Journal of Chromatography, 382 (1986) 225—231
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3254

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CILASTATIN IN BIOLOGICAL FLUIDS

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(First received February 10th, 1986; revised manuscript received May 12th, 1986)

SUMMARY

Cilastatin, a dehydropeptidase-I inhibitor, is coadministered with the β-lactam antibiotic imipenem. The described procedure was developed for quantification of cilastatin in human plasma and urine. The assay involved sample purification on a C_{18} extraction cartridge, reversed-phase high-performance liquid chromatography with post-column derivatization and fluorescence detection. Standard curves were linear from 0.75 to 75.0 μg/ml in plasma and from 2.5 to 200.0 μg/ml in urine. Intra-day mean coefficients of variation at concentrations within the standard curve range were 4.2 ± 2.4% and 3.1 ± 1.7% in plasma and urine, respectively. The inter-day coefficients of variation for analyses of cilastatin in plasma (1.0 and 50.5 μg/ml) were less than 10% after 31 days of analysis while those for urine (5.0 and 74.1 μg/ml) were less than 11% after 44 days of analysis. The limits of reliable detection were 0.75 and 2.5 μg/ml in plasma and urine, respectively. This procedure met the sensitivity and specificity requirements for the analysis of samples from clinical pharmacokinetic studies

INTRODUCTION

Cilastatin (Fig. 1), a potent inhibitor of the renal dipeptidase dehydropeptidase-I, is coadministered with the β-lactam antibiotic imipenem [1—7]. Earlier quantification of cilastatin in plasma and urine was based on an enzyme assay of the inhibitory titer against porcine renal dehydropeptidase-I. However, that method did not differentiate between cilastatin and a subsequently identified active metabolite [1, 8]. Consequently, an analytical procedure, specific for the determination of cilastatin, was required for the

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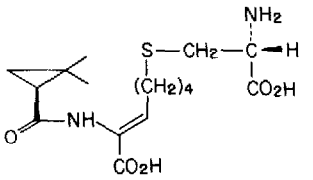

Fig. 1. Structure of cilastatin.

analysis of samples from clinical studies. A high-performance liquid chromatographic (HPLC) method was developed for the analysis of human plasma and urine samples which contained cilastatin either alone or combined with imipenem.

EXPERIMENTAL

Methanol and isopropanol (HPLC grade) were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Sodium hydroxide and HPLC-grade phosphoric acid and potassium phosphate monobasic were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Ethylene glycol and 4-morpholinepropanesulfonic acid (MOPS) were bought from Aldrich (Milwaukee, WI, U.S.A.). Equal volumes of 1.0 *M* MOPS, pH 6.8, and ethylene glycol were mixed to prepare urine stabilizer (see *Stability*). The *o*-phthalaldehyde (OPA) reagent solution was purchased from Pierce (Rockford, IL, U.S.A.). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.) and a Baker-10 extraction system from J.T. Baker (Phillipsburg, NJ, U.S.A.). Human control heparinized plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ, U.S.A.). All reagents and standard solutions were prepared with Milli-Q reagent-grade water (18 MΩ cm^{-1} resistivity).

The cilastatin standard and the internal standard, S-(*p*-methylbenzyl)-L-cysteine, were obtained from Merck Sharp & Dohme Research Labs. (Rahway, NJ, U.S.A.). Solutions of cilastatin which ranged from 3.75 to 375 μg/ml were prepared by the appropriate dilutions of a stock solution of cilastatin (1 mg/ml) and were used for the plasma standard curve. Another stock solution of cilastatin (2 mg/ml) was diluted to prepare solutions which ranged from 25 to 2000 μg/ml for the urine standard curve. These solutions were stable for three months at 5°C.

Two solutions of the internal standard, 30 and 100 μg/ml, were used in the analysis of plasma and urine samples, respectively.

