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Simple method for determination of the cephalosporin DQ-2556 in biological fluids by high-performance liquid chromatography

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ABSTRACT

A sensitive method for the determination of DQ-2556 by high-performance liquid chromatography was established. The limits of detection for serum and urine were 0.1 and 2 μg/ml, respectively. Two clean-up procedures for serum samples were developed. In the first, deproteinization with 10% trichloroacetic acid was used and the recovery was 68.5%. In the other, ultrafiltration under acidic conditions was employed and the recovery was 85.1%. The former procedure is economical but complicated, whereas the latter is simple and labour-saving, but a special ultrafiltration tube is required. This situation offers a flexible choice, depending on the conditions of the laboratory.

INTRODUCTION

DQ-2556, (6*R*,7*R*)-7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido]-3-[4-(oxazol-5-yl)-1-pyridinio]methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (**I**), a new cephalosporin, has a broad antibacterial spectrum, including activity against *Pseudomonas aeruginosa*[1], and is currently under clinical trials in Japan. It is difficult to develop a method for the determination of **I** in biological samples because of its high hydrophilicity. As a part of a study on the pharmacokinetics of the drug, a sensitive high-performance liquid chromatographic (HPLC) method was developed for routine use. A large number of samples must be analysed in a short period in clinical trials to evaluate the pharmacokinetics of a drug and to plan subsequent trials. This paper offers both a traditional and a labour-saving procedure for the clean-up of serum samples.

EXPERIMENTAL

Chemicals and reagents

The sulphate of compound **I** was synthesized by the Research Institute, Daiichi Pharmaceutical (Tokyo, Japan)[2] and *S*-*p*-nitrobenzyl-L-cysteine (**II**) was prepared by

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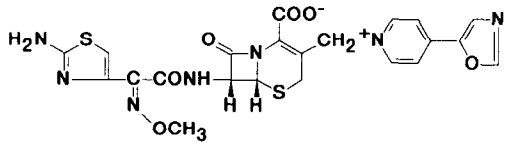
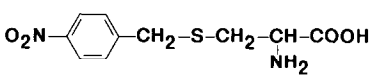
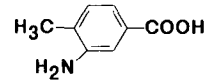


DQ-2556 (I)



S-p-Nitrobenzyl-L-cysteine
(II)



3-Amino-4-methylbenzoic acid
(III)

Fig. 1. Structures of DQ-2556 and internal standards.

the method of Berse *et al.*[3] with slight modifications. The sources of other materials used were as follows: 3-amino-4-methylbenzoic acid (**III**) and trichloroacetic acid from Tokyo Kasei Kogyo (Tokyo, Japan), ultrafiltration tubes (Ultrafree C3TK, molecular weight cut-off 30 000 Da) from Nihon Millipore (Tokyo, Japan) and lyophilized human serum (Consera) from Nissui Seiyaku (Tokyo, Japan). The lyophilized serum was dissolved in distilled water just before use as a control serum. Serum was obtained from healthy volunteers by a doctor with informed consent. Chemicals and solvents were of analytical-reagent grade.

HPLC apparatus and conditions

HPLC analysis was performed with an SP8700 solvent delivery system (Spectra Physics, San Jose, CA, U.S.A.), equipped with a Type AS-48 automatic sample injector, fitted with a 50-μl loop (Tosoh, Tokyo, Japan) and a Model 638-41 variable-wavelength UV detector (Hitachi, Tokyo, Japan). The detector was coupled with a FACOM S-3300 computer system (Fujitsu, Tokyo, Japan). The chromatographic system was equipped with a precolumn, fitted with an MPLC New Guard Cartridge RP-8 (Brownlee Labs., Santa Clara, CA, U.S.A.) and a stainless-steel column (15 cm × 4.6 mm I.D.) packed with 5-μm octadecylsilica (TSK gel ODS-80TM) (Tosoh). The mobile phase was acetonitrile–0.02 *M* acetic acid (8:92, v/v) and the flow-rate was 1.2 ml/min for serum. The retention times were 9, 18 and 14 min for **I**, **II** and **III**, respectively. For urine, the mobile phase was acetonitrile–water (11:89, v/v) and the flow-rate was 0.8 ml/min. The retention times were 8 and 17 min for **I** and **II**, respectively.

