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Journal of Chromatography A, 812 (1998) 191–196

JOURNAL OF CHROMATOGRAPHY A

Short communication

On-line solid-phase extraction of cephalosporins

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Abstract

Using a single, on-line solid-phase sample clean-up procedure, serum concentrations of ten cephalosporins (cefaloridine, cefalotin, cefamandole, cefazolin, cefodizime, cefoperazone, cefoxitin, ceftizoxime, ceftriaxone and cefuroxime) were assayed on a C_{18} column, using UV detection. Serum samples diluted with water were directly injected, without any sample preparation, into the extraction column (alkylamino packing) where the drugs were separated from the serum components. Using an on-line column-switching system, cephalosporins were transferred and separated on the analytical column by ion-pair high-performance liquid chromatography. The proposed method was rapid and efficient: it requires a small serum volume (10 μl) and it can be automated. Recovery was nearly complete. Detection limits varied from 0.5 to 2 μg/ml from extraction of 10 μl of serum. The method was highly specific even in the presence of other drugs commonly found in clinical samples and provides a rapid, simple technique suitable for use in routine microbiological practice.

Keywords: Sample preparation; Cephalosporins; Antibiotics; Lactams

1. Introduction

Cephalosporins have assumed a prominent role in modern antimicrobial therapy due to their enhanced intrinsic microbiological activities and favorable safety profile.

Determination of these drugs is often performed by microbiological assay procedures. Such methods lack chemical specificity and require incubation for many hours. Nowadays, high-performance liquid chromatography (HPLC) is widely used for the assay of antibiotics in biological fluids [1]. HPLC is rapid, specific and it allows the simultaneous determination of several different drugs or their metabolites. Several papers concerning the analysis of individual cephalosporins have been published (for reviews see Refs. [1,2] as well as analysis as a group by HPLC [3–12]. A majority of the methods use laborious solvent extraction techniques to separate the drug from the serum. Solid-phase sample extraction (SPE) has also been employed [4,13].

HPLC analysis of drugs in biological matrices usually involves treatments of the sample before injection into the chromatograph. Typical treatments include precipitation, liquid–liquid extraction and off-line SPE. Such conventional procedures may involve tedious, complex and time-consuming steps. Because of the extensive sample handling, contamination and sample loss are not unusual. Direct sample injection may solve these problems. With a column-switching technique, the manual sample preparation steps are drastically reduced, or even eliminated [14]. In addition to being fast, efficient and easily automated, SPE is a clean analytical procedure. This technique is particularly attractive since it allows the simultaneous removal of matrix

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0021-9673/98/$19.00
PII: S0021-9673(98)00382-3

components and preconcentration of the analytes. SPE is becoming the preferred method for isolation of organic components from predominantly aqueous samples due to the availability of very efficient particles [15].

As far as we know, however, there have been few publications describing HPLC assay for the determination of individual cephalosporins using a column-switching technique as on-line sample clean-up [16–19].

In this paper we describe a single, simple, rapid and selective on-line solid-phase clean-up procedure applied to ten cephalosporins in serum (cefaloridine, cefalotin, cefamandole, cefazolin, cefodizime, cefoperazone, cefoxitin, ceftizoxime, ceftriaxone and cefuroxime) and the subsequent analysis on a reversed-phase column with UV detection. These semi-synthetic antibiotics have similar structures based on a 7-aminocephalosporanic nucleus. The extraction proposed depends on the carboxylic group at the C-3 position. The assay permits the determination of these antibiotics in serum without interferences from endogenous substances or most of the commonly co-administered drugs. The technique can be easily adapted to the analysis of other β-lactam antibiotics.

Furthermore, this assay may be preferable to other methods because the same on-line sample clean-up procedure, the same analytical column and flow-rate, and a readily adjustable three-solvent mobile phase (two similar mobile phases) were used for all ten cephalosporins in a small sample volume (10 μl of serum).

