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High-performance liquid chromatographic determination of cilastatin and its major metabolite N-acetylcilastatin in rat plasma, urine and bile

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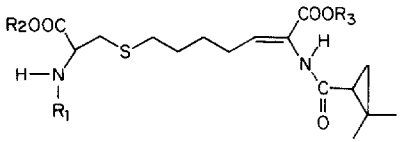
ABSTRACT

A new high-performance liquid chromatographic method coupled with solid-phase (C_8) sample extraction has been developed for the simultaneous quantification of cilastatin and its major metabolite N-acetylcilastatin in rat plasma, urine and bile The method is linear, reproducible and reliable with a detection limit of 1 μg/ml in all three fluids Plasma concentrations of cilastatin and N-acetylcilastatin at selected time intervals and biliary and urinary recoveries of cilastatin and N-acetylcilastatin following an intravenous dose of 10 mg/kg cilastatin are presented

Cilastatin, monosodium-(*Z*)-(S)-[6-carboxy-6-[[(2′,2′-dimethylcyclopropyl) carbonyl]amino]-5-hexenyl-L-cysteine] (I, Fig. 1), is known to inhibit renal dipeptidase-I, dehydropeptidase-I (DPDH-I), which is located in the brush border of the proximal tubular cells [1]. The enzyme is responsible for inactivation of the potent antimicrobal agent imipenem [2–4]. In humans, the urinary recovery of imipenem varied from 12 to 42% of the dose when the drug was given alone, but increased to 64 to 75% when co-administered with cilastatin at a 1:1 ratio [5,6]. N-Acetylcilastatin (II, Fig. 1) was identified as the major metabolite in human urine [7], and in rat and dog bile, urine and plasma [8] after administration of cilastatin.



I, R_1=H R_2=H R_3=Na
II, R_1=CH_3CO R_2=H R_3=Na
III, R_1=CH_3CH_2CO R_2=H R_3=Na

Fig. 1 Structures of cilastatin (I), N-acetylcilastatin (II) and N-propionylcilastatin (III).

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A reversed-phase high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection has been reported for the isolation and quantification of N-acetylcilastatin in human urine [7]. Another HPLC method [9] which involved liquid–solid phase extraction, reversed-phase chromatography, post-column derivatization and fluorescence detection has also been described for the determination of cilastatin in biological fluids. Neither procedure is capable of simultaneous measurement of both cilastatin and N-acetylcilastatin. Inclusion of measuring N-acetylcilastatin is critical for studying elimination kinetics of cilastatin in rats since radioactive histograms of cilastatin-dosed rats revealed that N-acetylcilastatin was the major metabolite in plasma, bile and urine. Biliary and urinary recoveries of N-acetylcilastatin in rats after administration of cilastatin were found to be 33.8 and 47.3%, respectively [10]. N-Acetylcilastatin was also found to have twice the DPDH-I inhibitory potency of the parent compound [11]. A method which can simultaneously quantify cilastatin and N-acetylcilastatin in biological fluids is needed for a proper study of elimination kinetics of cilastatin.

The present method utilizes reversed-phase HPLC with UV detection following C_8 (octylsilane) liquid–solid sample extraction. N-Propionylcilastatin was used as an internal standard. Application of the developed method was demonstrated by quantifying both cilastatin and N-acetylcilastatin in rat plasma, urine and bile samples following intravenous administration of 10 mg/kg cilastatin.

EXPERIMENTAL

Materials

Cilastatin, N-acetylcilastatin and N-propionylcilastatin (internal standard) were all obtained from Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). Acetonitrile, methanol, phosphoric acid (85%), hydrochloric acid and sodium phosphate were products of Fisher Scientific (Fair Lawn, NJ, U.S.A.). Ion-pairing reagents (PIC B-5, B-6, B-7 and B-8) were obtained from Waters Assoc. (Milford, MA, U.S.A.) Bond-Elut cartridges (C_8, 200 mg per 3 ml) were obtained from Analytichem International (Harbor City, CA, U.S.A.).

Standard preparation

Working standards for cilastatin and N-acetylcilastatin were prepared by dilutions of 10 mg/ml of stock standards to 8, 4, 2, 1, 0.5, 0.25, 0.1 mg/ml. The internal standard stock solution was prepared by dissolving N-propionylcilastatin in acetonitrile at 1.0 mg/ml.

