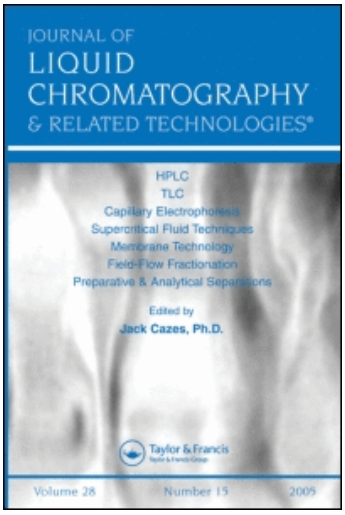

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DETERMINATION OF CEFPIRAMIDE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH INTERNAL SURFACE REVERSED-PHASE SILICA COLUMN

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ABSTRACT

We investigated the isolation of cefpiramide (CPM), cephem antibiotics, from human plasma proteins by high-performance liquid chromatography with a new column, Pinkerton® column (Regis Chemical Co., Illinois, U.S.A.), and discussed whether the column is applicable to routine clinical determination. In condition of

our studies, CPM could be analyzed without any pretreatment. The chromatograms of CPM obtained were single, sharp peaks. Proteins and endogenous compounds in plasma did not interfere with the assay. And, the precise free fraction of CPM could be obtained. The effect on the isolation by the different flow direction was also studied since the column could be used reversibly. There were significant differences ($p < 0.01$) between two slopes of the calibration curves by the different flow direction. It is suggested that calibration curves for both flow directions are required. The analytical procedures outlined in this study may be applicable to routine clinical use since this is a simple and rapid method.

INTRODUCTION

Cefpiramide sodium (CPM; (6R,7R)-7-[(R)-2-(4-hydroxy-6-methyl-3-pyridylcarboxamido)-2-(p-hydroxyphenyl)acetamido]-3-[[(1-methyl-1H-tetrazol-5-yl)-thio]methyl]-8-oxo-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2-carboxylate)is a recently developed antibiotic (1). The determination of the plasma concentration of antibiotics has been usually performed by bioassay. However, this method entails complicated procedures and is time consuming. Wilson has performed plasma CPM determinations by high-performance liquid chromatography (HPLC), which is a simpler method, and the results demonstrated good agreement with those by bioassay (2). The HPLC method generally involves many steps for pretreatment (deproteinization, extraction and so on). Demotes-Mainard et al. reported that CPM could be determined by an automatic HPLC technique without any pretreatment (3). However, this method also had problems due to the short life of the precolumn, and the expense of automated pretreatment equipment.

Recently, a new HPLC column, the Pinkerton^{R} column packed with internal surface reversed-phase (ISRP) silica supports, has been developed to accommodate the analysis of drugs in plasma by direct injection (4-6). This column can exclude large molecular protein-like substances due to the small pore diameter of the ISRP supports, while hydrophobic drug molecules, including their metabolites, can penetrate the particulate matter and interact with the internal partitioning phase. Pinkerton et al. have reported that the column can reduce pretreatment procedures, especially in the determination of drug levels in blood, and that it showed good precision and reproducibility (5,6). It has been shown that plasma samples can be injected without any pretreatment, and also that the column can be injected reversibly, which extends the life of the column (7).

The present study was performed to evaluate the precision and reproducibility of the column in the determination of plasma CPM. If the column can be used in this way, it would aid the rapid determination and/or therapeutic drug monitoring.

MATERIALS AND METHODS

Materials

CPM and carbamazepine (CBZ) were kindly supplied by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan) and Ciba-Geigy Japan, Ltd. (Takarazuka, Japan), respectively. Normal human plasma was obtained from the Japanese Red Cross Society (Tokyo, Japan). The Pinkerton^{R} column (25 cm x 4.6 mm ID; Regis Chemical

Co., Illinois, U.S.A.) was kindly supplied by Koken Co. Ltd. (Tokyo, Japan). Other chemicals and reagents, purchased from commercial sources, were of analytical grade and were used without further purification.

Samples

CPM was dissolved in plasma or pH 7.4 phosphate buffer to yield a spiked sample having concentrations between 0.5 and 50 μg/ml. CBZ was also added, in some cases, to the CPM-spiked sample as an internal standard at a concentration of 5 μg/ml. All samples were filtered through a 0.22 μm membrane filter (Millex^{R} GV; Nihon Millipore Ltd., Osaka, Japan) prior to injection.

