[Chem. Pharm. Bull.
30(2) 622—627 (1982)]

High Performance Liquid Chromatographic Assay of Cefsulodin, Cefotiam and Cefmenoxime in Serum and Urine

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(Received July 13, 1981)

A high performance liquid chromatographic method for the analysis of cefsulodin (CFS), cefotiam (CTM) and cefmenoxime (CMX) in human blood or urine samples was established. The procedure is simple, and includes deproteinization with methanol, separation of the supernatant by centrifugation and/or filtration, and injection onto a reversed phase column. The extent of recovery of added drugs and the reproducibility of the procedure demonstrated that the method could replace the conventional biological assay. The minimum detectable amounts of cephalosporins in serum are 0.05 μg/ml for CMX, 0.1 μg/ml for CTM and 0.2 μg/ml for CFS. In urine samples, cephalosporins can be detected at levels of 2 μg/ml or more. The sensitivity seems to be good enough for clinical application of the method considering the usual dosages of the drugs. Examples of the clinical application of the method are also reported.

Keywords——high performance liquid chromatography; cephalosporin; cefsulodin; cefotiam; cefmenoxime; serum; urine

Cephalosporins are widely used for the treatment or prevention of infectious diseases and as a consequence of extensive research and development, a number of derivatives have become available in the clinic. To find the optimal dosage regimen for newly synthesized drugs and to minimize side effects, especially those due to overdosage, monitoring of the drug concentration in patients' blood or urine and the pharmacokinetic study of its time-dependent change are very important. Analysis of the blood concentration of cephalosporins can be conveniently performed by microbioassay using a bacterium sensitive to the administered cephalosporin. However in the case of combination therapy, some ambiguity in the analytical results may arise. Furthermore, metabolites of the drug cannot be determined by the conventional bioassay. Therefore, for clinical application, a speedier and simpler method is needed even at the sacrifice of sensitivity. To overcome these problems, a chromatographic technique seemed to be most appropriate.

In the present paper, we describe a revesed-phase high performance liquid chromatographic analysis for the recently introduced cephalosporins, cefsulodin(CFS) and cefotiam(CTM), as well as cefmenoxime(CMX).[1] The method proved to be useful for drug concentration analysis in human blood and urine specimens.

Experimental

Reagents and Materials——CFS, CTM and CMX were prepared in our company and cefuroxime (CXM), ceftezole (CTZ) and cefacetrile (CEC), used as internal standards, were purchased from Hoechst AG, Hoechst Japan and Ciba-Geigy Japan, respectively (Chart 1). Acetate buffer, 0.1 M (pH 4.4), was prepared from 0.1 M acetic acid and 0.1 M sodium acetate (2: 1, v/v). Acetonitrile was used for high performance liquid chromatography (HPLC), and the other reagents were of special grade (Wako Pure Chemicals, Ltd.). Human serum and urine used as blank specimens were obtained from healthy male volunteers, while those of patients who had received cephalosporins were frozen at −20°C soon after collection and thawed just before analysis.

Apparatus and Chromatographic Conditions——HPLC was performed on an ALG/GPC 204 (Waters Assoc.) machine equipped with a UV detector at 254 nm. A stainless steel column (15 × 0.4 cm, i.d.) was packed with Nucleosil $5C_{18}$ (Macherey Nagel Co.) and to this was attached a precolumn (1 × 0.4 cm, i.d.) with the same filler to protect the main column from deterioration due to serum or urine components. Mix-