Apparatus

The high-performance liquid chromatograph consisted of three components: a WISP Model 710B automatic sample injector with 96-sample card reader (Waters Assoc.), 8700 XR gradient pump with SP8500 dynamix mixer (Spectra-Physics, San Jose, CA, U.S.A.) and SP8733 XR UV variable-wavelength detector oper-

ating at 210 nm. A 100 mm × 4.6 mm I.D. column packed with 5-μm Partisil ODS-3 RAC 2 (Whatman, Clifton, NJ, U.S.A.) was used with an in-line guard column of 5 μm, 30 mm × 4.6 mm I.D RP-18 (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase used was a mixture of acetonitrile–0.05 *M* sodium phosphate monobasic buffer containing 0.005 *M* PIC B-8 ion-pairing reagent; the pH was adjusted to 4.0 with 85% phosphoric acid (12:88, v/v). The flow-rate was set at 1.2 ml/min.

Sample preparation

Plasma or urine sample was prepared by adding 100–400 μl of plasma or 10 μl of urine and 10 μl of internal standard stock solution (1.0 mg/ml) to a 75 mm × 12 mm culture test tube. The sample was diluted to 1.0 ml with distilled water and acidified with a drop of concentrated hydrochloric acid. After vortexing, the diluted plasma or urine sample was applied to an Analytichem C_8 extraction cartridge which had been prewashed with 1.0 ml methanol followed by 2.0 ml of distilled water. The compounds of interest were eluted by 1.0 ml of a mixture of methanol–water (80:20, v/v). The eluent was collected into a 75 mm × 10 mm culture test tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted by adding 1.0 ml of mobile phase, and 50–100 μl of the final solution were injected for HPLC analysis.

For bile sample, 10 μl were diluted to 1.0 ml with mobile phase, followed by addition of 10 μl of internal standard stock standard. The mixture was vortexed for 30 s and centrifuged at 1500 *g* for 5 min, and 50–100 μl of the supernatant were injected into the HPLC system.

Preparation of the standard curve

Calibration standards were prepared by adding 10 μl of an appropriate working standard to 100 μl of plasma, 10 μl of urine or 10 μl of bile. Six calibration concentrations of cilastatin and N-acetylcilastatin were used for standard curves (1.0, 2.5, 5.0, 10, 20 and 40 μg/ml) in each matrix. Sample extraction and quantification were carried out as described above. Concentrations of cilastatin and N-acetylcilastatin were calculated from the linear regression equation of the daily calibration curve constructed by plotting the peak areas of cilastatin and N-acetylcilastatin to the peak area of the internal standard.

Biological sample

Adult male Sprague–Dawley rats, weighing 300–400 g, with free access to food and water were used in this study. Under light pentobarbital anesthesia (40 mg/kg intraperitoneally), a cannula for blood sampling was implanted in the right jugular vein one day before the experiment. In all animals, the enterohepatic circulation was interrupted by bile duct cannulation just prior to the experiment. The bile duct was isolated through a 2-cm abdominal mid-line incision and cannulated with polyethylene tubing (PE-10) under methoxital anesthesia (37.5 mg/kg).

Each rat was placed in a metabolic cage prior to full recovery. Cilastatin was administered by intravenous injection at a dose of 10 mg/kg 30 min after bile duct cannulation, and blood samples were drawn for time intervals of 5, 10, 15, 20, 25, 30, 35 and 40 min. Urine and bile samples were collected from 0 to 4 h. All samples were stored at −15°C until the day of analysis.

RESULTS AND DISCUSSION

An HPLC method with UV detection combined with a C_8 sample extraction cartridge has been developed for the simultaneous determination of cilastatin and its major metabolite N-acetylcilastatin in rat plasma, urine and bile. The effects of pH, molarity and ion-pairing reagents on the retention times of cilastatin, N-acetylcilastatin and N-propionylcilastatin were investigated in order to optimize chromatographic conditions suitable for sample analysis. The optimization processes were carried out by alternately varying molarity (0.01–0.1 n*M*), pH value (3–5) of sodium phosphate monobasic buffer and four different ion-pairing reagents (B-5, B-6, B-7 and B-8). The pH was found to have a dramatic effect on the retention times of cilastatin, N-acetylcilastatin and N-propionylcilastatin, *i.e.*, decreasing the pH from 5 to 3 in the mobile phase caused a five- to twenty-fold increase in the retention times of all three compounds (Fig. 2). It is also interesting to note that the elution order of cilastatin and N-acetylcilastatin was reversed when the pH of the mobile phase was changed from 4 to 5. The effect of the molarity of sodium phosphate monobasic buffer and ion-pairing reagents was negligible in the range 10–100 m*M* and five to eight carbon chain-length, respectively (Fig. 2). However, it was necessary to add the ion-pair reagent octanesulfonic acid (B-8) to the mobile phase in order to increase the retention time of cilastatin and to separate it from the solvent front.