HPLC system

Reversed-phase chromatography was combined with post-column derivatization of the primary amine in each analyte with OPA—mercaptoethanol and fluorescence detection of the resulting isoindole derivatives [9, 10]. The HPLC system consisted of two 6000A pumps, a WISP 710B autosampler, a 720 system controller, and an M730 data module, all from Waters Assoc. The detector was a Perkin-Elmer 650-10S fluorescence spectrophotometer (Norwalk, CT, U.S.A.) with Model 150 xenon power supply (335 nm excitation, 455 nm emission). The analytical column was a Bio-Sil ODS-10 reversed-

phase column (250 × 4.0 mm I.D.) and the guard column was an ODS-10 reversed-phase cartridge (40 × 4.6 mm I.D.), both from Bio-Rad Labs. A stainless-steel column (260 × 4.6 mm I.D.) was hand-packed with Whatman 40-μm glass beads and used as a mixing column for post-column derivatization [11, 12]. The mobile phase for plasma analysis was 10.9% isopropanol in 0.2% orthophosphoric acid, pH 3. The mobile phase for urine analysis was 6% isopropanol in 0.2% orthophosphoric acid, pH 3. Mobile phase was filtered through a 0.2-μm Nylon-66 filter (Rainin), then degassed by stirring under vacuum before use. The mobile phase flow-rate was 2.0 ml/min, while the derivatizing reagent flow-rate was 1.0 ml/min. The derivatizing reagent and auxiliary pump should always be kept in a hood due to stench.

Sample preparation

Plasma. To 0.5 ml plasma, 75 μl of 30 μg/ml internal standard solution and 2 ml of 0.5 *M* KH_2PO_4, pH 3, were added. The sample was vortexed at high speed and loaded onto a C_{18} cartridge which was activated according to the procedure recommended by the manufacturer. The cartridge was rinsed with 20 ml of 0.001 *M* orthophosphoric acid. Methanol (1.5 ml) eluted the cilastatin and internal standard from the cartridge. The collected eluate was diluted with 1.0 ml water, vortexed. and a 50—200 μl sample injected. The standard curve was prepared by spiking control plasma with varying amounts of cilastatin (0.375—37.5 μg) and following the described procedure.

Urine. To 1 ml of stabilized urine, 50 μl of 100 μg/ml internal standard solution and 2.5 ml of 0.02 *M* orthophosphoric acid were added. The vortexed sample was loaded onto an activated C_{18} cartridge. The cartridge was then rinsed with 20 ml of 0.001 *M* orthophosphoric acid, followed by 1.5 ml of methanol for elution of cilastatin and internal standard. The collected eluate was diluted with 1.0 ml water, vortexed, and a 25—75 μl sample injected. The standard curve was prepared by mixing 0.5 ml urine stabilizer, 0.5 ml pooled control urine, and varying amounts of cilastatin (1.25—100 μg), and following the described precedure.

Peak quantification

Peak-height (for plasma) or peak-area (for urine) ratios (cilastatin derivative/internal standard derivative) were calculated. Sample concentrations were calculated from the equation $y = mx + b$ determined by the weighted (weight α $1/y$ for plasma; weight α $1/y^2$ for urine) linear regression analysis of the daily standard curve.

RESULTS AND DISCUSSION

Sample preparation

Endogenous components in plasma and urine co-eluted with the cilastatin and internal standard derivatives. Therefore, sample clean-up prior to chromatography was necessary. Cilastatin and internal standard were retained to a greater extent on C_{18} extraction cartridges, relative to the interfering substances affording a suitable clean-up procedure.

Chromatography

Typical chromatograms of cilastatin in plasma are shown in Fig. 2. Fig. 3 provides representative chromatograms of cilastatin in urine.

Linearity

Standard curves were linear from 0.75 to 75.0 μg/ml in plasma and from 2.5 to 200.0 μg/ml in urine. Mean standard curves of cilastatin ($n = 6$ for each concentration) in plasma and urine were described by $y = 0.071569x - 0.006625$,