High-performance liquid chromatography

An LC-5A chromatographic system (Shimadzu Co., Kyoto, Japan) consisting of an LC-5A liquid pump with a Pinkerton^{R} column, an SPD-2A UV spectrophotometric detector and a Chromatopac C-R2A data processor was used. To change the flow direction within the column, a changeover valve was developed and inserted between the injector and the detector. The mobile phase was a mixture of 0.1 M KH_2PO_4 having a pH of 6.8 - isopropanol (98 : 2) and the flow-rate was adjusted to 1 ml/min. The elutions were carried out at ambient temperature (25 $\pm$ 2 ^{o}C) and the effluent was detected at the wavelength of 273 nm. A sample volume of 10 μl was injected in all the studies.

Calibration curves

Triplicate measurements of the respective spiked samples having a known concentration of CPM were made and calibration curves of CPM in plasma and phosphate buffer were obtained. With regard to the direction of flow within the column, the curves for the forward and reverse directions were both obtained in the respective cases. The peak heights were plotted against the concentrations and the calculations were made by the least-squares method.

Recovery and precision

The recovery of the drug was assessed by 6 replicate analyses with a spiked sample of 30 μg/ml and was calculated as the percentage of the determined concentration to the known concentration of the sample. The within-day and between-day precisions were examined. For the latter, spiked samples were assayed once a day for 9 successive days. The free concentration of CPM was determined after ultrafiltration of the spiked sample, as mentioned below, and the analytical precision was also examined.

Ultrafiltration

To assay the free fraction of the drug in plasma, the free CPM in plasma was separated with an ultrafiltration device (MPS-1^{R} [MPS]; Amicon Co., Ltd., Denver, U.S.A.) (8). A 0.5 ml volume

of sample was placed in the device and centrifuged at 2000 xg (Hitachi centrifuge SCR20B, Hitachi Koki Co., Ltd., Tokyo, Japan) for 15 min at room temperature. The ultrafiltrate was injected into the chromatograph to assay the free drug concentration. The free fraction (FF) was calculated as the percentage of free drug in the total concentration in the respective samples. Preliminary experiments indicated that the adsorption of CPM to the device was not more than 1 % and this amount is not regarded as significant.

Statistical analysis

Statistical analysis was conducted by both the analysis of variance (ANOVA) and Student's t-test. Values of p less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Chromatogram

Recently, Nakagawa and coworkers reported that the isolation pattern of the chromatogram obtained with the Pinkerton^{R} column is affected by pH, the ionic strength, and the concentration of organic modifiers in the mobile phase (9). Trials looking for the optimum conditions were made and isopropanol was found to be the appropriate organic solvent to separate the CPM peak from other peaks of endogeneous substances. In the region of an isopropanol concentration of less than 2 % in the mobile phase,

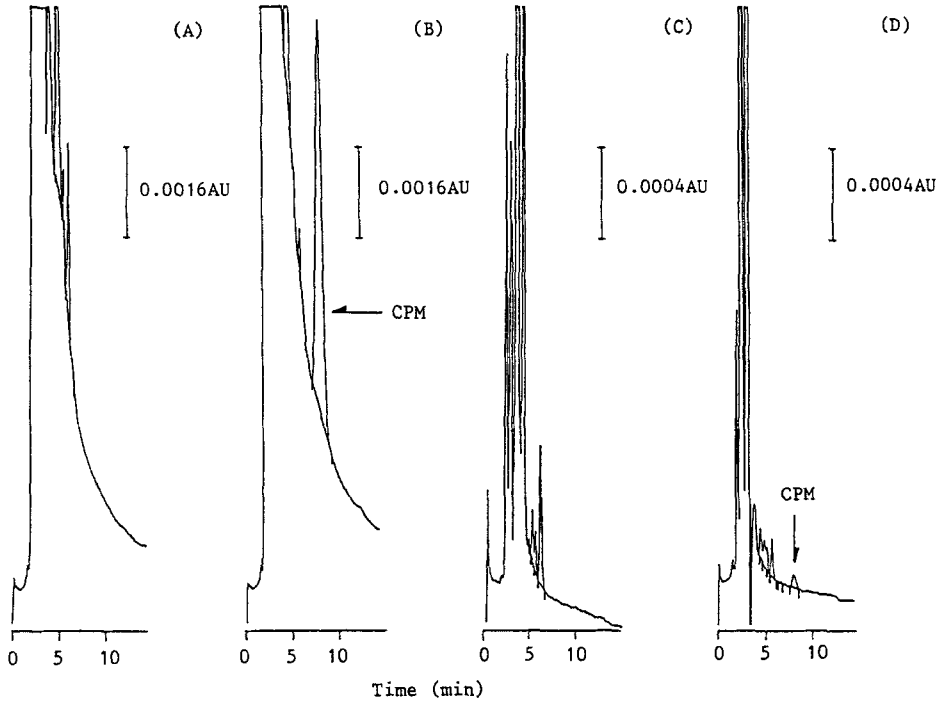

FIGURE 1: Chromatograms of (A) intact plasma, (B) cefpiramide in plasma, (C) blank ultrafiltrate, (D) cefpiramide in ultrafiltrate.