Sample preparation

Serum. In procedure A, to 0.5 ml of serum in a 10-ml glass centrifuge tube, 0.5 ml of a 10% solution of trichloroacetic acid, containing **II** at a concentration of 100 μg/ml, was added and mixed on a vortex mixer for 1 min. After centrifugation at 1500 *g* at 4°C for 10 min, the supernatant was transferred to another centrifuge tube. The supernatant was extracted with 7 ml of diethyl ether and centrifuged (1500 *g*, 10 min, 4°C). The organic layer was discarded and the aqueous layer was transferred to 1-ml

sample tube. The tube was set on the automatic sample injector and a 50-μl portion was analysed by HPLC.

In procedure B, a 0.2-ml portion of serum was transferred to the inner cell of the ultrafiltration tube, the bottom of which was made of an ultrafiltration membrane, and acidified with 0.2 ml of 3 *M* acetic acid containing 150 μg/ml of **III** as the internal standard. After centrifugation for 2 h at 5000 *g* at 4°C, the inner cell was discarded and the outer tube, which contained the collected filtrate at the bottom, was set on the tray of an automatic sample injector.

Urine. Urine samples were diluted 5-fold with distilled water, if necessary, and centrifuged. To 0.25 ml of urine, 0.25 ml of a 40 μg/ml (for samples in which the concentrations of **I** were $\leqslant 80$ μg/ml) or 400 μg/ml (for samples in which the concentrations of **I** were >80 μg/ml) solution of **II** was added and a 50-μl portion of the mixture was injected into the HPLC system.

Calibration graph

The standard serum samples were prepared by dissolving **I** in control serum at concentrations of 0.30, 0.59, 1.19, 2.37, 4.74, 9.48, 18.96, 37.93, 75.86, 151.7 and 303.4 μg/ml. Three 0.5-ml portions of standard sample at each concentration were processed as described in the sample preparation section. A calibration graph was obtained by plotting the peak-area ratios (**I/II** or **I/III**) against the concentrations of **I** in the standard samples.

Standard urine samples were prepared by dissolving **I** at concentrations of 2.54, 5.07, 10.14, 20.29, 40.58 and 81.15 μg/ml or 25.4, 50.7, 101.4, 202.9, 405.8 and 811.5 μg/ml. Three 0.25-ml portions of the standard samples were treated as described in the sample preparation section. The calibration graph was obtained in the same way as for serum.

Recovery in serum sample preparation

The absolute recovery in the serum sample clean-up procedure A was measured as follows. Blank serum was spiked with **I** at concentrations in the range 0.3–303.4 μg/ml. A 0.5-ml volume of each sample was processed as described in the sample preparation section. An aqueous solution with the same concentration of **I** as the serum was processed in the same manner. The peak area of **I** observed in the chromatogram of the serum sample was compared with that observed in the chromatogram of the aqueous solution.

For procedure B, the recovery was examined as follows. Blank serum was spiked with **I** in the same manner as in procedure A, and 0.2 ml of each sample was processed as described in procedure B in the sample preparation section. The peak area of **I** observed in the chromatogram of the serum sample was compared with that of the standard solution. The standard solution was a mixture of equal volumes of 3 *M* acetic acid and an aqueous solution of **I** in which the concentration of **I** was the same as that of the spiked serum sample. The recovery was also examined in the case in which 3 *M* acetic acid in the procedure was replaced with 0.2 *M* acetate buffer of different pH or with different concentrations of acetic acid.

RESULTS AND DISCUSSION

Chromatographic conditions

Compound **I** showed tailing in the chromatogram when Nucleosil $5C_{18}$ was used as the stationary phase. It has both a quaternary amine group and a carboxyl group in the molecule, and electrostatic interactions between these ionized functional groups and silanol groups on the stationary phase are considered to be responsible for the tailing. The stationary phase was then changed to end-capped octadecylsilica, TSK gel ODS-80TM, in which silanol groups are fully silylated. Compound **I** was eluted as a sharp and symmetrical peak (Figs. 2 and 3). Serum samples were analysed by HPLC with an acidic eluent in order to elute the interfering substances in serum earlier than **I**. Urine samples could also be analysed in neutral medium with a mixture of acetonitrile and water as eluent. Modification of the eluent with acidic buffer was not effective, because urinary components were eluted near the retention time of **I**.

