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DETERMINATION OF CEFTRIAXONE, A NOVEL CEPHALOSPORIN, IN PLASMA, URINE AND SALIVA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON AN NH_2 BONDED-PHASE COLUMN

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SUMMARY

A high-performance liquid chromatographic method has been developed for the determination of a new cephalosporin antibiotic in plasma, urine and saliva (mixed saliva) using normal-phase technique and an NH_2 bonded-phase column. The eluent mixture was a combination of acetonitrile and an aqueous solution of ammonium carbonate. The rapid method involved precipitation of protein from fluids by means of acetonitrile followed by automatic injection of the supernatant. The detection limit was 0.4 μg/ml for plasma, 3 μg/ml for urine and 0.03 μg/ml for saliva using UV detection.

INTRODUCTION

The determination of cephalosporin antibiotics in biological fluids is often performed by microbiological assay procedures [1—4]. In recent years, high-performance liquid chromatography (HPLC) has proved to be a powerful analytical tool for measuring such drugs in biological fluids, because of its specificity, rapidity and sensitivity. HPLC methods for the determination of cephalosporins have been reported [5—7]; some of these deal with the use of ion-exchange columns [8], others with reversed-phase HPLC on C_{18} columns [9—12]. For the analysis of cephalosporins which are weak acids ($pK_a > 2$), reversed-phase chromatography with ion suppression is usually the method of choice [13]. For stronger acids ($pK_a < 2$), the ion-pairing technique is preferable [14—16].

According to various laboratory experiences [16, 17], ion-pair reversed-phase HPLC has some practical limitations: the salt content of the mobile phase can cause blocking in the pump and/or injector; R_4N^+ salts can be destructive to packing materials (even if buffered at pH 7), producing shrinkage

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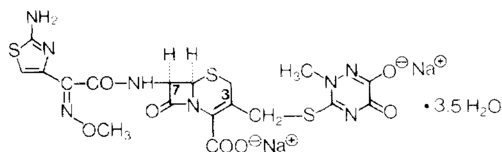

Fig. 1. Formula of ceftriaxone (Ro 13-9904).

of the reversed-phase material with deterioration of column performance. When low concentrations of ion-pairing salts are used, the time required for column—mobile phase equilibrium is often several hours at least; on the other hand, high ion-pair reagent concentrations are to be avoided because of possible column damage.

For pharmacokinetic studies with a new "third generation" parenteral cephalosporin, ceftriaxone (Ro 13-9904) (6*R*, 7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-{[(2,5-dihydro-6-hydroxy-2-methyl-5-oxo-*as*-triazin-3-yl)-thio]methyl}-8-oxo-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2-carboxylic acid disodium salt (Fig. 1) with a potent in vitro activity against a wide range of bacteria [17—19] and long biological half-life [20, 21], we have developed a new HPLC method and compared it with the ion-pair HPLC method of Trautmann and Haefelfinger [16]. The method involves "normal-phase" HPLC performed on a polar alkylamino bonded-phase column (NH_2), connected to a UV spectrophotometric detector.

EXPERIMENTAL

Materials and reagents

Acetonitrile, HPLC grade was from E. Merck (Darmstadt, G.F.R.), ammonium carbonate, analytical reagent grade, from Carlo Erba (Milan, Italy); aqueous solutions of ammonium carbonate and the mobile phase for chromatography were prepared with HPLC-grade water produced by the Milli-Q system (Millipore, Bedford, MA, U.S.A.). Ceftriaxone (Ro 13-9904), pharmaceutical grade, was from Hoffmann-La Roche (Basel, Switzerland).

Stock solution of ceftriaxone

The solution contained 1 mg of ceftriaxone (the disodium salt, with 3.5 molecules of water of crystallization, Fig. 1) per ml of water and was stored at 0—5°C in the refrigerator. This solution was freshly prepared every week.

HPLC equipment and operating conditions

The analysis was carried out on a chromatographic system made up as follows: Model 410 constant-flow pump from Kontron (Zürich, Switzerland); Pye-Unicam variable-wavelength detector, Model 4020 (Cambridge, Great Britain) operating at a wavelength of 274 nm; ASI Model 45 sample processor (Kontron) with Rheodyne automatic injector (Berkely, CA, U.S.A.), a loop capacity of 20 or 50 µl; analytical column, 25 × 0.4 cm, Hibar® type filled with LiChrosorb (NH_2), 5 µm (Merck); precolumn (between the pump and

the injector) was 10 × 0.4 cm, filled with Corasil 37—50 μm from Waters Assoc. (Milford, MA, U.S.A.); guard column (between the injector and the analytical column), 3 × 0.4 cm from Merck, filled with Corasil 37—50 μm.