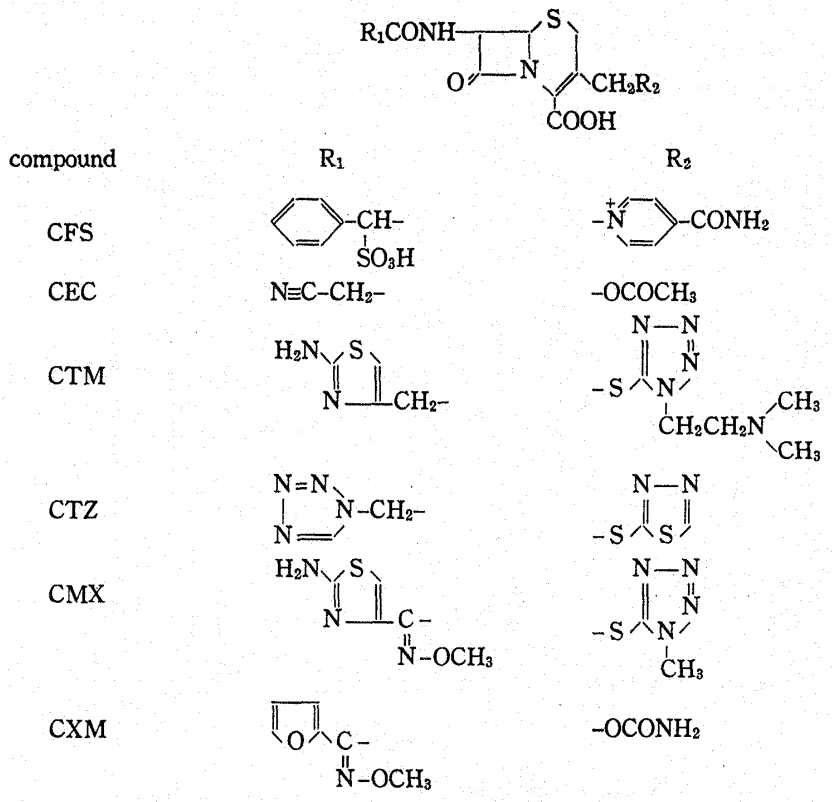

Chart 1

tures of 0.1 M acetate buffer and acetonitrile were used as eluting solvents for CFS and CTM in mixing ratios of 92: 8 and 95: 5 (v/v), respectively. In both cases, the flow rate was controlled at 0.8 ml/min. For the analysis of CMX, a three-solvent system, water, acetonitrile and acetic acid (50: 10: 1, v/v), was employed at a flow rate at 0.7 ml/min. The chromatographic data were directly acquired with a Hewlett-Packard laboratory automation system, model 3354; the peak areas were recorded digitally.

Sample Preparation——Serum: A 1 ml aliquot of a methanol solution of the internal standard was added to 0.5 ml of serum. The mixture was shaken for about 30 sec on a microthermomixer. The subsequent centrifugation at 3000 rpm for 3 min deproteinized the solution and the supernatant was further clarified through a 0.5 μm Millipore filter (FH type, Millipore Corp.).

CEC, CTZ and CXM were used as internal standards for the analysis of CFS, CTM and CMX, respectively, and the drug concentration in methanol was adjusted to nearly that expected for the drug in the sample to obtain comparable chromatogram peaks of the internal standard and the administered drug.

To prepare the sample solution for injection into the chromatographic column, the filtrate obtained above was diluted with water. In the cases of CFS and CTM, water equivalent to twice the volume of the filtrate was added, whereas an equal volume of water was added with CMX. The diluted solution was stored in an ice-water bath until use to prevent deterioration. A 50-μl sample was injected with a microsyringe into the HPLC column.

Urine: As in the method used for the serum sample, 1 ml of the methanolic internal standard solution was added to 0.5 ml of the urine sample and the mixture was filtered immediately after mixing. The succeeding procedures were the same as those for the serum.

Results and Discussion

Separation

Figs. 1 and 2 show the chromatograms obtained from the human blank serum and urine in comparison with those obtained from the same sample when the drug and the internal standard were added, 4 μg/ml to the serum and 50 μg/ml to the urine. As is clear from the figures, all drugs and internal standards were eluted within 15 min without overlapping with biological components, which indicates that the method is suitable for the measurement of a large number of samples in a short time.