The chromatogram was monitored at 306 nm because the solution of **I** had an absorption maximum at this wavelength and the baseline was clear, whereas at 254 nm some interfering peaks were detected in the chromatogram.

Clean-up procedures for serum samples

Procedure A. The most frequently used clean-up procedures for cephalosporins in biological samples are extraction with organic solvents and precipitation of serum proteins by an organic solvent or trichloroacetic acid[4]. As the attempt to extract **I** was not successful on account of its high hydrophilicity, a precipitation procedure was chosen for clean-up. Precipitation of serum proteins with methanol or acetonitrile was not satisfactory, giving a fluffy precipitate in the supernatant when it was allowed to stand in the automatic sample injector. Therefore, a procedure consisting of precipitation with trichloroacetic acid and diethyl ether wash was developed. The internal standard for this procedure was expected not only to absorb at 306 nm but also

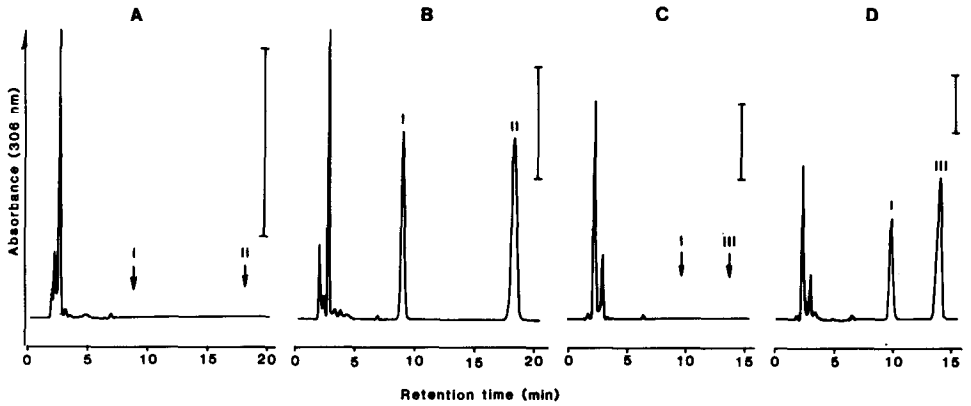


Fig. 2. Chromatograms of serum samples. (A) and (C) control; (B) spiked with 19.0 μg/ml of DQ-2556 and 100 μg/ml of *S*-*p*-nitrobenzyl-L-cysteine (**II**); (D) spiked with 20.6 μg/ml of DQ-2556 (**I**) and 150 μg/ml of 3-amino-4-methylbenzoic acid (**III**). Samples were processed by procedure A [(A) and (B)] or by procedure B [(C) and (D)]. Bars represent 0.1 absorbance.

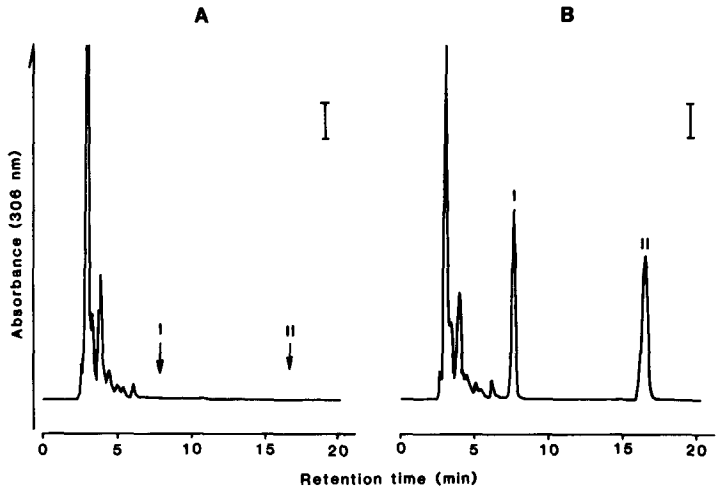


Fig. 3. Chromatograms of urine samples. (A) Control; (B) spiked with 101 μg/ml of DQ-2556 (**I**) and 400 μg/ml of *S*-*p*-nitrobenzyl-L-cysteine (**II**). Bars represent 0.1 absorbance.

