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DETERMINATION OF THE MONOCYCLIC β-LACTAM ANTIBIOTIC CARUMONAM IN PLASMA AND URINE BY ION-PAIR AND ION-SUPPRESSION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A sensitive and selective high-performance liquid chromatography method has been developed for the determination of the new monocyclic β-lactam antibiotic carumonam in plasma and urine. The method for plasma involves protein precipitation with acetonitrile and removal of lipids with dichloromethane; urine is diluted with buffer. Separation and quantification are achieved using a mobile phase based on either ion-suppression or ion-pair chromatography on a reversed-phase column with UV detection. The limit of determination is 0.5 μg/ml plasma, using a 0.5-ml specimen, and 25 μg/ml urine, using a 50-μl specimen. The inter-assay reproducibility is generally better than 4% when an internal standard is used. Since β-lactam antibiotics may degrade on storage, close attention must be paid to the stability of these drugs in biological fluids; novel measures to prevent degradation on storage are described. The assay has been successfully applied to the analysis of several thousand samples from pharmacokinetic studies, including a study involving patients with impaired renal function.

INTRODUCTION

Carumonam (C, Fig. 1, (2*S*,3*S*)-3-[(*Z*)-2-(amino-4-thiazolyl)-2-[(carboxymethoxy)imino]acetamido]-2-[(carbamoyloxy)methyl]-4-oxo-1-acetidinesulphonic acid, disodium salt) is a new synthetic monocyclic N-sulpho β-lactam antibiotic, which is presently under joint development by Takeda Chemical Industries (AMA 1080) and F. Hoffmann-La Roche & Co. (Ro 17-2301). Carumonam belongs to a novel class of monocyclic β-lactam antibiotics discovered independently, but almost simultaneously, by scientists at Takeda ("sulfaze-

*Preliminary results were presented at the 13th International Congress on Chemotherapy, Vienna, August 28–September 2, 1983 [9].

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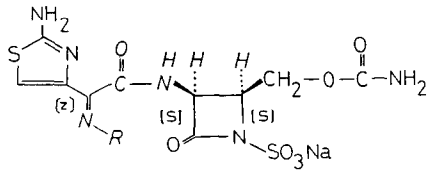

C = Ro 17-2301 (AMA 1080) R= -O-CH_2-COONa

I.S. II = Ro 17-1950 (AMA 1071) R= -O-$C(CH_3)_2$-COONa

I.S. I = Ro 17-2125 R= -O-CH_3

Fig. 1. Structural formulae of carumonam (C) and the internal standards.

cins") and at the Squibb Institute ("monobactams") [1,2]. As is already known for the nocardicins [3], these compounds lack the classical bicyclic structural principle of the penicillins and the cephalosporins. Of the many compounds reported, aztreonam (SQ 26,776) and carumonam are presently under clinical development.

Carumonam exhibits exceptionally high activity against a variety of aerobic gram-negative bacteria, e.g. *Enterobacteriaceae, Pseudomonas aeruginosa, Haemophilus, Neisseria* and *Branhamella* [4–6]. Owing to its remarkable stability against a wide range of chromosomally and plasmid-mediated bacterial β-lactamases, it is active against pathogens that are often resistant to aminoglycosides and even third-generation cephalosporins [5]. Since carumonam is much less toxic than aminoglycosides, it could be considered for the treatment of infections caused by highly resistant aerobic gram-negative bacteria.

Pharmacokinetic features of carumonam, including a kinetic study from patients with impaired renal function, have been published elsewhere [7,8]. A bioassay was used to generate the first kinetic parameters [9]; later, a high-performance liquid chromatographic (HPLC) method was preferred to the bioassay, the latter being cumbersome and not capable of differentiating between a drug and its active metabolites, or degradation products and other co-administered antibiotics. Possible pitfalls encountered with the bioassay have been described by several authors [10,11].

The method reported here has been applied to the analysis of several thousand plasma and urine samples, including a study with patients with impaired renal function, after intramuscular, subcutaneous and intravenous application. Close attention is paid to the known problem of instability of β-lactam antibiotics in biological fluids: novel measures to prevent degradation on storage have been used.