2. Experimental

2.1. Reagents and chemicals

Acetonitrile and methanol (both HPLC grade) were obtained from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogensulfate (TBA HSO_4) was purchased from Sigma (St. Louis, MO, USA). Cefaloridine, cefalotin, cefamandole, cefazolin, cefoperazone, cefoxitin, ceftriaxone and cefuroxime were obtained from Sigma; cefodizime (Modivid, Hoechst) and ceftizoxime (Eposerin, Pharmacia) were commercially available preparations for injection. Most of these cephalosporins were in the form of their sodium salts. Analytical grade, filtered water was obtained daily from an Elgastat UHQ PS apparatus (ELGA, High Wycombe, UK). Drug-free serum for the preparation of standard solutions was obtained from healthy volunteers.

2.2. Apparatus

The liquid chromatograph consisted of a Beckman Model 110B HPLC pump (Beckman, Fullerton, CA, USA), a Beckman 126 programmable solvent delivery module and a Beckman 166 programmable ultraviolet detector. The injector was a Rheodyne Model 7725i manual injection valve, fitted with a 20-μl sample loop. The chromatograms were integrated with a System Gold Laboratory Data System (Beckman). The coupled-column system was operated by a pneumatic, six-port, automated switching valve (Valco, Schencon, Switzerland) controlled by the HPLC system.

2.3. Chromatographic conditions

All analyses were performed at room temperature. The extraction column was 50 mm×2.1 mm I.D., dry filled with an NH_2 40-μm silica (Supelclean LC-NH_2 from Supelco) (Supelchem, Milano, Italy). The analytical column was a Supelcosil LC-18 (15 cm×4.6 mm I.D., particle size 3 μm) from Supelco. Wash solution consisted of a mixture of 5% methanol in 0.01 *M* phosphate buffer (pH 7.0). Two analytical mobile phases were used: mobile phase I was composed of methanol–acetonitrile–0.01 *M* phosphate buffer (pH 7.0) (20:15:65) and 5 m*M* TBA HSO_4; mobile phase II was similar to mobile phase I except that acetonitrile was omitted and 30% of methanol was used.

Sample aliquots of 20 μl (serum–purified water, 1:1, v/v) were injected directly onto the precolumn. Wash solution, at a flow-rate of 0.3 ml/min, was used to eliminate the biological matrix components from the extraction column. The flow-rate for the analytical column was set at 1 ml/min. The effluent from the analytical column was monitored by UV.

Analyses of a composite standard were performed at a fixed 267 nm UV wavelength. Solutions of individual compounds were injected to confirm the identity of the peaks in the mixture. Moreover, in

order to investigate the linearity, reproducibility, recovery and detection limit, each particular cephalosporin was detected using a wavelength chosen so as to maximize the sensitivity.

2.4. Column switching procedure

In order to decrease sample viscosity, serum was diluted 1:1, v/v, with purified water. A 20-μl aliquot of the diluted sample was injected directly into the chromatograph. The sample was brought onto the column by wash solution delivered by pump 1 and directed to waste, while pump 2 delivered the analytical mobile phase to the analytical column. The bulk of the serum matrix was removed whilst cephalosporins were trapped in the column. The flow-rate of the wash-step was set at 0.3 ml/min. After a flushing period of 1.3 min, the pre-column was connected with the analytical column via the switching valve and analytical mobile phase from pump 2 eluted the analytes of interest to the analytical column. The connection time of the extraction column to the analytical column was 1.3 min. At this stage, the valve was switched to the initial position and pump 1 delivered the wash solution to the extraction column to prepare it for the next sample, while pump 2 maintained the flow of analytical mobile phase through the analytical column where cephalosporins were separated and detected by UV. The flow-rate of analytical column was set at 1 ml/min for 18 min.

2.5. Quantification

Quantification was based on peak areas, as measured by the System Gold Laboratory Data System. The detection limit was defined as the analyte concentration yielding a peak three times the noise level. A stock solution of individual drugs was prepared by dissolving the drugs in purified water. The working composite standard of different concentrations of cephalosporins containing cefaloridine, cefalotin, cefamandole, cefazolin, cefodizime, cefoperazone, cefoxitin, ceftizoxime, ceftriaxone and cefuroxime, was prepared by combining an appropriate aliquot of each stock solution and diluting with drug-free serum. Standard curves were prepared from serum spiked with antibiotic concentrations in the range 2 to 250 μg/ml. The calibration data of peak area against the concentration of the drug were fitted to a linear, unweighted model. Reproducibility was tested at 5 and 100 μg/ml by six repeated analyses of spiked serum and the relative standard deviation (RSD) of peak area was calculated. Recovery experiments were performed on all ten cephalosporins at a concentration of 50 μg/ml in serum. They were based on the comparison of peak areas of extracted spiked serum samples against unextracted aqueous standards at the same drug concentration.