to be hydrophilic so as not to be extracted with diethyl ether. Among many compounds tested, L-γ-glutamyl-*p*-nitroanilide, sulphathiazole and **II** were found to have acceptable chromatographic characeristics. Although the first two were commercially available, they were not sufficiently stable in the samples, even at low temperature (8°C). The UV spectrum of **II** showed a maximum absorbance at 280 nm, but even at 306 nm the absorbance was fairly large. This compound was stable for at least 2 days in acidic solutions of the prepared samples.

Procedure B. As a large number of samples must be analysed in a short period in clinical trials, it is better if the clean-up procedure is more labour saving. Ultrafiltration is a simple and frequently used method for deproteinization, like precipitation with trichloroacetic acid[5]. However, the bound fraction of the drug to serum protein should

TABLE I

RECOVERY OF DQ-2556 AND 3-AMINO-4-METHYLBENZOIC ACID (**III**) FROM SERUM UNDER DIFFERENT ACIDIC CONDITIONS AFTER ULTRAFILTRATION

The serum was made acidic by the addition of an equal volume of acetate buffer or of acetic acid. Concentrations in the serum: DQ-2556 = 30 μg/ml; **III** = 150 μg/ml.

Added solution	*Recovery (mean ± S.D.) (%)*	
	DQ-2556	*III*
0.2 *M* acetate buffer (pH 5)	64.5 ± 2.6	51.1 ± 0.7
0.2 *M* acetate buffer (pH 4)	65.0 ± 1.1	51.1 ± 1.2
0.2 *M* acetate buffer (pH 3)	68.1 ± 0.4	56.8 ± 0.9
0.2 *M* acetic acid	70.2 ± 2.3	57.5 ± 0.6
1.0 *M* acetic acid	77.0 ± 2.7	89.1 ± 1.8
3.0 *M* acetic acid	85.1 ± 1.7	99.1 ± 1.1

be dissociated prior to ultrafiltration. Haginaka *et al.*[6] showed that cephalosporin bound to serum proteins is dissociated by acidification of the serum.

The recovery of **I** from human serum at various pH values is summarized in Table I. It increases as the serum is made more acidic. The recovery of **I** reached 85.1% on addition of an equal volume of 3 *M* acetic acid to the serum, and the pH of the filtrate was less than 3 under these conditions. When solutions of **I** and **II** in distilled water were treated in the same way as serum, the recoveries were 94.6% and 101.4%, respectively. These data showed that a small amount of **I** (< 6%) might be adsorbed on the ultrafiltration membrane. Although a higher recovery was expected under more acidic conditions, these conditions were employed because the acidity of sample recommended by the maker of the column was pH 2 or above. The internal standard did not have to be hydrophilic, because this procedure involved no extraction steps. When **III**, which is commercially available, was used as the internal standard, the analysis time was shortened, as its retention time was shorter than that of **II**.

Linearity, recovery and precision

It was expected that serum samples in clinical trials would contain widely different concentrations of **I**. Therefore, calibration graphs were developed at two levels of sensitivity for **I** (0.30–5 and 5–300 μg/ml) in procedure A. Linear relationships were observed in both ranges, and the correlation coefficients for low and high levels were 0.9998 and 0.9997, respectively. In procedure B, the calibration graph was linear in the range 0.3–330 μg/ml and the correlation coefficient was 0.9991. The relative