the duration of the retention time was increased and the peak width was also expanded. On the other hand, at concentrations of greater than 2 %, the peak overlapped the peaks of endogenous substances. Thus, the concentration of isopropanol in the mobile phase was selected as 2 %. FIGURE 1 shows the typical chromatograms obtained under conditions which were found to be optimal for the determination of CPM in plasma. The chromatograms of intact plasma and its ultrafiltrate are shown in

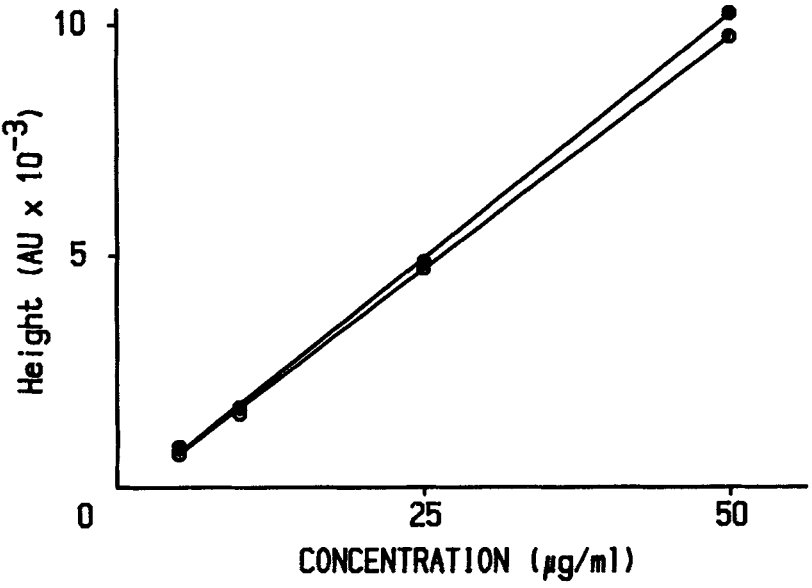

FIGURE 2: Calibration curve for cefpiramide. (○) CPM spiked in plasma, A → B flow direction. (●) CPM spiked in plasma, B → A flow direction. Each plot is the mean of three determinations.

FIGs. 1(A) and 1(C), respectively. In FIGs. 1(B) and 1(D), CPM peaks are shown from the spiked plasma and its ultrafiltrate. The contour chromatograms of CPM were single, sharp peaks. The peak heights were consistent with the respective known concentrations of CPM in the spiked samples.

Calibration

The relation of the CPM concentration in the plasma versus peak height showed good linearity within the range of 5 - 50 μg/ml (FIGURE 2). However, two different regression lines were

obtained depending on the direction of the flow within the column. The regression equation and the correlation coefficient in the forward direction were $Y = (0.200 \times 10^{-3})X - (0.300 \times 10^{-3})$[n = 12, r = 0.999] and those in the reverse direction were $Y = (0.210 \times 10^{-3})X - (0.314 \times 10^{-3})$[n = 12, r = 0.999], and the difference was statistically significant (10). This difference was also obtained with an internal standard method using CBZ. The heights equivalent to a theoretical plate (HETP) were also significantly different for the different flow directions in the column. These effects of the flow direction on the calibration equations were also evident with phosphate buffer, which was used for the determination of the ultrafiltrate. The regression equation and the correlation coefficient in the forward direction were $Y = (0.288 \times 10^{-3})X - (0.020 \times 10^{-3})$[n = 12, r = 0.999] and those in the reverse direction were $Y = (0.304 \times 10^{-3})X - (0.013 \times 10^{-3})$[n = 12, r = 0.999](FIGURE 3). These respective curves showed good linearity but the slope and the intercept at the vertical axis were different. In addition, the regression lines obtained from the plasma and the phosphate buffer were not identical. This may be due to differences in the range of concentrations or to the effects of the peaks of endogenous substances in the plasma. These results suggested that, although the column is reversible, calibration curves should be prepared for the respective flow directions and according to the status of the sample. The detection limit of CPM with this method was low as 0.5 μg/ml.