The eluent mixture containing acetonitrile—water—ammonium carbonate solution (10%, w/v) (70:26:4) was filtered through a 0.5-μm filter before use. The procedure was carried out at constant flow-rate of 1.5 ml/min, corresponding to a pressure of ca. 100 bar.

The UV detector was coupled to a chromatographic computer (Sigma 10, Perkin-Elmer, Norwalk, CN, U.S.A.) for the integration of peak areas and subsequent calculations, using the external standard method. The chromatograms were recorded on a PM 8252 recorder (Pye-Unicam). Under these conditions, the retention time of ceftriaxone was ca. 7.5 min.

During the night, the entire system was flushed with a mixture of acetonitrile—water (80:20, v/v) at a flow-rate of 0.1 ml/min; a mixture of methanol—water (90:10, v/v) was used to store the analytical column during periods of non-use, e.g., a weekend, and for long periods *n*-hexane is preferable.

Procedure for plasma samples

Test plasma (0.25 ml) was mixed with water (0.75 ml) either in a screw-capped test tube (PTFE-lined caps; Sovirel 13) for manual injection or in a cone-shaped vessel (3-ml vessels, cat. No. 3-39296, Supelco, PA, U.S.A.) for automated injection, and the mixture was homogenized by slow agitation. Acetonitrile (2 ml) was then added and the vial was shaken on a vortex mixer for about 30 sec. After centrifugation at 2200 *g* for 3 min, 20—50 μl of the clear supernatant were injected into the chromatographic system.

Specimens of control plasma (0.25 ml) and some (3—5) 0.25-ml specimens of the control which had been spiked with an aqueous solution of ceftriaxone giving plasma concentrations ranging from 5 to 250 μg/ml were processed along with unknown samples in the same way.

Procedure for urine samples

Urine samples were diluted with water (1—5 ml) and then processed as the plasma samples.

Procedure for saliva samples

Test saliva (0.5 ml) was mixed with acetonitrile (1 ml) in a cone-shaped vessel (3 ml) and shaken on a vortex mixer for about 30 sec. After centrifugation at 2200 *g* for 3 min, 100 μl of the clear supernatant were injected into the chromatographic system.

Specimen control saliva (0.5 ml) and some (2—3) 0.5-ml specimens of the control which had been spiked with an aqueous solution of ceftriaxone giving saliva concentrations ranging from 0.03 to 1 μg/ml were processed along with unknown samples in the same way.

The conditions for the saliva analysis had to be modified as follows: mobile phase, acetonitrile—water—ammonium carbonate (5% w/v) (70:26:4); UV detection wavelength 245 nm; loop capacity 100 μl. Under these conditions, the retention time of ceftriaxone was ca. 6.5 min.

Calculation

The Sigma 10 system computed peak areas; the corresponding concentrations of ceftriaxone were obtained by reference to an external standard calibration curve.

RESULTS AND DISCUSSION

Linearity

Using the external standard method, a linear correlation was obtained between the amount of ceftriaxone chromatographed and the relative peak area in the range 0.6—500 μg/ml of plasma ($n = 8$); $r = 0.999972$.

Sensitivity

The detection limit for plasma, urine and saliva samples was 0.4 μg/ml, 3 μg/ml and 0.03 μg/ml, respectively, with a signal-to-noise level of ca. 3:1.

Accuracy and precision

Accuracy experiments were performed on control human plasma and urine spiked with the drug. The accuracy for plasma, defined as [(the amount found)/(the amount added)] × 100, was found to be about 100% over a wide range of concentrations as shown in Table I. A similar extensive study has not yet been carried out for urine, although initial results were similar to those for plasma.

