer (pH 5) containing cefazolin were mixed with 500 μl of acetonitrile, mixed in a vortex mixer for 30 s and centrifuged for 2 min at 8700 **g** (Beckman Microfuge). The clear supernatant was transferred to another conical centrifuge tube containing 500 μl of chloroform, mixed in a vortex mixer for 30 s and centrifuged for 2 min. A 5–20 μl portion of the upper aqueous phase was injected on to the chromatograph.

Results and Discussion

Representative chromatograms of plasma and urine samples are illustrated in Fig. 2. The retention times of ceftriaxone and cefazolin were 4.2 and 6.8 min, respectively. Endogenous plasma or urine components did not give any interfering peaks. The potential interference of some other cephalosporins and drugs was investigated (Table 1). Cefotaxime was co-eluted with ceftriaxone, but it was possible to separate the two compounds by reducing the proportion of methanol in the mobile phase. The use of

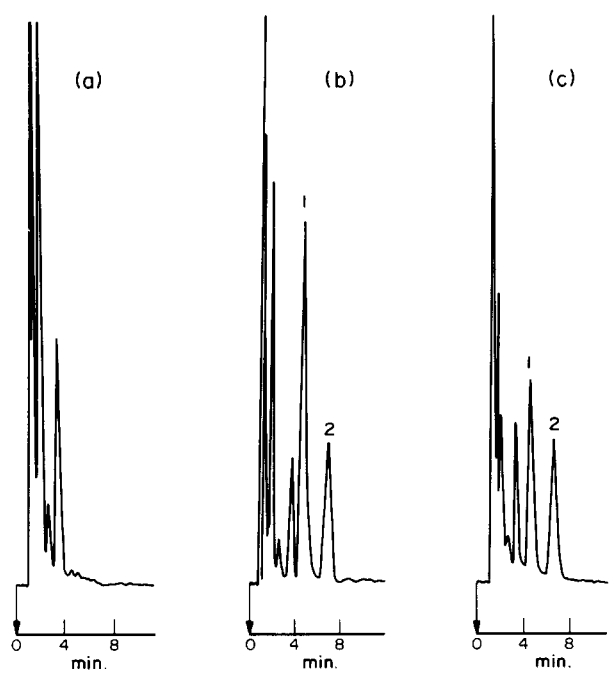

Figure 2
Chromatograms of (a) human drug-free plasma; (b) human plasma (125 μg ml^{-1}); (c) human urine (80 μg ml^{-1}). Peaks: 1 = ceftriazone; 2 = cefazolin.

Table 1
Retention times of some drugs

Drugs*	Relative retention time†
Cefotiam	0.27
Cefsulodin	0.37
Desacetylcefotaxime	0.44
Cefonicid	0.50
Ceforanide	0.58
Cefadroxil	0.62
Ceftazidime	0.66
Aztreonam	0.66
Latamoxef	0.70
Cephalexin	0.81
Cephaloridine	0.85
Ceftriaxone	1.00
Cefotaxime	1.01
Cefazolin	1.44
Ciprofloxacin	1.58
Cefpiramide	2.16
Cefoperazone	2.88
Cephalothin	No response
Apalcillin	No response

* 1 μg of each drug was injected on the column.
† Relative to ceftriaxone: 4.2 min.

methanol instead of acetonitrile in the mobile phase reduced trailing of the peak, which ceftriaxone always exhibits in reversed-phase liquid chromatography [9]. In addition, because of the acidic properties of ceftriaxone, no retention was achieved when the pH of the mobile phase exceeded 3.

The detection limit (signal-to-noise ratio >2) was 0.5 μg ml^{-1} for both plasma and urine. Under these chromatographic conditions, ceftriaxone showed maximum UV absorbance at 270 nm. This limit of detection could only be improved by increasing the injection volume. But if this volume exceeded 40 μl, peak trailing became excessive and could affect resolution, sensitivity and quantitation.

The reproducibility (intra-day assay, $N = 10$) and repeatability (inter-day assay, $N = 10$) of the HPLC procedure were tested with plasma and urine of a subject who received intravenously 1 g of ceftriaxone (Table 2). Accuracy and precision were determined on blank human plasma samples spiked with ceftriaxone at different concentrations (15 and 150 μg ml^{-1}). Accuracy was defined as (amount found/amount added) × 100. The within-day precision data had a relative standard deviation (RSD) of 3.4% for 15 μg ml^{-1} (accuracy = 100.8%) and 3.8% for 150 μg ml^{-1} (accuracy = 103.0%). The between-day data gave a RSD of 3.8% for 15 μg ml^{-1} (accuracy = 99.7%) and 4.2% for 150 μg ml^{-1} (accuracy = 103.4%). There was a linear relationship between concentration and response up to 400 μg ml^{-1} in plasma samples ($r = 0.9990$, $N = 8$) and diluted urine samples ($r = 0.9992$, $N = 8$).

The stability of ceftriaxone during sample storage, sample pretreatment and chromatography was studied. Two plasma samples containing 40 and 155 μg ml^{-1} of ceftriaxone were kept for one month at −20°C. No degradation of ceftriaxone was noted (Fig. 3).

