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Development and validation of a MEKC method for the direct determination of cefozopran in human serum

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Abstract

A method for determining the concentration of cefozopran, a cephem anti-microbial agent which has a broad spectrum, in human serum using micellar electrokinetic capillary chromatography (MEKC) by serum direct injection is developed and the validation of the assays of this method is performed. A borate buffer (25 mM; pH 10.0) containing sodium dodecyl sulfate (SDS) (50 mM) is used as a run buffer. The electrophoresis of serum samples is carried out at 25 kV and the detection of cefozopran at 244 nm as its absorption maximum at the cathodic side of the capillary. The migration time of cefozopran is 6.5 min. Linearity (0–200 mg/l) is good and limit of quantification is 0.5 mg/l at a signal-to-noise ratio of 3. Coefficient of variation (CV) of intra-day precision and that of inter-day precision are 2.4–4.0% (7.3–92.0 mg/l) and 2.9–7.7% (22.5–71.4 mg/l), respectively, and the recovery rate is 92–109%. The detection results of 12 other cephem anti-microbial agents under the analytical conditions of this method show that the migration time of cefmetazole is identical with that of cefozopran, making it impossible to separate these two anti-microbial agents. This method is characterized by the fact that simple and economic determination can be achieved by directly injecting the serum samples of micro-quantities into the capillary.

Keywords: Cefozopran; Antibacterial agent; Serum cefozopran concentration; Capillary electrophoresis; Direct human serum injection

1. Introduction

Cefozopran (CZOP, SCE-2787) is a cephem anti-microbial agent synthesized and developed by Takeda Chemical Industries, Ltd. Its chemical formula is shown in Fig. 1 [1]. CZOP has a broad spectrum, its action mechanism is the inhibition of bacterial cell wall (peptidoglycan) synthesis and its action is sterilizing. The reason why this drug exhibits strong anti-microbial activity against gram-positive bacteria as well as gram-negative bacteria is assumed to be that it is stable against β-lactamase and that its action of inhibiting cell wall peptidoglycan cross-bridge formation is strong because it has a powerful affinity for penicillin binding proteins 1 and 2 of *Staphylococcus aureus* as well as for penicillin binding protein 3 of *Escherichia coli* and *Pseudomonas aeruginosa* [2]. Its blood concentration half-life is observed to extend as renal function is deteriorated and this tendency is especially obvious in patients who are of old age or who have renal dysfunction. Additionally, serious side effects such as shock, anaphylactoid symptoms

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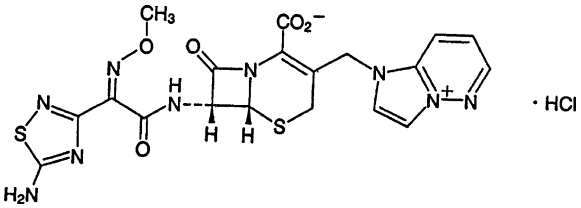

Fig. 1. The chemical structure of cefozopran.

and acute renal insufficiency have been reported [3,4]. Its excretion rate into urine up to 24 h after its administration is 82–94% and no active anti-microbial metabolite other than CZOP is observed in urine [5–7]. Therefore, a simple, easy and reliable CZOP determination method is required in order to prevent serious side effects and to monitor its concentration in blood. Traditionally, bioassay has been used in pharmacological research of CZOP. As a clinical laboratory method, a separation and analysis method that enables specific analysis is recommended, but only a few have been reported so far [8]. Borner [9] and Borner and Borner [10], using high-performance liquid chromatography (HPLC), determined a sample of CZOP extracted with dichloromethane following the de-proteinization of serum with acetonitrile. Yamashita et al. [11], using a column switching HPLC method, performed analysis by diluting serum with a phosphate buffer into concentrations 10–20 times as low as the original one and injecting them into a column without further pretreatment. Yamashita et al., in their method, performed analysis with an extremely simple and easy pretreatment compared to the method used by Borner [9] and Borner and Borner [10], but problems with their method is that the whole system is complicated and that analysis time including time for pretreatment becomes long. With the plasma protein binding rate of CZOP being low as 6.2–8.3% [12,13], the determination of free CZOP by isocratic HPLC after ultra filtration can be another possibility, but it makes determination cost higher. HPLC is an easy analytical method, but a HPLC column is a little expensive and comparatively low in durability. However, a capillary tube used for this method rarely has problems such as blinding or deterioration, is highly durable, and requires extremely tiny volumes of a run buffer and samples. Recently, CE has been recognized as an alternative to HPLC because CE requires less sample volumes, and solvent waste is negligible. Capillary electrophoresis (CE), because of the advantages of very high resolution and minute sample volume requirement, has been applied to the analysis of a wide range of biological substances [14]. Attention was given to the advantages of a fused-silica capillary tube used for CE over a separation column packed with material used for HPLC [15]. It offered a higher theoretical plate number as well as higher durability and enabled a determination with samples of micro-quantities and therefore, we used it for the CZOP determination. The purpose of this research is to develop a new CZOP determination method by direct serum injection using micellar electrokinetic capillary chromatography (MEKC). The assay validation of this method is also reported herein.