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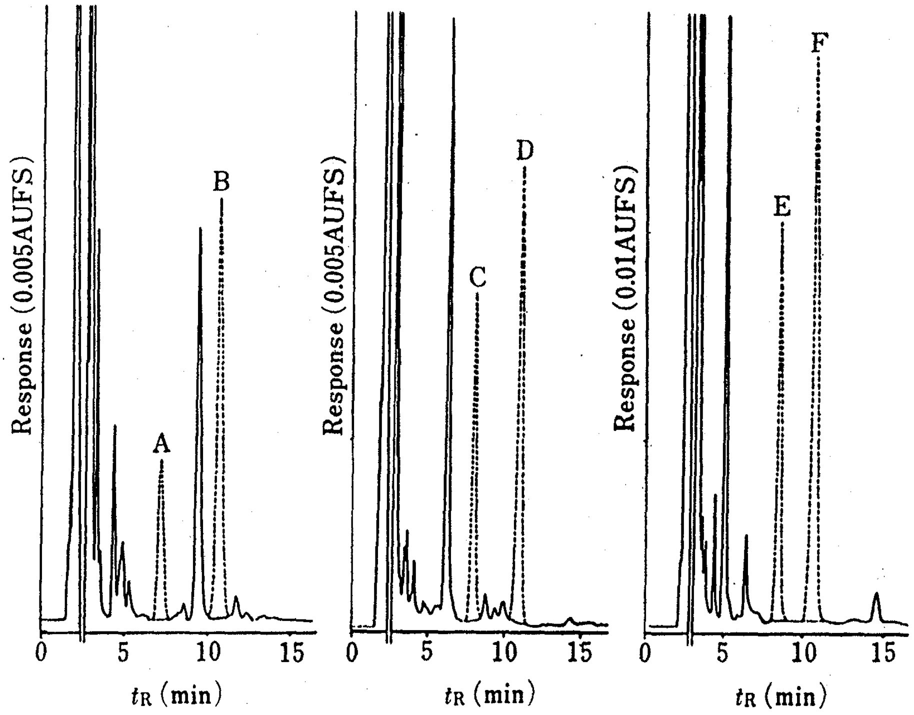

Fig. 1. Chromatograms of Blank Serum and Serum Containing 4 μg/ml of Drug and 10 μg/ml of Internal Standard (IS)

——, blank serum; ---, serum containing drug and IS; A, CFS; B, CEC (IS); C, CTM; D, CTZ (IS); E, CMX; F, CXM (IS).