One plasma sample containing 10 μg ml^{-1} was kept for two days, either at +4°C or at ambient temperature. In each case, 16 determinations were made during 48 h; the RSD

Table 2
Reproducibility and repeatability of the HPLC assay of plasma and urine samples from a subject to whom ceftriaxone was administered intravenously

Sample		Intra-day assay ($N = 10$) Mean ($\mu g\ ml^{-1}$)	RSD (%)*	Inter-day assay ($N = 10$) Mean ($\mu g\ ml^{-1}$)	RSD (%)*
Plasma	1	1.5	4.7	1.5	6.1
	2	39.3	1.6	38.9	4.4
	3	157.3	2.0	155.4	2.5
Urine	1	3.9	4.0	3.8	6.2
	2	127.0	2.7	129.6	3.7

*RSD = relative standard deviation.

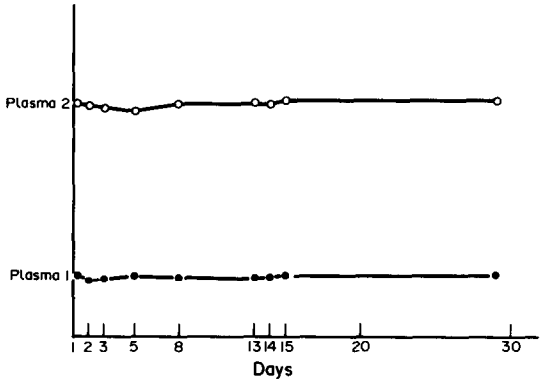

Figure 3
Stability of plasma samples during 30 days at −20°C. (●) plasma 1 spiked with 40 $\mu g\ ml^{-1}$ of ceftriaxone and (○) plasma 2 spiked with 155 $\mu g\ ml^{-1}$. Each value was the mean of two determinations.

($N = 16$) was 6% (mean ±SD = 10.0 ± 0.6 $\mu g\ ml^{-1}$) for the sample kept at ambient temperature and 6.5% (mean ±SD = 9.8 ± 0.6 $\mu g\ ml^{-1}$) for the sample kept at +4°C.

After sample pretreatment, ceftriaxone was present in an aqueous phase, which had to be of a suitable pH to prevent its degradation. By analogy with previous studies [11, 12], samples were diluted in a 0.1 M acetate buffer (pH 5.0) containing the internal standard. Stability in the final aqueous layer was studied with a human plasma sample spiked with 10 and 100 $\mu g\ ml^{-1}$ of ceftriaxone. These plasma samples, treated as described above, were kept at ambient temperature for 6 h. Twice each hour, an aliquot of the aqueous phase was injected on the chromatograph and the peak heights of ceftriaxone and cefazolin were measured. RSD values ($N = 12$) were 4.0 and 5.3%, respectively, for ceftriaxone and the internal standard (plasma 10 $\mu g\ ml^{-1}$), and 2.7 and 4.1% (plasma 100 $\mu g\ ml^{-1}$). These results confirmed the stability of the compounds in the final aqueous layer at pH 6.5.

This assay was used for quantitation of ceftriaxone in plasma of a patient who had received 1 g of the drug intravenously (Fig. 4). Five minutes after injection the plasma concentration was 243 $\mu g\ ml^{-1}$, 32 $\mu g\ ml^{-1}$ after 12 h and 10 $\mu g\ ml^{-1}$ after 36 h.

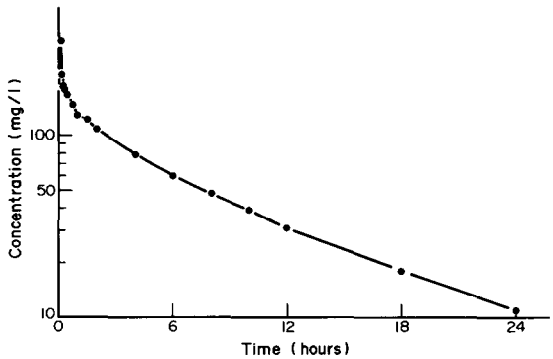

Figure 4
Plasma concentrations of ceftriaxone in a patient who received 1 g of drug intravenously.

Conclusion

The clinical efficacy of ceftriaxone can be enhanced by the ability to determine concentrations accurately and rapidly in biological fluids thereby ensuring that bactericidal levels are achieved and maintained. This method is useful where only small volumes of sample are available in paediatric subjects and in patients with renal failure. Patel [14] suggested that plasma concentrations of ceftriaxone must be monitored in anephric patients so to enable dosage adjustments to be made.

No change was observed in the chromatographic retention times of ceftriaxone and the internal standard in one day. Much greater variations were observed using the ion-pair method for determination of cephalosporins [3]. The life-time of the column was excellent as the performance did not deteriorate over 12 months. Cefazolin was selected as the internal standard since it has a chemical structure related to that of ceftriaxone. The presence of an internal standard was not absolutely necessary since there was no extraction of the drug from biological fluids, but its use seemed important in giving information on the stability of the chromatographic system. This micromethod is simple and convenient for clinical monitoring and pharmacokinetic studies. No stability problems were observed with ceftriaxone during the experimental procedure.

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