Interferences studies were carried out with many compounds that could be coadministered with the cephalosporins (paracetamol, theophylline, fluconazole, aciclovir, digoxin, ranitidine, vancomycin and teicoplanin).

3. Results and discussion

In the optimization of the sample clean-up step, various sorbents were tried. The stationary phase was selected according to the following criteria: (i) efficient removal of matrix interferents using a minimum of wash solvent and (ii) retention of all analytes on the extraction column before complete transfer to the analytical column using HPLC mobile phase. Three types of solid-phase extraction material were evaluated for the extraction of cephalosporins from serum: C_8, C_{18} and NH_2 phases. Both C_8 and C_{18} gave satisfactory retention of the compounds, but the alkylamino phase also gave reduced background serum interferences in the resultant chromatograms when evaluated against those obtained after extraction with C_8 and C_{18}. The chromatograms obtained with alkylamino extraction phase are shown in Fig. 1. For comparison, chromatograms obtained with C_8 and C_{18} extraction packings are also shown in Fig. 1. The NH_2 phase has a pK_a value of 9.8; the functional group is positively charged at neutral pH and it can bind carboxylic groups, as those of cephalosporin molecules, negatively charged. After retaining the isolates, the column was washed for 1.3 min with 5% methanol in 0.01 *M* phosphate buffer (pH 7.0), which washed off most unwanted material and did not remove the compounds of interest from the packing. The trapped drugs can then be eluted from the extraction column with a mobile phase

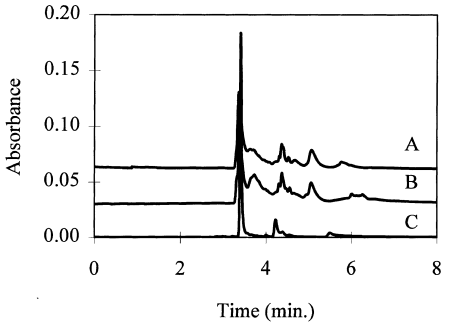

Fig. 1. Chromatograms of blank serum from extraction with (A) C_8, (B) C_{18} and (C) NH_2 packing.

containing a stronger, similarly charged, competing counterion (TBA^+) which displaces the analytes from amino phase by ion-pair coupling with the carboxylic acid group. Moreover, ion-pair formation between the anionic form of the cephalosporin molecules and TBA enhanced the selectivity and allowed better resolution of the peaks. In order to determine optimum operating conditions, aqueous solutions (50 μg/ml) of cephalosporins were assayed separately and in combination, using a variety of methanol and acetonitrile concentrations in the mobile phase. The conditions determined for the assay of aqueous mixtures of cephalosporins were then used as a basis for subsequent assay of these drugs in serum. The use of 5 m*M* TAB HSO_4 in a 30% methanol (or 20% methanol+15% acetonitrile) in 0.01 *M* phosphate buffer (pH 7.0) mobile phase in combination with a C_{18} analytical stationary phase gave a reliable system for analysis. Cephalosporins that had unacceptably long retention times when using mobile phase II, were assayed with mobile phase I. In fact the addition of acetonitrile solved the problem. The concentration of acetonitrile at 15% was judged to give the optimum separation of the peaks with a reasonable chromatographic time. Fig. 2 shows a typical chromatographic separation of the ten cephalosporins obtained by assaying 10 μl of serum sample with mobile phase I, mobile phase II and blank extracts (UV detection at 267 nm). In spiked serum samples the different drugs were well resolved from one another with minimal tailing. The analysis time varied between 4 and 13 min, depending on the antibiotic being assayed and the mobile phase used. To optimize separation, improve peak