2. Experimental

2.1. Chemicals and reagents

CZOP hydrochloride was obtained from Takeda Chemical Industries Ltd. (Osaka, Japan). Sodium dodecyl sulfate (SDS), boric acid, sodium tetraborate decahydrate and sodium hydroxide (0.1N) were purchased from Wako Pure Chemicals (Osaka, Japan). Cefadroxil, cefatrizine, cefazolin, cefotaxime, cefoperazone, cefoxitin, ceftriaxone, cefmetazole, cefuroxime, cefsulodin and cephalothin were purchased from Sigma. Flomoxef was provided by Shionogi Pharmaceutical (Osaka, Japan). “Interference check A plus”, a kit for evaluating interference from bilirubin, hemoglobin and chyle (Sysmex, Kobe, Japan) was used. All solvents and chemicals used were at least of an analytical grade.

2.2. Preparation of standard solutions and calibration standards

CZOP stock standard solution was prepared and adjusted by dissolving 26.74 mg of CZOP in 25 ml of distilled water (1.0 g/l), which was further diluted with distilled water into working standard solutions of various concentrations. This stock standard solution was stable for 3 months even when stored at 4 °C. Serum standard solution was prepared by adding the CZOP standard solution by 5% (v/v) to drug-free human

serum. CZOP serum standard solutions of 0, 6.25, 12.5, 25.0, 50.0, 100.0 and 200.0 mg/l were prepared to be used as CZOP calibration standard solutions.

2.3. CE instrumentation

A P/ACE system MDQ CE made by Beckman Coulter (Fullerton, CA), an untreated fused-silica capillary tube of a 670 mm length (effective length 500 mm), and a 75 μm bore were used.

2.4. MEKC conditions

A sodium tetraborate decahydrate (25 mM)/sodium hydroxide (0.1N) buffer (pH 10.0) containing SDS (50 mM) was used as a run buffer. This run buffer was passed through a 0.45 μm membrane (Millipore, Bedford, MA, USA) and de-aired ultrasonically for 5 min before actual use. Sample injection was performed at the anodic side of the capillary for 5 s (0.5 Pa), MEKC at 25 kV (25 °C) and detection at 244 nm. The capillary was rinsed with sodium hydroxide (0.1N) for 15 min (20 Pa) before analysis was started. Additionally, the capillary was rinsed under the pressure of 20 Pa after the analysis of each sample, with sodium hydroxide (0.1N) for 3 min, with distilled water for 2 min and with the run buffer for 5 min and then a serum sample was injected into it directly.

2.5. Specificity and linearity

The effects of endogenous substances in normal person's serum on CZOP determination were evaluated by comparing chromatograms of drug-free human serum containing no CZOP and a CZOP-added serum sample. Because serum was injected directly in this method, the effects of various interfering substances in serum on the determination were evaluated. In other words, the concentration series of direct bilirubin (0–39.6 mg/dl), indirect bilirubin (0–40.8 mg/dl), hemolytic hemoglobin (0–914 mg/dl) and chyle (0–4940 formazin turbidity) were prepared, the CZOP standard solution was added to them to make 50 mg/l of CZOP-added serum samples and their CZOP concentrations were measured, so that the effects of each interfering substance could be evaluated. Other cephem anti-microbial agents (all in the evaluation concentration of 2 g/l) were determined with this method and their effects were evaluated by comparing the migration times of these agents and CZOP. The CZOP calibration serum standard solutions described in Section 2.2 were measured to obtain linearity and the results were plotted against the peak height and concentration of CZOP to obtain linear regression.