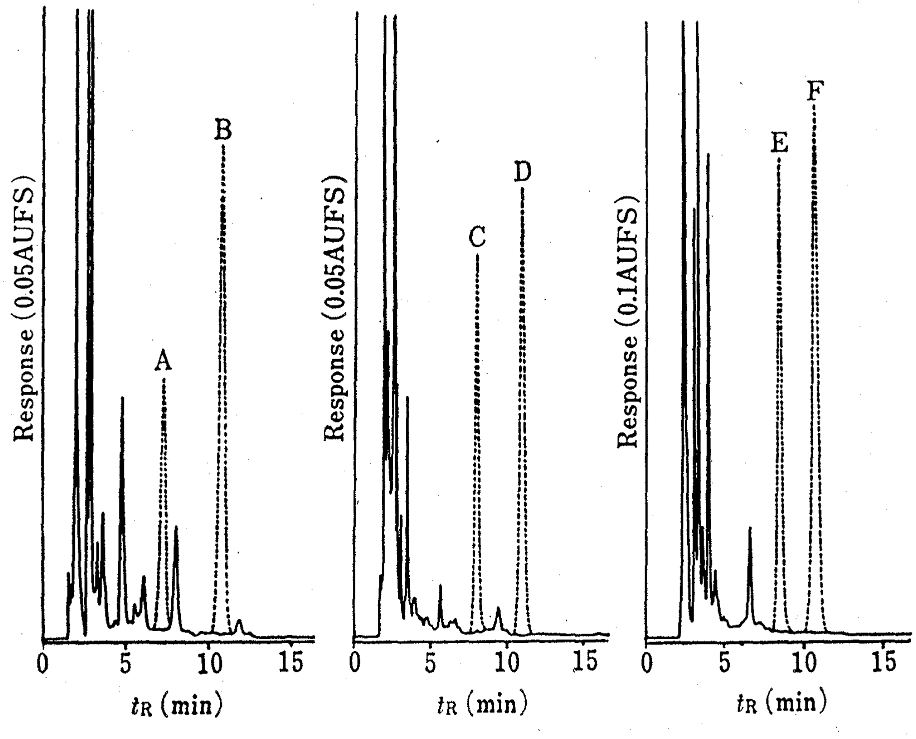

Fig. 2. Chromatograms of Blank Urine and Urine Containing 50 μg/ml of drug and 100 μg/ml of Internal Standard (IS)

——, blank urine; ---, urine containing drug and IS; A, CFS, B, CEC (IS); C, CTM; D, CTZ (IS); E, CMX; F, CXM (IS).

Deproteinization

One of the most important processes in the analysis of drug concentration in serum by HPLC is deproteinization. The life of the chromatographic column depends strongly on the protein content in the injected samples. Trichloroacetic acid, perchloric acid, methanol, ethanol and acetonitrile have been frequently used for deproteinization.[2] However, acids are not appropriate for this purpose since some cephalosporins suffer degradation. Actually, the recovery after deproteinization with 0.5 ml of 6% trichloroacetic acid or 0.33 N perchloric acid to 0.5 ml of serum containing 5 μg of CMX was about 85% in each case. One reason for the low recovery may be the occlusion of CMX in the precipitate. Deproteinization with an organic solvent does not affect the pH of the sample solution, but the precipitate produced on addition of acetonitrile was too sticky to be handled, adhering to the centrifuge tube. In contrast, the protein separated with methanol or ethanol was woolly and easily removable by centrifugation. In fact, trial experiments conducted similarly to those described above, except that 1 ml of methanol or ethanol was used in place of trichloroacetic acid or perchloric acid, resulted in quantitative recoveries of the drugs and internal standards. The injection of a highly alcoholic solution may transiently disturb the resolution of the column, however. For instance, injection of 50 μl of the supernatant obtained from the deproteinized solution containing 0.5 ml of serum and 1 ml of methanol or ethanol broadened the cephalosporin peaks on the chromatogram, although the peak areas and retention times remained unchanged. The broadening was more marked with ethanol than methanol. Dilution of the supernatant with an equal amount of water prevented this effect in the case of CMX analysis but twice the volume of water was required to obtain sharp drug peaks in the CFS and CTM analyses.

Recoveries and Calibration Curves

To 0.5 ml of serum or urine obtained from untreated healthy volunteers was added 0.25 to 4.0 μg or 5.0 to 50 μg of a cephalosporin (CFS, CTM or CMX). After deproteinization with 1 ml of the methanolic solution of the internal standard, the solution was worked up as described in the experimental section to prepare calibration curves and also to determine the recoveries within the drug concentration range being studied. All three drugs were recovered quantitatively from both serum and urine, independent of the amount added to the biological specimens (Table I). The calibration curves thus obtained were linear, passing through the origin of the coordinates.