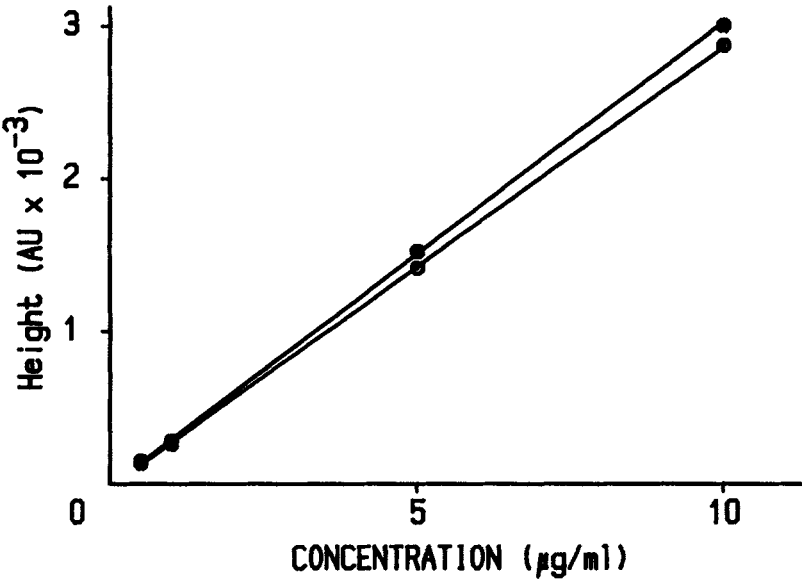

FIGURE 3: Calibration curve for cefpiramide. (○) CPM spiked in phosphate buffer, A → B flow direction. (●) CPM spiked in phosphate buffer, B → A flow direction. Each plot is the mean of three determinations.

Recovery and precision

The results for recovery and precision studies on this method are shown in TABLE 1. The recovery of the drug in plasma and its ultrafiltrate seemed to be complete. The within-day precisions varied less than 3 % and the between-day precisions were within approximately 5 % expressed as the coefficients of variation. These results suggested the applicability of this method to the determination of CPM in biological fluids.

In addition, another advantage of this method is the small volume of the sample. Published methods using HPLC and bioassay

TABLE 1: Within-day and between-day precisions and the recovery of cefpiramide (CPM)

Column	CPM*	n	Coefficient of variation(%)		Recovery(%)** [mean ± S.D.]
			Within-day	Between-day	
A → B	total	9	1.17	4.85	
	free	9	3.70	0.54	
B → A	total	9	1.36	4.98	
	free	9	2.48	1.54	
	total	6			100.81 ± 4.51
	free	6			103.28 ± 5.64

* The total CPM was added to normal plasma to give a concentration of 30 μg/ml. Free CPM was prepared by centrifugal ultrafiltration.

** Recovery (%) = $\frac{\text{determined concentration of CPM}}{\text{known concentration of added CPM}} \times 100$

require a large volume of sample and many steps of pretreatment (11-13). On the other hand, the present method using the Pinkerton^{R} column allows direct injection of the plasma sample reducing the procedures of pretreatment and the sample volume from subjects.

Binding study

CPM is highly protein bound drug and its biological activity has been proven to parallel the free fraction (FF) in the bioassay medium (14,15). This supports the belief that the free

TABLE 3: Free fraction of cefpiramide

Conc.(μg/ml)	Free fraction(%)
5	2.82 ± 0.18
10	3.14 ± 0.08
15	3.48 ± 0.18

Values are the mean ± S.D. of three determinations.
* There were no significant differences between the free fractions of different concentration by the one way layout analysis of variance.

drug is pharmacologically active. Thus, the free fraction of the drug is what should be monitored during the course of treatment. This method also involved the determination of the free drug concentration in the sample. The FFs obtained throughout the present studies are presented in TABLE 2. These values are in good agreement with those obtained by bioassay. Since the FF of CPM was constant in spite of changing the concentration, the protein binding of CPM was concentration - independent in the range of 5 to 15 μg/ml.

In conclusion, HPLC using the Pinkerton^{R} column as presented here enables the determination of CPM levels using a smaller plasma sample volume and without any pretreatment. In addition, it is suggested that calibration curves for both flow directions are required, although the column is reversible which prolongs its use. The analytical procedures outlined in this study may be

applicable to routine clinical use since this is a simple and rapid method.

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