2.6. Limit of quantification

Using the CZOP serum standard solution, the average of concentrations measurable at a signal-to-noise ratio of back ground noise of 3 and the coefficient of variation (CV) were calculated so that detection limit could be determined. The signal amplitude (the height) rather the area was used.

2.7. Precision and accuracy

As for intra-day precision, the CZOP-added serum samples of 7.0, 20.0, 40.0, 80.0 and 100.0 mg/l were prepared and each of them was measured five times in a consecutive manner. As for inter-day precision, the CZOP-added serum samples of 23.5, 40.0 and 70.0 mg/l were prepared and each of them was measured five consecutive days. The samples were stored at −20 °C during this process. Accuracy (recovery rate) was calculated by comparing the measurement values of CZOP standard solutions whose concentrations were identical with those of the CZOP-added serum samples.

3. Results and discussion

3.1. Method development

CZOP, highly polarized and having a comparatively high serum sample measurement concentration rage (5–150 mg/l) [6] as well as strong UV absorption, can be determined by direct serum injection. This method is characterized by the ability to allow the determination of CZOP concentration without serum pretreatment. A direct serum injection method usually requires no use of an internal standard. Furthermore, with this method, the precision and accuracy of the determination was good and errors in injection and variations of the EOF had little effect on the determination. Thus, this method allowed the determination

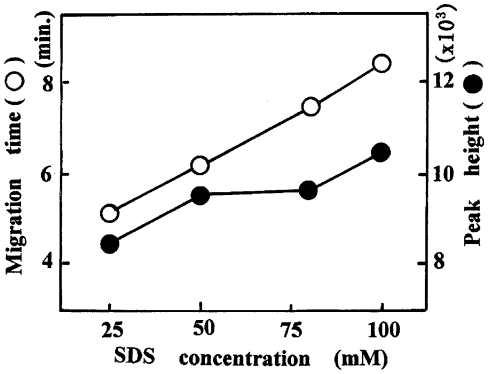

Fig. 2. Effect of SDS concentration in borate buffer (25 mM, pH 10.0) on the migration times and peak height of cefozopran. Sample: standard serum (50 mg/l).

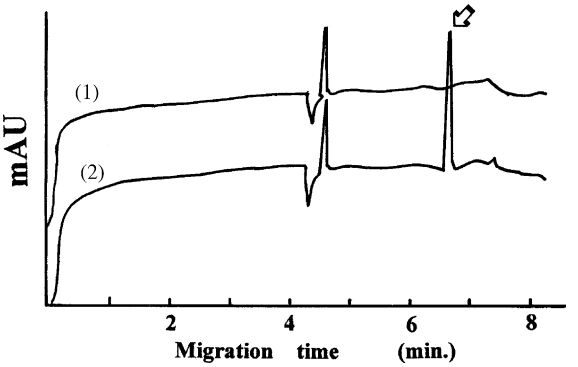

Fig. 3. Typical chromatograms of cefozopran in (1) blank human serum, (2) human serum spiked with 20.0 mg/l cefozopran.

without the use of an internal standard. The evaluation results of the optimal pH of a run buffer using a borate buffer showed there was not a big difference in migration time within the range of pH 8.0–11.0 while the maximum of sensitivity (peak height) was obtained at pH 8.5. At pH 10.0, the sensitivity decreased by 20% from the maximum, but it was sufficient for practical use. Also, considering interference from serum impurities on the determination, baseline noise level, etc., pH 10.0 was optimal. The evaluation results of the optimal concentration of the borate buffer to be used as a run buffer showed the more the molarity of the borate buffer increased, the longer the migration time became, while the sensitivity lowered. However, considering the effects of serum impurities, etc., 25 mM was used for this method. Fig. 2 shows the evaluation results of the optimal concentration of SDS to be added to the run buffer. Regarding a MEKC method where negative ionic micelle (SDS) is used under either a neutral or alkaline condition, it is reported that, in general, as the SDS concentration in a run buffer increases, the migration time of the substance to be measured becomes longer and determination sensitivity increases [16]. The same results as those were obtained by using this method. To achieve a rapid and highly sensitive determination, 50 mM SDS was used. The ultraviolet region absorption wavelength of CZOP (λ_{max}) was observed in the ranges under 200 and 244 nm. However, determination could not be carried out under 200 nm because of interference from serum impurities, while good separation was obtained at 244 nm. Up to 20 s of injection time of serum samples into the capillary (sampling time) was allowed and with this method, injection for 5 s at the anodic side of the capillary enabled analysis with sufficient sensitivity. Twenty-five kilovolts was used for this method because lowered load voltage on the capillary caused the migration time to become extremely long.

