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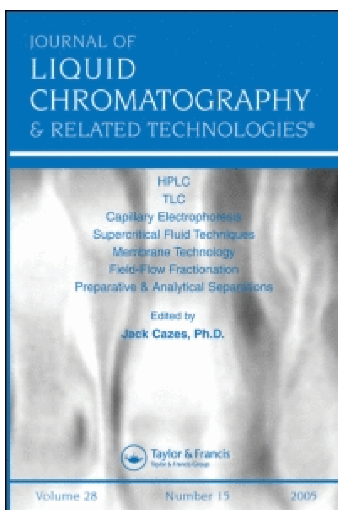

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SEPARATION, IDENTIFICATION, AND QUANTIFICATION OF N-ACETYL CILASTATIN IN HUMAN URINE

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ABSTRACT

A new high-performance liquid chromatographic method coupled with anion-exchange sample extraction has been developed for the isolation and quantification of N-acetyl cilastatin from human urine samples. N-Acetyl cilastatin, isolated from urine after I.V. administration of cilastatin, was converted to dimethyl esters, and characterized by thin-layer chromatography and mass spectrometry. The detection limit of the assay was 5 μg/ml in urine. The method was shown to be linear, reproducible and reliable for the quantification of N-acetyl cilastatin in urine from four human subjects given I.V. doses of cilastatin alone and together with 250 and 1,000 mg of imipenem.

INTRODUCTION

Cilastatin (monosodium-(z)-(s)-[6-carboxy-6-{[(2',2'-dimethyl-(s)-cyclopropyl)carbonyl]amino}-5-hexenyl-L-cysteine), I, is known to inhibit the renal dipeptidase, dehydropeptidase-I (DPDH-I) responsible for the metabolism and inactivation of the potent antimicrobial agent imipenem (1). The urinary recovery of

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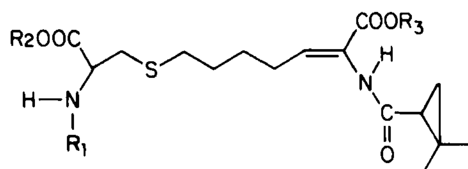

I, R_1=H R_2=H R_3=Na
II, R_1=CH_3CO R_2=H R_3=Na
III, R_1=CH_3CH_2CO R_2=H R_3=Na
IV, R_1=CH_3CO R_2=R_3=CH_3

imipenem varied between 12% and 42% of the dose when imipenem was administered alone to human subjects. Coadministration with cilastatin resulted in an increase in the urinary recovery of imipenem to about 70% in all subjects (2).

The urinary recovery of cilastatin was 70 to 80% of the dose when I with or without imipenem was administered to man (3).

A method for the detection and quantification of N-acetyl cilastatin, II, in man was sought because it was detected and characterized in the chimpanzee and the rhesus monkey (4). N-Acetylation of amino acids, sulfonamides and some drugs is known to be a major metabolic route (5).

A method utilizing reversed-phase high-performance liquid chromatography (HPLC) coupled with anion-exchange sample extraction for the isolation and quantification of II from human urine samples was developed. II, isolated from urine after I.V. administration of I, was converted to dimethyl esters and characterized by thin-layer chromatography and mass spectrometry. Application of the developed method was demonstrated by quantifying II in urine from human subjects given I.V. doses of I alone and together with 250 and 1,000 mg of imipenem.

MATERIALS AND METHOD

Chemicals and Materials

N-Acetyl-cilastatin, N-propionyl cilastatin (III, internal standard), cilastatin and imipenem standards were all obtained

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from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). Acetonitrile and methanol were purchased from Burdick and Jackson Laboratories Inc. (Muskegon, MI). Sodium chloride, phosphoric acid (85%), triethylamine and other chemicals were all analytical reagent grade. Anion-exchange (SAX, 500 mg) cartridges were obtained from Analytichem International (Harbor City, CA). A SEP-PAK cartridge rack was obtained from Waters Associates (Milford, MA). N-Nitrosomethylurea was used to generate diazomethane in diethyl ether (0.05M) and obtained from Pfaltz & Bauer, Inc. (Stamford, CT). Silica gel GF (250 microns) thin-layer chromatography plates were purchased from Analtech, Inc. (Newark, DE).

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Apparatus

The high-performance liquid chromatography consisted of four components: Series 4 LC, ISS-100 autosampler, 3600 data station and 660 printer (Perkin-Elmer Corp., Norwalk, CT). A Spectroflow 757 UV detector (Kratos Analytical Inst., Ramsey, NJ) operating at 210 nm was used to monitor the eluent.

A 25 cm x 4.6 mm i.d. column packed with 5 µm Ultrasphere ODS (Altex Scientific, Inc., Berkeley, CA) was used with an in-line guard column of 5 µm RP-18 (Brownlee Laboratories, Santa Clara, CA). The mobile phase used was a mixture of methanol/acetonitrile/0.85% H_3PO_4 solution, adjusted to pH = 4.0 with triethylamine (8:2:15, V/V/V). The flow rate was set at 1.0 ml/min at ambient temperature. A Model 201 fraction collector (Gilson Medical Electronics, Middleton, WI) was used to collect the fraction. Low resolution mass spectra were obtained with a Model 7035 VG mass spectrometer (VG Instruments Inc., Stamford, CT).