EXPERIMENTAL

Materials

Carumonan and the internal standards I.S. I (Ro 17-2125) and I.S. II (Ro 17-1950) were from authorized batches (F. Hoffmann-La Roche, Basle, Switzer-

TABLE I

CONCENTRATION OF CARUMONAM IN WORKING SOLUTIONS AND PLASMA STANDARDS

Working solutions (μg/ml)	Plasma standards (μg/ml)
4000	80
2000	40
500	10
100	2
50	1
25	0.5

land). Ammonium sulphate, methanol, glacial acetic acid, sulphuric acid and sodium hydroxide solution (1 *M* Titrisol) (all p.a. from E. Merck, Darmstadt, F.R.G.), tri-N-hexylamine (HPLC grade, Fisons, Loughborough, U.K.), and acetonitrile (HPLC grade S, Rathburn, Walkerburn, U.K.) were used to prepare the mobile phases. Dichloromethane, used in the sample preparation step, potassium dihydrogenphosphate and disodium hydrogenphosphate (all p.a.) for the preparation of the Sörensen phosphate buffer were from E. Merck. The water was distilled twice, and the dichloromethane redistilled before use. All other solvents were used without further purification.

Solution and standards

For the preparation of plasma standards, either fresh frozen plasma or plasma obtained by centrifugation of sodium citrated human blood (Blutspendezentrum SRK, Basle, Switzerland) at ca. 1000 *g* for 20 min, thereafter stored at −20°C was used after being tested for the absence of endogenous components that would interfere with the analysis of carumonam and/or the internal standards.

The plasma pH was adjusted to ca. 6.0 by adding 1% (v/v) 0.5 *M* citric acid before calibration standards and quality control samples were prepared.

The disodium salt of carumonam (87.55 mg, equivalent to 80 mg free acid) was dissolved in 20 ml of 0.1 *M* Sörensen phosphate or citrate buffer (pH 5.0) by ultrasonication to give the stock solution. The working solutions were prepared by diluting appropriate aliquots of the stock solution with 0.1 *M* phosphate or citrate buffer (pH 5.0).

The plasma standards were prepared by diluting 0.2 ml of the corresponding working solution with human blank plasma to 10 ml (Table I). The plasma standards were divided into aliquots of 0.6 ml into 1.5-ml Eppendorf tubes and buffered with 0.3 ml of 0.1 *M* potassium dihydrogenphosphate or citrate buffer (pH 3.0). The buffered plasma standards were stored frozen (−20°C) until required for analysis.

Sample collection and storage

Blood samples were obtained using special citric acid-containing vacutainers® (Becton & Dickinson Vacutainer systems, Rutherford, NJ, U.S.A.) adjusting the

blood pH immediately to ca. 6.5 [12]. Within 30 min of collection, each blood sample was centrifuged at 1000 *g* for 15 min. The plasma was then transferred immediately to glass tubes and stored at either −70°C or −20°C (in the latter case for no longer than one month).

Urine samples were collected and titrated immediately to pH 5.0–6.0 by adding citric acid crystals. All urine samples were frozen at −70°C or −20°C.

Sample work-up

Pretreatment. To avoid degradation of carumonam due to multiple thawing and freezing of the samples, two different procedures are recommended: each 0.6-ml sample was pipetted into 0.7-ml polypropylene microtubes (two tubes for duplicate analysis, one tube in reserve) and refrozen immediately. As an alternative, each 0.6 ml was pipetted into 1.5-ml Eppendorf tubes, mixed immediately with 0.3 ml of 0.1 *M* phosphate or citrate buffer (pH 3.0) and 0.1 ml of internal standard solution and then frozen until needed.

Work-up for plasma samples. Unless already treated as described above, 0.25 ml of 0.1 *M* phosphate or citrate buffer (pH 3.0) and 0.05 ml of internal standard solution were added to 0.5 ml of citrated plasma in a tapered tube and vortexed for 10 s. Protein was precipitated by adding 1 ml of acetonitrile to 0.7 ml of the diluted sample. The vial was vortexed twice for 15 s and centrifuged for 10 min at 1000 *g*. The clear supernatant was transferred to a narrow tube containing 5 ml of dichloromethane. The vial was vortexed for 15 s and centrifuged for 10 min at 1000 *g*. About 200 μl of the clear aqueous phase were transferred to a tapered glass tube containing 50 μl of mobile phase; any dichloromethane still dissolved in the aqueous phase was removed by evaporation under reduced pressure (5 min at 200 Torr) using a rotary evaporator (Model RE 120, Büchi, Flawil, Switzerland), equipped with a spider connection for six cylindrical tubes. A portion (usually 40 μl) of the aqueous phase was injected onto the column.