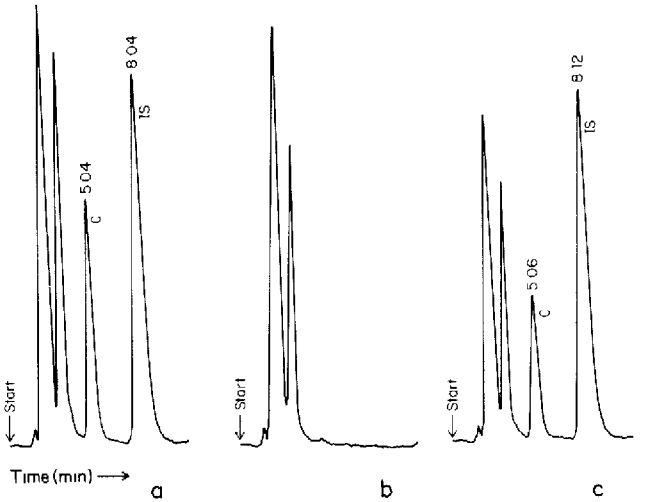

Fig. 2. Chromatograms of cilastatin in plasma. (a) Standard (10 μg/ml); (b) control plasma blank; (c) volunteer sample 1.5 h after imipenem/cilastatin sodium administration. Peaks: C = cilastatin; IS = internal standard.

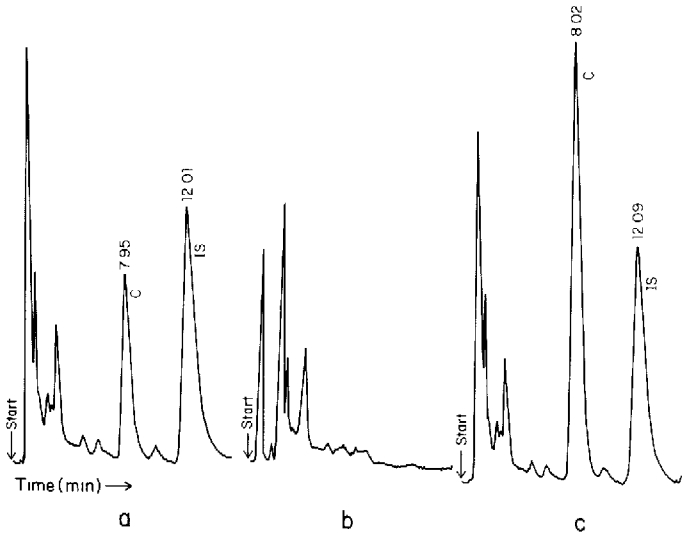

Fig. 3. Chromatograms of cilastatin in stabilized urine. (a) Standard (50 μg/ml); (b) stabilized control urine blank; (c) volunteer sample 1—2 h after imipenem/cilastatin sodium administration. Peaks: C = cilastatin; IS = internal standard.

$r = 0.99933$ and $y = 0.02129x - 0.00528$, $r = 0.99956$, respectively. The limits of reliable detection were 0.75 μg/ml in plasma and 2.5 μg/ml in urine (signal-to-noise ratio of 3).

Accuracy and precision

Individually prepared replicate ($n = 6$) standards were analyzed to assess intra-day reproducibility. All concentrations included in the daily standard curves were tested. Table I lists mean calculated concentrations and coefficients of variation for plasma analysis, while similar data for urine analysis are provided in Table III. The coefficients of variation were 4.2 ± 2.4% for plasma analyses and 3.1 ± 1.7% for urine analyses.

Inter-day reproducibility was assessed through the daily analysis of quality-control (QC) standards. Large volumes of QC standard solutions were prepared, assayed, divided into aliquots, and stored at the recommended temperatures (see *Stability*). The coefficients of variation for 0.98 and 50.46 μg/ml QC standards of cilastatin in plasma were 8.1 and 4.5%, respectively, after 31 days of analyses (see Table II). Coefficients of variation for 5.04 and 74.07 μg/ml QC standards of cilastatin in urine were 10.9 and 7.1%, respectively, after 44 days of analyses (see Table IV).