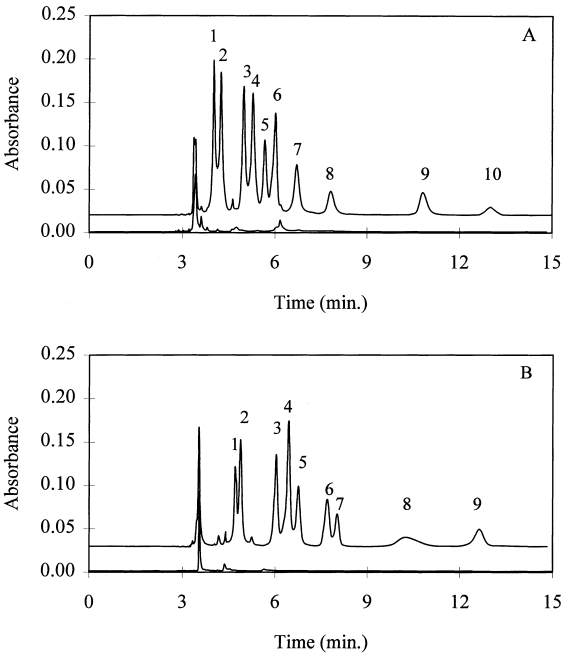

Fig. 2. Chromatograms of extracted drug-free serum and extracted cephalosporin-spiked serum using (A) mobile phase I and (B) mobile phase II (detection 265 nm). Peaks: 1=cefaloridine, 2=ceftizoxime, 3=cefazolin, 4=cefuroxime, 5=cefodizime, 6=cefoperazone, 7=cefoxitin, 8=cefamandole, 9=ceftriaxone, 10=cefalotin.

shape and improve analysis time, the mobile phase must be varied based upon the affinity for the column of the particular cephalosporin being analyzed.

The retention times of cephalosporins analyzed by mobile phase I and mobile phase II are indicated in Table 1. Based on a 20-μl sample volume, the sensitivity achieved with the assay system described varied from 0.5 to 2 μg/ml for the ten drugs tested. A larger injection volume would increase the sensitivity of the system.

The column-switching procedure gave a nearly complete recovery of cephalosporins from serum. Values obtained are shown in Table 2. The method was linear for all cephalosporins in the concentration range 2–250 μg/ml. Values for precision and accuracy, tested at concentrations of 5 and 100 μg/ml, are shown in Table 2.

A number of drugs were evaluated for potential interferences. Serum standards containing cephalo-

Table 1
Retention times of cephalosporins

	Retention time (min)		λ
	Mobile phase I	Mobile phase II	
Cefaloridine	4.0	4.7	252
Ceftizoxime	4.3	4.9	240
Cefazolin	5.0	6.0	273
Cefuroxime	5.3	6.4	260
Cefodizime	5.7	6.8	263
Cefoperazone	6.0	7.8	240
Cefoxitin	6.8	8.0	254
Cefamandole	7.8	10.3	269
Ceftriaxone	10.8	12.6	274
Cefalotin	13.0		240

sporins were spiked with each potentially interfering drug and carried through the extraction and chromatographic procedure. No interference was detected from these compounds as evidenced by the lack of change in the concentration of cephalosporin standards when compared with the standard analyzed without these compounds. Of the tested drugs, only ranitidine exhibited any possible interference, appearing as a shoulder on the ceftizoxime peak. HPLC analysis of drugs in biological fluids usually involves treatment of the sample before injection into the chromatograph. Extraction procedures are often complex and time-consuming, requiring a large volume of sample. Contamination and sample loss are not unusual. Multi-assay systems have been described for the analysis of cephalosporins, but they involve more or less complex extraction procedures. The method described here is fast, efficient, easy to operate. Manual sample preparation steps are eliminated by use of the column-switching technique. The assay requires minimal sample volume and only dilution of serum before injection into the chromatograph. The technique can be easily automated. This simple procedure can be applied with only minor modifications to the analysis of a range of other β-lactam antibiotics.

4. Conclusions

There is a need in the clinical laboratory for a simple HPLC procedure that can be applied with only minor modifications to the analysis of a range of cephalosporins. In this paper we describe such a method. In addition to being fast, efficient, simple and easily automated, the method described requires small sample volumes. Manual off-line sample preparation was restricted to the absolute minimum.The method requires only dilution of the sample before an assay can be performed. SPE provides excellent background serum interference removal and quantitative antibiotic recovery. Ten cephalosporins were analyzed on a single C_{18} reversed-phase column using two similar mobile phases. This simple procedure can be applied with only minor modifications to the analysis of a range of other β-lactam antibiotics. This assay may represent an interesting alternative to other procedures for routine analysis and for pharmacokinetic studies.