TABLE I. Recoveries of CFS, CTM and CMX from Spiked Human Serum and Urine

Drug	Concentration added to serum (μg/0.5 ml)	Recovery (%)	Concentration added to urine (μg/0.5 ml)	Recovery
CFS	0.25	96	5	99
	0.5	102	10	96
	1.0	99	25	97
	2.0	98	50	100
	4.0	99		
CTM	0.25	94	5	96
	0.5	100	10	98
	1.0	99	25	98
	2.0	98	50	97
	4.0	100		
CMX	0.25	100	5	100
	0.5	102	10	100
	1.0	102	25	100
	2.0	100	50	99
	4.0	99		

Reproducibilities and Detection Limits

The reproducibility of the procedure was tested with 0.5-ml samples of serum and urine containing 1 and 25 μg of cephalosporins, respectively. Variation coefficients were 1.4%

for CFS, 1.2% for CTM and 0.7% for CMX with serum and 1.0% for CFS and 0.8% for CTM and CMX with urine. The detection limits of this method were *ca.* 0.05 μg/ml serum for CMX, *ca.* 0.1 μg/ml serum for CTM and *ca.* 0.2 μg/ml serum for CFS. The urine values seem to be variable, depending on the individual and the time of collection. A precise detection limit has not been determined, but cephalosporins at 2 μg/ml urine are detectable. These limits seem to be low enough for clinical application of this method if the normal dosage of the drugs is taken into consideration.

Stabilities of Drugs and Internal Standard Substances

Cephalosporins are rather unstable drugs in solution when compared with other lipid-soluble drugs. Degradation of cephalosporins in the course of analytical processes is usually not negligible. In the present analytical method, however, time-consuming processes, such as extraction and concentration, are excluded to minimize drug decomposition. To estimate the decomposition during the processes from deproteinization with methanol to injection into HPLC, which consume the longest time in this method, the stability of the drugs at 25°C in a mixture of methanol and water (2: 1, v/v) was determined at the concentration of 2 μg/ml. The amount of unchanged cephalosporins was linearly related to the elapsed time, suggesting a pseudo first-order reaction for the degradation process. The time required for 5% degradation, $t_{0.95}$, was estimated on the basis of this assumption as shown in Table II. The stabilities

TABLE II. Stabilities of Cephalosporins in a Mixture of Methanol and Water (2: 1, v/v) at 25°C

Compound	k_{obs} (h^{-1})	$t_{0.95}$ (h)
CFS	5.8×10^{-2}	0.8
CTM	1.3×10^{-2}	3.8
CMX	1.1×10^{-2}	4.7
CEC	1.2×10^{-2}	4.4
CTZ	7.4×10^{-3}	7.2
CXM	8.8×10^{-3}	5.9

TABLE III. Serum and Urine Concentration of CFS, CTM and CMX in Human Subjects after a Single Intravenous Administration

Drug	Patient	Dose (mg/kg)	Time (min)	Serum Concentration (μg/ml)	Time (h)	Urine Concentration (μg/ml)
CFS	A	17	10	89	0—2	2200
			20	65	2—4	900
			30	54	4—6	320
			60	37	6—8	150
			120	25		
			240	10		
			360	4.1		
CTM	B	18	30	77	0—2	5800
			60	36	2—4	1800
			120	22	4—6	260
			180	7.0		
			240	5.5		
			420	0.8		
CMX	C	20	10	92	0—2	2600
			20	66	2—4	680
			30	45	4—6	55
			40	36		
			50	26		
			60	20		

of CFS, CTM and CMX were a little lower in methanol–water than in water alone but degradation during the period of the deproteinization process was negligible.[3] The biological specimens should be stored at 0°C until use, since under this condition no detectable degradation occurs.

Application

The method was applied to the measurement of CFS, CTM and CMX concentrations in the serum and urine of patients dosed with the drugs. Patient A was given CFS 17 mg/kg *i.v.*, patient B CTM 18 mg/kg *i.v.*, and patient C CMX 20 mg/kg *i.v.* The analytical results are shown in Table III. Though the number of clinical examples is still limited, the results obtained are clear-cut and show a reasonable agreement with the published pharmacokinetic data obtained by the bioassay method.[4]

Acknowledgement We are grateful to Drs. E. Ohmura and M. Nishikawa of this division for their encouragement throughout this work.

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