TABLE I

ACCURACY AND PRECISION RESULTS FOR PLASMA SAMPLES SPIKED WITH CEFTRIAXONE

Quantity added (μg/ml)	n	Accuracy (%)	Intra-assay precision* (± %)	Instrument reproducibility** (± %)
225.0	6	96.4	0.7	0.4
112.5	2	97.3	—	—
45.0	6	95.5	0.6	0.45
22.5	2	94.7	—	—
12.0	2	99.0	—	—
6.0	6	100.2	1.2	1.0
2.4	2	102.4	—	—
0.6	6	100.0	4.0	2.5

*Refers to the standard deviation obtained after analysing six plasma samples having the same nominal concentration during one day.

**Refers to the standard deviation obtained by chromatographing each plasma extract six times over a one-day period.

The inter-assay precision, evaluated by analysing spiked plasma samples on different days over one week ($n = 5$), was found to be ± 0.7% and ± 5% for samples of nominal concentration 45 μg/ml and 0.6 μg/ml, respectively.

For saliva the overall accuracy was found to be 98% in the range 0.03—2 μg/ml ($n = 6$). The intra-assay precision was determined by analysing five

different spiked saliva samples on the same day containing 0.2 and 0.04 μg/ml; a relative standard deviation (C.V.) of ± 2% and ± 8%, respectively, was obtained.

The inter-assay precision was determined by analysing one specimen from each concentration (0.2 and 0.04 μg/ml) over five days within a period of two weeks; a relative standard deviation (C.V.) of ± 3.5% and ± 7.5%, respectively, was obtained.

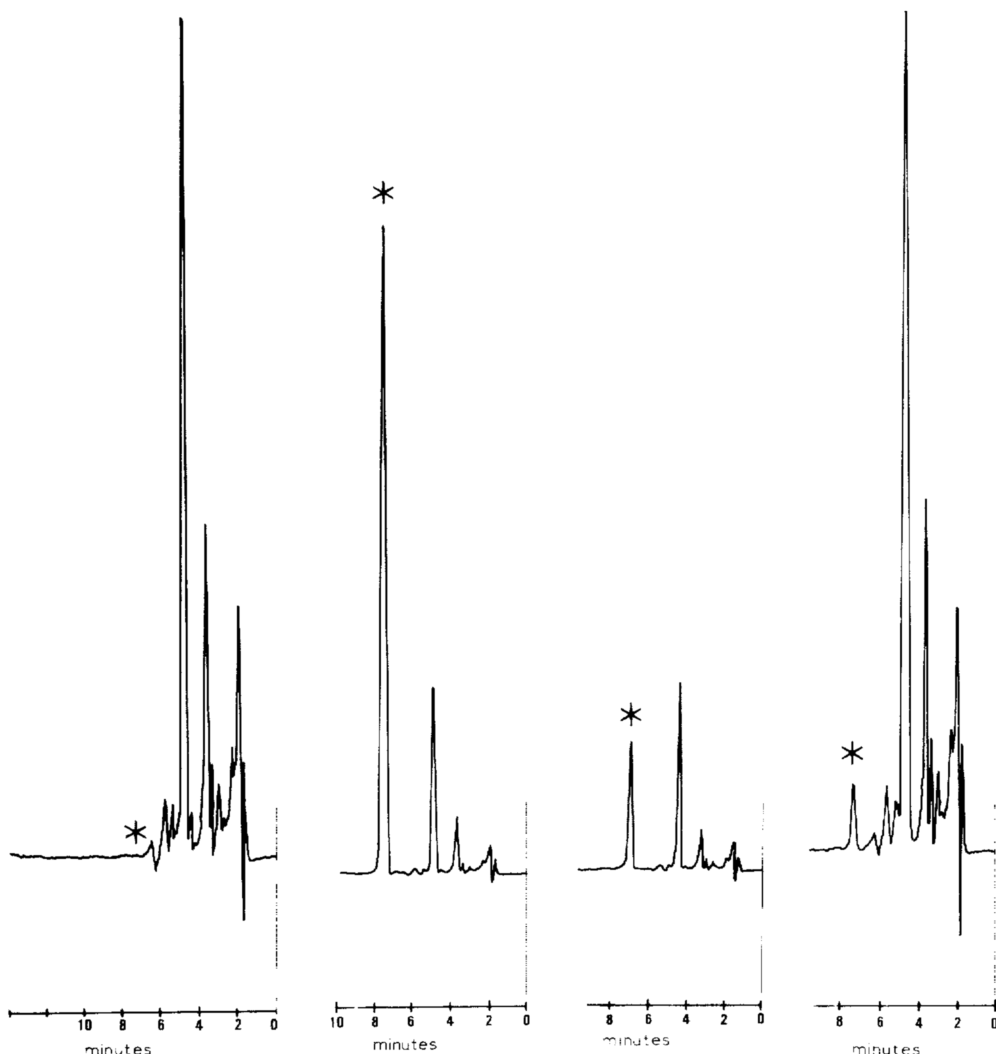

Fig. 2. Chromatogram of human drug-free plasma extract. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