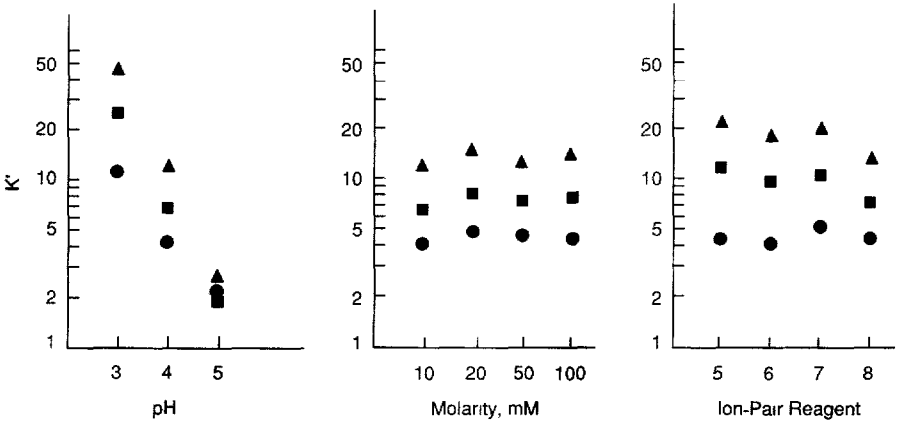

Fig. 2 Effect of pH, molarity and ion-pairing reagent on the retention time of cilastatin (●), N-acetylcilastatin (■) and N-propionylcilastatin (▲)

Under the chromatographic conditions described in the Experimental section, cilastatin, N-acetylcilastatin and N-propionylcilastatin were completely separated with respective retention times of 4.6, 8.2 and 15.1 min. Fig. 3 shows representative chromatograms of control (B) and dosed (A) rat plasma, urine and bile samples. No interfering peaks at the retention times of cilastatin, N-acetylcilastatin and N-propionylcilastatin were seen in control samples of any matrix.

The isolation method using a C_8 extraction cartridge was found to be superior to the conventional liquid–liquid extraction method since it has the advantages of yielding cleaner chromatograms, better recoveries, and is less time-consuming. It also gave better recoveries of cilastatin and N-acetylcilastatin than the anion-exchange cartridge method used for the isolation of N-acetylcilastatin in human urine [7].

The recoveries of cilastatin and N-acetylcilastatin added to control rat plasma and urine at concentrations between 10 and 75 μg/ml were all $>80\%$ (Table I).

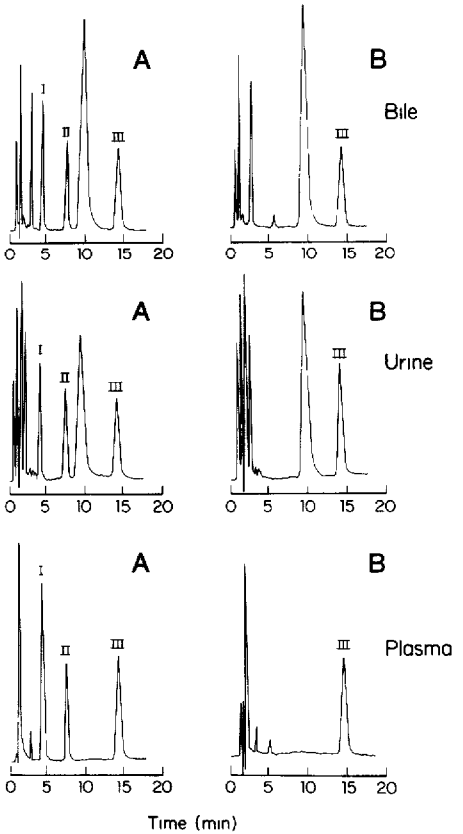

Fig 3 Typical chromatograms for cilastatin (I), N-acetylcilastatin (II) and N-propionylcilastatin (III) in rat bile, urine and plasma. (A) Samples containing 10 μg/ml III after intravenous administration of 10 mg/kg cilastatin; (B) control sample