Clinical Samples

Four healthy male volunteers received I.V. doses of cilastatin (250 mg) alone and together with imipenem (250 mg, 1,000 mg). Urine was collected for time intervals of -1-0, 0-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-10 and 10-24 hours. Urine samples were stored at -70° C until the day of analysis.

Sample Preparation

Urine was prepared by placing 2 ml of urine, 0.1 ml of III (4 mg/ml), and 20 µl of triethylamine in a glass culture tube. After vortexing, the urine sample was applied to an Analytichem anion exchange cartridge, which was prewashed with 3 ml of methanol followed by 3 ml of water. The cartridge was washed with 5 ml of methanol followed by 1 ml of water. Two ml of 1M sodium chloride solution was passed through the cartridge and the eluent was collected into a glass culture tube. After transferring to a 2 ml autosample vial, the sample was injected into the HPLC system.

Separation, Derivatization and Characterization

A Gilson Model 201 fraction collector was programmed to collect the fraction (0.5 ml) corresponding to the retention time of authentic standard II under the HPLC conditions described. This collecting procedure was repeated until sufficient amounts of endogenous II in human urine extract was collected. All the fractions were pooled and placed in a 100-ml, round-bottomed flask. Methanol and acetonitrile were evaporated using a rotovapor-evaporator under the water aspirator vacuum. The aqueous solution was then acidified with 6N HCl and transferred to a 100 ml separator funnel. II was extracted by shaking the mixture with 25 ml of ethyl acetate. The ethyl acetate layer was evaporated to dryness and the residue was dissolved in 0.25 ml of methanol. Esterification was carried out by reacting II in methanol with 1 ml of 0.05M diazomethane in diethyl ether. Organic solvents were evaporated to dryness under a stream of dry N_2. The residue was reconstituted in 0.15 ml of methanol. The product, N-acetyl cilastatin dimethyl esters, IV, was spotted on a silica gel GF TLC plate, developed by a solvent mixture (hexane:ethyl acetate:glacial acetic acid = 5:10:1, V/V/V) and visualized in an I_2 chamber. Mass spectra were obtained by inserting IV directly into a VG mass spectrometer using the electron-ionization mode, with 30 e.v. ionization energy and 0.2 mA filament current.

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Quantification

Calibration standards were prepared by adding different amounts (20-1,000 μg) of II, 400 μg of III, 20 μl of triethylamine to 2 ml of human control urine. Sample extraction and HPLC analysis were carried out as described previously. Concentrations of II were calculated from the linear regression equation of the daily calibration curve constructed by plotting the peak height ratio of II to the internal standard, III.

RESULTS AND DISCUSSION

Reversed-phase HPLC combined with anion-exchange sample extraction was utilized to selectively and preparatively isolate II in human urine from subjects given I.V. doses of I. Thin-layer chromatography confirmed the identity of II by matching the R_f values of endogenous II (0.38) and its dimethyl esters IV (0.47) with those of authentic reference standards. Mass spectrum of IV derivatized from endogenous II with diazomethane was compared favorably with that of authentic standard. Molecular ion at m/z 428 and characteristic ions at m/z 396, 331, 252 and 188 were observed for structural confirmation of IV.

An HPLC method using UV detection has been developed for the quantification of II in urine. Figure 1 shows representative chromatograms of subject and control urine samples. No peaks interfered with the detection of II and III in control samples of urine. Under the chromatographic conditions utilized, retention times of I, II, III, and imipenem were 4.9, 7.5, 11.4, and 2.8 min, respectively.

The isolation method using anion-exchange cartirdge was found to be superior to conventional solvent extraction methods. It has the advantages of yielding cleaner chromatograms and better recover, and is less time-consuming. The recovery of II added to control urine with concentrations between 20 and 250 μg/ml was greater than 50%. Standard curves ranged from 10 μg/ml to 500 μg/ml with an intraday and interday (n = 5) correlation of r^2 = 0.9999 and 0.9998, respectively.

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TABLE 1

Individual and Total Urinary Recovery of N-Acetyl Cilastatin (mg) Following Intravenous Infusion of Imipenem/Cilastatin

		Time Interval (hr)										
Subject	Treatment[a]	Pre Dose	0-1	1-2	2-3	3-4	4-6	6-8	8-10	10-24	Total (mg)	% Dose[b]
1	A	0	13.74	6.81	3.22	0	0	0	0	0	23.77	9.0
	B	0	3.11	1.40	0	0	0	0	0	0	4.51	1.7
	C	0	9.65	3.52	1.23	0	0	0	0	0	14.40	5.5
2	A	0	26.88	8.18	4.58	1.73	0	0	0	0	41.37	15.7
	B	0	24.71	10.61	3.58	1.32	0	0	0	0	40.22	15.3
	C	0	18.87	7.46	2.71	0	0	0	0	0	29.04	11.0
3	A	0	20.60	7.19	3.08	1.12	0	0	0	0	31.99	12.2
	B	0	20.44	7.17	2.32	0	0	0	0	0	29.93	11.4
	C	0	21.80	8.36	1.79	0	0	0	0	0	31.95	12.1
4	A	0	24.23	8.72	6.80	0	0	0	0	0	39.75	15.1
	B	0	28.20	11.44	4.42	0	0	0	0	0	44.06	16.7
	C	0	27.16	10.45	4.26	1.23	0	0	0	0	43.10	16.4