Fig. 3. Chromatogram of authentic standard recovered from control plasma. Nominal concentration: 225 μg/ml. Loop = 20 μl, S = 0.08 a.u.f.s. (*), ceftriaxone.

Fig. 4. Chromatogram of authentic standard recovered from control plasma. Nominal concentration: 45 μg/ml. Loop = 20 μl, S = 0.08 a.u.f.s. (*), ceftriaxone.

Fig. 5. Chromatogram of authentic standard recovered from control plasma. Nominal concentration: 2.4 μg/ml. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

Stability

It has been reported that ceftriaxone is unstable in ethanol, and that sample solutions must be analyzed within two hours of preparation [16]. In the solution used in our method (acetonitrile—water) no stability problems were observed, either at 0—5°C after one week's storage or at room temperature after one day. In addition, these solutions were stable under normal laboratory lighting conditions.

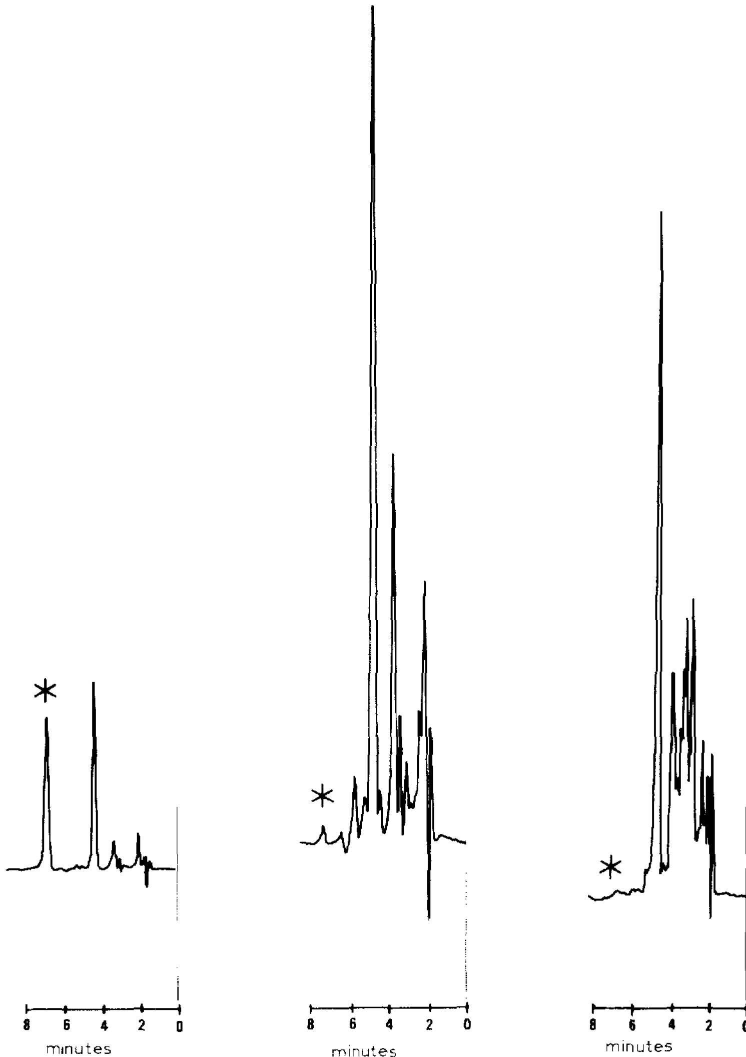

Fig. 6. Chromatogram of plasma extract from a patient receiving 1.0 g of ceftriaxone (intramuscularly) (first administration of a treatment), 8th-hour sample. Loop = 20 μl, S = 0.08 a.u.f.s. (*), ceftriaxone.