3.2. Validation of the assays

Fig. 3 shows the chromatograms of blank human serum and CZOP-added human serum. Good separation was obtained without interference from endogenous substances in serum on the determination. Linearity of 0–200 mg/l was good ($r = 0.9996$). The limit of quantification was 0.5 mg/l at S/N of 3. As for the limit of quantification for determination by HPLC, Borner and Borner [10], using samples extracted with dichloromethane from serum samples de-proteinized with acetonitrile, reported that of 0.6 mg/l, while Yamashita et al. [11], using a column switching method where serum samples diluted with a phosphate buffer were injected directly, reported that of 0.05 mg/l, which means the latter achieved determination sensitivity 10 times as high as our method. Assumed reason for this achievement is that, because samples from which almost all proteins accounting for the major part of the serum samples were removed in the pretreatment column of the column switching system were injected into an analytical column, the background of baseline became low, and therefore, highly sensitive determination became possible. On

Table 1
Intra-day and inter-day precision and in accuracy of cefozopran in serum

Concentration (mg/l)	Concentration found (mg/l)	S.D. (mg/l)	CV (%)	Accuracy (%)
Intra-day assay ($n = 5$)				
7.0	7.3	0.29	4.0	104
20.0	20.7	0.707	3.4	104
40.0	43.7	1.51	3.5	109
80.0	78.4	2.14	2.7	98
100.0	92.0	2.13	2.4	92
Intra-day assay (5 days)				
23.5	22.5	1.73	7.7	98
40.0	37.3	2.30	8.2	93
70.0	71.4	2.05	2.9	102

the other hand, with this method, because CZOP was measured together with proteins contained in serum, the increase of baseline noise was difficult to avoid and therefore, sensitivity somewhat decreased, yet it was sufficient for the determination. Table 1 shows the evaluation results of intra-day and inter-day precisions and those of recovery rates. Borner et al. [10], using HPLC, reported CV of intra-day precision as 0.7–7.7% (0.7–160 mg/l), that of inter-day precision as 10.6 and 3.4% (3.5 and 75 mg/l) and recovery rates as 84–103% (0.7–160 mg/l), while Yamashita et al. [11] reported recovery rates as 100.5% (2.5 mg/l) and 92.3% (0.75 mg/l). Our achievement of the accuracy of determination using this method excelled these reported achievement. Because serum was injected directly into the capillary in this method, interference from various endogenous substances in serum on the CZOP determination, which often poses a problem in clinical laboratory, was evaluated. As a result, no effect of direct bilirubin (up to 39.6 mg/dl), indirect bilirubin (up to 40.8 mg/dl), hemolytic hemoglobin (up to 914 mg/dl) and chyle (up to 4940 formazin turbidity) was observed. The effects of other cephem anti-microbial agents (concentration for evaluation: 2.0 g/l) on the determination were also evaluated and the calculation results as the ratio of the migration time of each anti-microbial agent to that of CZOP (relative migration time) were as follows: flomoxef, 0.962; cefoxitin, 1.017; cefazolin, 1.030; cefoperazone, 1.165; cephalothin, 1.179; cefadroxil, 1.287; ceftriaxone, 1.300. Chromatograms of cefatrizine, cefotaxime, cefsulodin and cefuroxime were not detected, while the migration time of cefmetazole coincided with that of CZOP and these two agents were difficult to separate. The total analysis time of this method was 17 min consisting of 10 min for the rinsing and conditioning of the capillary and 7 min for electrophoresis, which is fast even compared to the reported HPLC methods [10,11], and the whole operation is simple and easy, lowering the occurrence of measurement errors.

4. Conclusions

This method is a specific, highly accurate and economical determination method using MEKC by direct serum injection. The analysis of CZOP was impossible when cefmetazole was present and therefore, its administration together with cefmetazole requires caution. This method, even compared to traditional HPLC, is a simple and fast determination method requiring no pretreatment and the serum of only microquantities.

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