TABLE II

ACCURACY AND PRECISION OF THE PROPOSED METHODS FOR THE DETERMINATION OF DQ-2556 IN SERUM

Procedure	*Concentration (μg/ml)*		*Accuracy [(found/added) · 100]*	*Precision, R.S.D. (%)*
	Added	*Found (mean ± S.D.)*		
A	*Intra-assay (n = 5):*			
	0.59	0.61 ± 0.02	103.4	3.8
	2.37	2.43 ± 0.06	103.8	2.4
	9.48	9.42 ± 0.17	99.4	1.8
	37.93	36.74 ± 0.54	96.9	1.5
	151.71	149.89 ± 2.70	98.8	1.8
B	*Intra-assay (n = 5):*			
	0.32	0.31 ± 0.01	96.9	2.9
	1.29	1.31 ± 0.01	101.6	0.8
	5.15	5.10 ± 0.13	99.0	2.5
	20.60	20.48 ± 0.53	99.4	2.6
	82.40	80.85 ± 2.07	98.1	2.6
	Inter-assay (n = 10):			
	0.39	0.39 ± 0.02	98.9	6.1
	3.13	3.10 ± 0.11	98.9	3.5
	12.50	12.38 ± 0.17	99.1	1.4
	50.0	49.79 ± 0.73	99.6	1.5
	100.0	100.14 ± 0.34	100.1	0.3

TABLE III

ACCURACY AND PRECISION OF THE PROPOSED METHODS FOR THE DETERMINATION OF DQ-2556 IN URINE

Concentration (μg/ml)		*Accuracy [(found/added) · 100]*	*Precision, R.S.D. (%)*
Added	*Found (mean ± S.D.)*		
Intra-assay (n = 5):			
2.54	2.62 ± 0.13	103.1	5.1
5.07	5.11 ± 0.15	100.8	2.9
20.29	19.71 ± 0.54	97.1	2.7
40.58	39.53 ± 0.79	97.4	2.0
81.15	79.55 ± 2.05	98.0	2.6
202.9	206.7 ± 2.04	101.9	1.0
811.5	813.9 ± 5.58	100.3	0.7
Inter-assay (n = 9):			
3.13	2.96 ± 0.24	94.7	7.9
12.5	12.53 ± 0.23	100.2	1.8
50.0	50.53 ± 0.20	101.6	0.4
100.0	99.77 ± 0.18	99.8	0.2

recovery (accuracy) and the precision, defined as the relative standard deviation (R.S.D.), are summarized in Table II. The R.S.D. values were less than 4% in both procedures in intra-assay and less than 6% in inter-assay comparisons. The absolute recoveries in procedures A and B were 68.5 ± 1.1% and 85.1 ± 1.7%, respectively, at a concentration of 20 μg/ml and they were almost constant over the concentration range of the standard samples. The lyophilized serum could be used as a control serum in the preparation of standard samples without any difficulties after dissolution in distilled water.

Calibration graphs for urine were prepared at two levels of sensitivity for **I** (2.5–100 and 100–800 μg/ml) in a similar way as for serum. The plotted data were well described by linear relationships with correlation coefficients for low and high levels of 0.9998 and 0.9999, respectively. The R.S.D. values were less than 3%, except at low concentration (Table III). The limits of detection were 0.1 μg/ml in serum and 2 μg/ml in urine at a signal-to-noise ratio of 3.

Comparative evaluation of clean-up procedures

Two clean-up procedures for serum samples were developed. In the first precipitation of serum proteins with trichloroacetic acid is used. Although only ordinary reagents, common solvents and no expensive materials are used, careful handling of the sample is required. The second procedure is very simple and requires only the addition of serum and 3 *M* acetic acid to the ultrafiltration tube and centrifugation, but the tubes are expensive. Hence the choice depends on the conditions of the laboratory.

The applicability of procedure B to the determination of other β-lactam antibiotics was confirmed by preliminary experiments in which the recoveries of some carbapenem compounds were over 94%. Recently, a similar procedure was success-

fully applied in the HPLC assay of a new cephalosporin, BMY-28100[7]. This procedure might be useful for the assay of β-lactams, although the effects of extremely low concentrations of serum proteins in patients with hypoalbuminaemia and the strong protein binding of drugs on the recoveries remain to be clarified.

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