Fig. 7. Chromatogram from a spiked plasma sample (0.6 μg/ml) showing the detection limit of the method. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

Fig. 8. Chromatogram from a human drug-free urine extract. Loop = 50 μl, S = 0.02 a.u.f.s. (*), ceftriaxone.

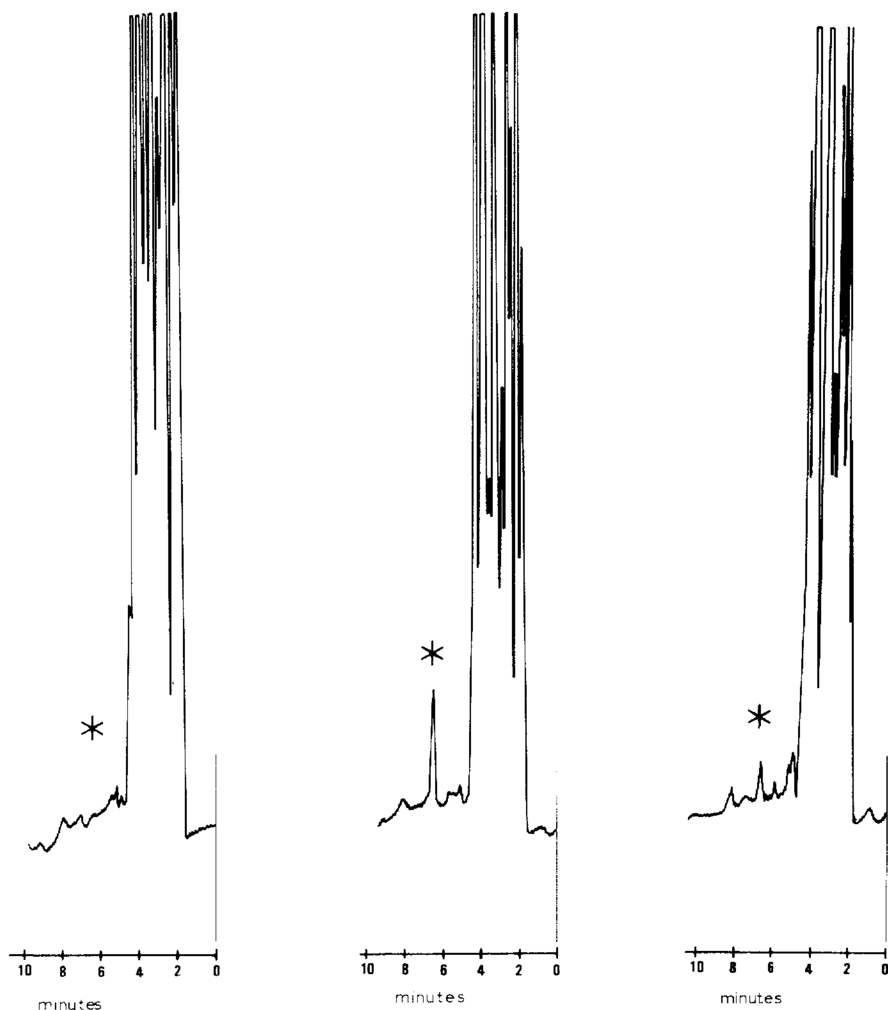

Fig. 9. Chromatogram of human drug-free saliva extract. Loop = 100 μl, S = 0.01 a.u.f.s. (*), ceftriaxone.

Fig. 10. Chromatogram of authentic standard recovered from control saliva. Nominal concentration: 0.2 μg/ml. Loop = 100 μl, S = 0.01 a.u.f.s. (*), ceftriaxone.

Fig. 11. Chromatogram of saliva extract from a volunteer receiving 1.5 g of ceftriaxone (intravenously); 8th hour after administration (concentration found: 0.06 μg/ml). (*), ceftriaxone.

Selectivity

A number of substances were investigated as possible internal standards. The following eluted with, or too close to, the solvent peak: sulfanilic acid, salicylic acid, 3,4,5-trimethoxybenzoic acid, nitrobenzoic acid, benzoic acid, phenoxyacetic acid, sulphanilamide, *p*-aminobenzoic acid. Salicylamide, barbituric acid, xanthanoic acid, 5-sulphosalicylic acid were retained on the column.