TABLE I

RECOVERIES OF CILASTATIN, N-ACETYLCILASTATIN AND N-PROPIONYLCILASTATIN IN RAT PLASMA AND URINE FROM A C_8 CARTRIDGE

Concentration (μg/ml)	Recovery (%)					
	Plasma			Urine		
	Cilastatin	N-Acetyl-cilastatin	N-Propionyl-cilastatin	Cilastatin	N-Acetyl-cilastatin	N-Propionyl-cilastatin
75	104 35 ± 11 18	99 61 ± 5 67	91 58 ± 2 77	82 01 ± 8 04	99 30 ± 3 06	92 17 ± 9 46
40	85 89 ± 4 54	92 94 ± 12 71	85 51 ± 13 21	86 52 ± 4 26	97 94 ± 4.48	83 25 ± 3 44
10	81 16 ± 3 24	83 36 ± 4 34	82 78 ± 6 62	84.19 ± 3 36	80 02 ± 5 45	71 45 ± 0 40

Standard curves ranged from 1 to 40 μg/ml with an intra-day and inter-day (n = 5) correlation of r = 0.990 and 0.991, respectively. Over this concentration range, the coefficients of variation (C.V.) (n = 5) for both cilastatin and N-acetylcilastatin ranged from 1.4 to 10.3% for intra-day precision and 1.2 to 10.2% for inter-day precision. The detection limit for both cilastatin and N-acetylcilastatin was 0.5 μg/ml if 200 μl of the final solution were injected.

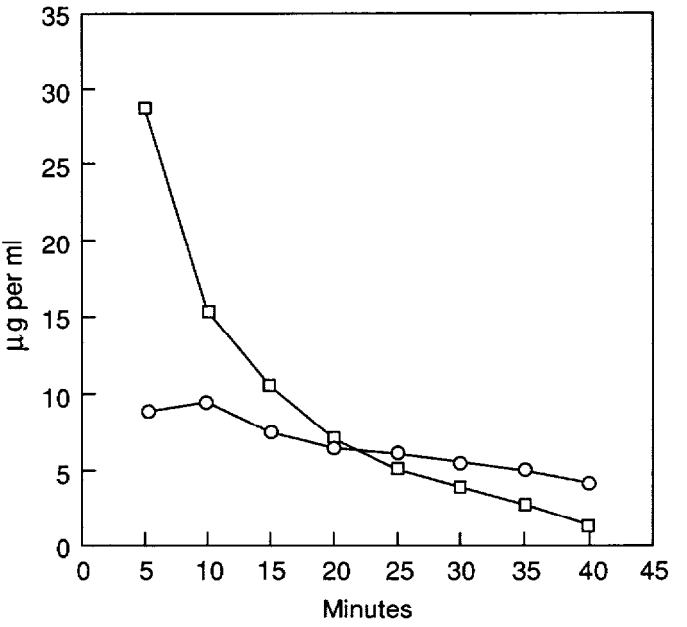

Fig 4. Plasma concentration of cilastatin (□) and N-acetylcilastatin (○) in rats following intravenous administration of 10 mg/kg cilastatin (n = 4).

The method described has been successfully applied to the quantification of both cilastatin and N-acetylcilastatin in rat plasma, urine and bile. Fig. 4 shows the plasma profiles of cilastatin and N-acetylcilastatin in rats following intravenous administration of 10 mg/kg cilastatin. Table II shows individual and total biliary and urinary recoveries of cilastatin and N-acetylcilastatin following intravenous administration of 10 mg/kg cilastatin. Individual biliary recovery of N-acetylcilastatin ranged from 27 to 41% with an average recovery of 33.8 %; only trace amounts of cilastatin were found in rat bile. The averaged urinary recoveries for cilastatin and N-acetylcilastatin were 11.4 and 47.3%, respectively [10].

TABLE II

BILIARY AND URINARY RECOVERY OF CILASTATIN AND N-ACETYLCILASTATIN IN RATS FOLLOWING INTRAVENOUS ADMINISTRATION OF 10 mg/kg CILASTATIN

Rat No	Percentage of dose					
	Bile		Urine		Total	
	Cilastatin	N-Acetyl-cilastatin	Cilastatin	N-Acetyl-cilastatin	Cilastatin	N-Acetyl-cilastatin
1	N.D [a]	36 9	6 8	37 4	6 8	74 3
2	N.D	30 4	10 7	48.2	10 7	78 6
3	N.D	26 9	13 3	62.1	13 3	89 0
4	N.D.	41 2	14 8	41 6	14 8	82 8
Mean		33 8	11 4	47.3	11 4	81 2
S D.		6 41	3 52	10.8	3 52	6 27

[a] N D. = not detectable (<0 05 μg).

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