[a]

Treatment	Imipenem	Cilastatin
A	0	250 mg
B	250 mg	250 mg
C	1,000 mg	250 mg

[b]

$$\frac{\text{Total mg } \underset{\sim}{\text{II}} \times \dfrac{\text{Molecular Weight } \underset{\sim}{\text{I}} \text{ (monosodium salt form)}}{\text{Molecular Weight } \underset{\sim}{\text{II}} \text{ (acid form)}}}{\text{Dose (250 mg)}} \times 100$$

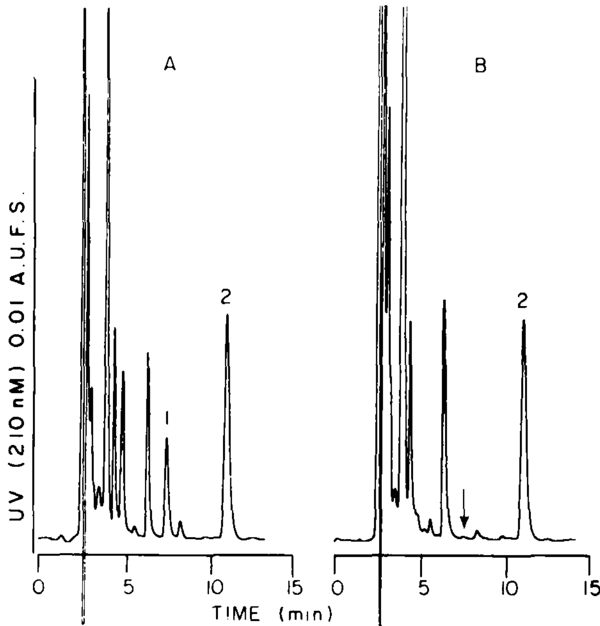

FIGURE 1. Representative chromatograms of (A) subject urine and (B) control urine. Peak 1: N-acetyl cilastatin, II. Peak 2: N-propionyl cilastatin, III. Conditions were as given in the Apparatus section.

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Over this concentration range, the CV values (n = 5) ranged from 0.4 to 7.0 for intraday precision and 0.5 to 6.8% for interday precision. The detection limit of II was 5 µg/ml of urine. This could be improved to 2.5 µg/ml by injecting larger samples into the HPLC system.

Quality control samples were prepared at the high and low ends of all standard curves. In urine, coefficients of variation (n = 5) were less than 10% for quality control samples. Analysis of stability control samples over a period of one month showed II to be stable when stored at -70° C.

The method described was successfully applied to the quantification of II in human urine samples from four subjects given I alone and together with 250 and 1,000 mg of imipenem.

Table 1 shows individual and total urinary recovery information of II. Individual urinary excretion of II ranged from 1.7% to 16.7% of the cilastatin dose between subjects, averaging 11.8% of the dose overall.

Utilization of triethylamine to adjust the pH of the mobile phase is critical for resolution of I and II under the chromatographic conditions described. At the pH range between 3 and 5, I could not be base-line separated from II when 1N sodium hydroxide was used for pH adjustment. The decrease in retention time of I may be due to the competition between triethylamine and the amino group of I for intermolecular interaction with residual Si-OH groups on the surface of the packing (6).

With modification in the mobile phase composition and the injection volume, this HPLC method can be adapted with a detection limit less than 1 μg/ml for the determination of II in human plasma from subjects given I.V. doses of I.

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REFERENCES

1. Kropp, H., Sundelof, J. G., Hajdu, R. and Kahan, F. M. Antimicrob. Agents Chemother. 22, 62, 1982.

2. Norrby, S. R., Alestig, K., Björnegård, B., Burman, L. A., Ferber, F., Huber, J. L., Jones, K. H., Kahan, F. M., Kahan, J. S., Kropp, H., Meisinger, M. A. P. and Sundelof, J. G. Antimicrob. Agents Chemother. 23, 300, 1983.

3. Norrby, S. R., Rogers, J. D., Zacchei, A. G., Ferber, F., Jones, K. H., Weidner, L. L., Demetriades, J. D., Gravallese, D. A. and Hsieh, J. Y-K. Submitted for publication.

4. Kahan, F. M., Kropp, H., Sundelof, J. G. and Birnbaum, J. J. Antimicrob. Chemother. 12, Suppl. D1-35, 1983.

5. Jakoby, W. B., Bend, J. R. and Caldwell, J. Metabolic Basis of Detoxification: Metabolism of Functional Groups. Academic Press, Inc., New York, 293, 1982.

6. Cooke, N. H. C. and Olsen, K. J. Chromatogr. Sci. 18, 512, 1980.