TABLE I

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF CILASTATIN IN PLASMA: INTRA-DAY REPRODUCIBILITY

Concentration (μg/ml)		Coefficient of variation (%)	Calculated versus nominal concentration (% difference)
Nominal	Calculated ($n = 6$)		
0.75	0.71	2.8	5 3
1.00	1.08	9.2	8.0
2.00	1.79	6.1	10.5
5.00	4.93	4.0	1.4
10.00	10.18	3.0	1.8
20.00	20.44	2.4	2.2
50.00	50.06	4.0	0.1
75.00	74.15	2.0	1.1
Mean		4.2	3.8

TABLE II

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF CILASTATIN IN PLASMA: INTER-DAY REPRODUCIBILITY

Mean calculated concentration (μg/ml)		Number of analyses	Number of days of analysis	Coefficient of variation (%)
Intra-day ($n = 6$)	Inter-day			
0.98	1.02	67	31	8.1
50.46	49.34	66	31	4.5

TABLE III

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF CILASTATIN IN URINE: INTRA-DAY REPRODUCIBILITY

Concentration (μg/ml)		Coefficient of variation (%)	Calculated versus nominal concentration (% difference)
Nominal	Calculated (n = 6)		
2.5	2.4	5.7	4.0
5.0	5.0	2.7	0.0
10.0	9.7	3.8	3.0
20.0	20.1	5.1	0.5
50.0	50.2	1.4	0.4
100.0	102.3	1.8	2.3
150.0	153.1	1.1	2.1
200.0	195.5	3.0	2.2
Mean		3.1	1.8

TABLE IV

ACCURACY AND PRECISION DATA FOR THE DETERMINATION OF CILASTATIN IN URINE: INTER-DAY REPRODUCIBILITY

Mean calculated concentration (μg/ml)		Number of analyses	Number of days of analysis	Coefficient of variation (%)
Intra-day (n = 6)	Inter-day			
5.04	5.06	79	44	10.9
74.07	73.30	86	47	7.1

Selectivity

The selectivity of the detection scheme was based on the derivatization of primary amines in the presence of OPA—mercaptoethanol to fluorescent isoindole derivatives [9, 10]. Consequently, the method was selective for cilastatin without interference from the N-acetyl metabolite. No interfering peaks were detected at the retention times of either the cilastatin derivative or the internal standard derivative when control drug-free samples were analyzed (see Figs. 2 and 3). Similarly, no interferences were detected when clinical samples which contained only imipenem were processed according to the cilastatin procedure.

Recovery

The comparison of peak heights (plasma) or peak areas (urine) in processed samples to those of injected standard solutions were used to determine recovery. The absolute recovery of cilastatin was 79.3 ± 8.5% from plasma and 63.5 ± 2.5% from urine. Relative recoveries of cilastatin from plasma and urine were 96.2 ± 5.1% and 92.1 ± 3.6%, respectively, when the volume of eluate was normalized to the volume of eluent added to the cartridge.

The absolute recovery of the internal standard was 75.6 ± 8.2% from plasma and 58.1 ± 2.1% from urine. Relative recoveries of the internal standard from plasma and urine were 90.9 ± 5.9% and 84.2 ± 3.1%, respectively.

Stability

Initial stability studies in vitro tested cilastatin alone in both plasma and urine. Recovery from plasma stored at −20 ± 5°C for eleven months was 93%. Recovery from urine samples stored at the same temperature for about four months was 102%.

Stability studies in vitro of cilastatin in combination with imipenem were conducted and remain ongoing. Results indicate that cilastatin was also stable when combined with imipenem in plasma stored at −15°C up to four months (102% mean recovery). However, cilastatin recovery decreased dramatically when both cilastatin and imipenem were present in urine stored at −15°C. Of the added cilastatin, 64% remained after seven days, while only 35% remained after 42 days.

Aliquots of urine collections submitted for imipenem assay are stabilized routinely by the addition of an equal volume of 1.0 *M* MOPS, pH 6.8—ethylene glycol (1:1) and storage at −75°C [13]. Similar treatment of urine samples which contained both imipenem and cilastatin prevented loss of cilastatin for as long as 42 days.

CONCLUSION

The described procedure was used to analyze approximately 4000 clinical samples. Two analysts who set up separate HPLC systems proved the durability and versatility of the procedure during one year of continuous analyses. Alternative integration systems, e.g. the Spectra-Physics 4100 computing integrator and the Hewlett-Packard 3357A laboratory automation system have also been used successfully.

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