Table 2
Precision, accuracy and recovery for cephalosporins (spiked serum)

Cephalosporin	Nominal value 5 μg/ml			Nominal value 100 μg/ml			50 μg/ml Recovery (%)
	Actual value (μg/ml)±SD	Precision (%)	Accuracy (%)	Actual value (μg/ml)±SD	Precision (%)	Accuracy (%)	
Cefaloridine	4.78±0.14	2.85	−4.50	103.99±4.78	4.78	3.99	97.5
Ceftizoxime	5.33±0.23	4.60	6.50	95.13±3.11	3.11	−4.87	102.3
Cefazolin	4.87±0.17	3.30	−2.60	103.66±2.87	2.87	3.66	97.8
Cefuroxime	5.2±0.15	2.92	3.90	104.55±3.64	3.64	4.55	98.5
Cefodizime	4.79±0.13	2.60	−4.28	102.22±2.45	2.45	2.22	101.4
Cefoperazone	5.26±0.18	3.50	5.10	104.88±5.11	5.11	4.88	98.3
Cefoxitin	4.77±0.21	4.20	−4.70	97.04±3.72	3.72	−2.96	99.2
Cefamandole	5.19±0.16	3.10	3.80	96.33±2.94	2.94	−3.67	97.8
Ceftriaxone	5.29±0.26	5.10	5.70	102.56±3.59	3.59	2.56	98.5
Cefalotin	5.15±0.14	2.80	3.00	104.83±2.88	2.88	4.83	97.7

Acknowledgements

This work was supported by a grant of MURST 60%.

References

[1] F. Jehl, C. Gallion, H. Monteil, J. Chromatogr. 531 (1990) 59.
[2] M.C. Rouan, J. Chromatogr. 340 (1985) 361.
[3] A.M. Brisson, J.B. Fourtillan, J. Chromatogr. 223 (1981) 393.
[4] C.E. Fasching, L.R. Peterson, Antimicrob. Agents Chemother. 21 (1982) 628.
[5] J.B. Lecaillon, M.C. Rouan, C. Souppart, N. Febvre, F. Juge, J. Chromatogr. 228 (1982) 257.
[6] K. Miyazaki, K. Ohtani, K. Sunada, T. Arita, J. Chromatogr. 276 (1983) 478.
[7] M.C. Rouan, F. Abadie, A. Leclerc, F. Jugé, J. Chromatogr. 275 (1983) 133.
[8] C. Nygard, S.K. Wahba Khalil, J. Liq. Chromatogr. 7 (1984) 1461.
[9] S.A. Signs, T.M. File, J.S. Tan, J. Antimicrob. Chemother. 26 (1984) 652.
[10] C.Y. Chan, K. Chan, G.L. French, J. Antimicrob. Chemother. 18 (1986) 537.
[11] F. Jehl, P. Birckel, H. Monteil, J. Chromatogr. 413 (1987) 109.
[12] D.E. Holt, J. de Louvois, R. Hurley, D. Harvey, J. Antimicrob. Chemother. 26 (1990) 107.
[13] P.M. Kovach, R.J. Lantz, J. Chromatogr. 567 (1991) 129.
[14] K.A. Ramsteiner, J. Chromatogr. 456 (1988) 3.
[15] P. Campinos-Falco, R. Herraez-Hernandez, A. Servillano-Cabeza, J. Chromatogr. 619 (1993) 177.
[16] Y.J. Lee, H.S. Lee, Chromatographia 30 (1990) 80.
[17] S. Bompadre, L. Ferrante, F.P. Alò, L. Leone, J. Chromatogr. B 669 (1995) 265.
[18] S. Bompadre, L. Ferrante, L. Leone, M. De Martinis, L. Ginaldi, D. Quaglino, J. Liq. Chromatogr. 18 (1995) 2895.
[19] H.T. Pan, P. Kumari, J. Lim, C.C. Lin, J. Pharm. Sci. 81 (1992) 663.