The selectivity of the method was tested with respect to other antibiotics.

Cefazolin, cefuroxim, desacetylcephalothin, 7-desacetylcephalothin, 7-aminocephalosporanic acid, cephalosporin C, and 7-desacetylaminocephalosporanic acid all eluted too close to, or with, the solvent peak. Ampicillin and 6-aminopenicillanic acid were retained.

Some examples of chromatograms are reported for plasma (see Figs. 2—7), urine (Fig. 8) and saliva (Figs. 9—11); these refer to the application of the method to control fluids, recovery studies and in vivo investigations.

Application of the method to biological specimens

The assay was applied to the quantitation of ceftriaxone in the plasma of a patient dosed (intramuscularly) with 1.0 g of ceftriaxone. In parallel, the determination of drug plasma levels was performed by ion-pair reversed-phase HPLC [16] (Table II). The results showed a good agreement between the two methods. No significant difference was found between the two methods

TABLE II

CEFTRIAXONE PLASMA LEVELS IN A PATIENT DOSED INTRAMUSCULARLY WITH 1.0 g OF CEFTRIAXONE

Time (h)	Concentration (μg/ml)	
	Normal-phase HPLC	Ion-pair HPLC
0.25	25.0	26.4
0.5	44.2	45.8
1.0	63.9	60.9
2.0	75.0	74.2
4.0	70.5	67.3
6.0	58.7	59.8
8.0	50.0	51.1
12.0	37.0	39.2
24.0	25.2	26.0

TABLE III

CEFTRIAXONE PLASMA AND SALIVA LEVELS IN A VOLUNTEER DOSED INTRAVENOUSLY WITH 1.5 g OF CEFTRIAXONE

Time (h)	Concentration (μg/ml)	
	Plasma	Saliva
0.25	236.8	0.35
0.5	211.8	0.31
1.0	181.2	0.27
2.0	143.3	0.20
4.0	112.5	0.16
6.0	84.4	0.11
8.0	71.0	0.12
12.0	45.7	0.08
24.0	25.9	0.05
36.0	8.0	0.03
48.0	2.0	Not detectable

at the 95% confidence level performing Student's t-test; the regression coefficient was $r = 0.99678$.

Another example is reported (Table III), dealing with plasma and saliva levels of a volunteer dosed (intravenously) with 1.5 g of ceftriaxone; in this case only our method was used since the ion-pair reversed-phase method [16] was not sensitive enough for saliva samples. Until now, very few antibiotics have been determined in mixed saliva [22] mainly because of the inadequate sensitivity, accuracy, and precision of the analytical methodology [23].

Tests performed on an NH_2 column and general comments

The NH_2 phase is frequently used for the separation of saccharides using an acetonitrile—water mixture [24]; in fact the NH_2 group displays a preferential interaction with the hydroxyl group of carbohydrates [25].

In so far as the retention of ceftriaxone is reduced with increasing water content in the mobile phase, the column is of the normal-phase type. In addition, increasing the proportion of ammonium carbonate in the eluent mixture reduced the retention time as reported (Table IV).

Many advantages resulted from the use of the proposed method in comparison to ion-pair HPLC methods; the column could be conditioned rapidly and it retained its performance over a three-week period, during which time 40 biological samples per day were analysed. No blocking of frits, phase shrinkage, or back-pressure problem was experienced.

TABLE IV

EFFECT OF AMMONIUM CARBONATE CONTENT IN THE MOBILE PHASE ON RETENTION TIME OF CEFTRIAXONE

Water (%)	Acetonitrile (%)	Ammonium carbonate (g per 100 ml of mixture)	Retention time (min)
25	75	—	Retained on column
25	75	0.1	Retained on column
25	75	0.2	22
25	75	0.3	14
25	75	0.4	12.5
25	75	0.5	11.5

After a month of non-use, the column was re-tested by analysing plasma and saliva specimens; the performance of the column had not deteriorated.

The method is rapid; no time-consuming sample preparation operations are required and the protein denaturation step is performed using an aqueous acetonitrile solution completely compatible with the mobile phase.

The retention of time of ceftriaxone did not change by more than 3% in one day. In our hands, much greater variation was observed using the ion-pair method.

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