Micromethod for plasma. To 200 μl of citrated plasma in a 1.5-ml Eppendorf tube, 100 μl of 0.1 *M* phosphate or citrate buffer (pH 3.0) were added and vortexed for 10 s. Then 20 μl of internal standard solution were added and vortexed for 10 s. Protein was precipitated by adding 200 μl of acetonitrile. The vial was vortexed and centrifuged for 2 min at 2000 *g*. The clear supernatant was transferred to another tube containing 500 μl of dichloromethane. The vial was vortexed twice for 15 s and centrifuged for 10 min at 2000 *g*. The clear aqueous phase was transferred to a tapered glass tube and processed as described above.

Work-up for urine samples. For the standard curve, 1 ml of mobile phase was mixed with 200 μl of 0.01 *M* phosphate buffer containing a known amount of carumonam and 50 μl of control urine. For the unknown samples, 50 μl of urine were mixed with the same volume of buffer and mobile phase as for the calibration samples. All samples were vortexed, and 20 μl were injected into the chromatograph.

Chromatographic systems

The modular HPLC system consisted of a single-piston reciprocating pump (Model 414-T, Kontron, Zürich, Switzerland), an autosampler (Model MSI 660-T, Kontron), an injection valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.), a

variable-wavelength UV detector (Model Spectroflow 773, Kratos, Ramsey, NJ, U.S.A.,or Model LCD 725, Kontron), and a computing integrator (Model SP-4200, Spectra Physics, San José, CA, U.S.A.).

For the analysis of plasma from patients with renal insufficiency, a column-switching system (Model 70-00A, Rheodyne) with precolumn backflush was used, controlled by an integrator via a laboratory-made interface (U. Timm, F. Hoffmann-La Roche, Basle, Switzerland).

Three different sets of chromatographic systems were used. In system I, for the analyis of plasma and urine, the mobile phase I was 0.3% ammonium sulphate solution–methanol–glacial acetic acid (97:2:1, v/v/v) [13], and the columns used were Merck Hibar® Empty Column R 125-4 packed with Hypersil ODS, 5 μm (Shandon, Runcorn, U.K.) and Spherisorb ODS-2, 5 μm (Phase Separations, Queensferry, U.K.); the internal standard was I.S. I.

In system II, also for the analysis of plasma and urine, mobile phase II was prepared by dissolving 1.08 g of trihexylamine in 200 ml of acetonitrile, before slowly adding 2.5 ml of 50% sulphuric acid and 800 ml of water. The mobile phase was adjusted to an apparent pH of 3.0 with 5 *M* sodium hydroxide solution. Interference from endogenous components could be overcome by modifying the pH on the mobile phase slightly. Apart from the dimensions (100×3 mm I.D.), the same packing materials were used as for system I, and the internal standard was I.S. II.

System III was used for the analysis of plasma from patients with impaired renal function. In this case, 1.35 g of trihexylamine were dissolved in 300 ml of acetonitrile before slowly adding 1.0 ml of 50% sulphuric acid and 700 ml of water. The mobile phase was adjusted to an apparent pH of 4.5 with 1 *M* sodium hydroxide solution. The columns used were: analytical column, R 125-4 packed with Hypersil ODS, 5 μm; precolumn, R 30-4 packed with Hypersil MOS, 5 μm. The precolumn was backflushed with mobile phase after 3.5 min (Fig. 2).

For all three systems, the flow-rate was adjusted to 1 ml/min, and UV detection was at 293 nm. The temperature of the columns was ca. 20°C.

Calibration and calculation

Five to seven standard samples, covering the expected concentration range, were processed and analysed together with the unknown and quality control samples. The concentrations of carumonam were determined using a calibration curve, obtained by a weighted (factor $1/y^2$) least-squares regression of either the peak-height ratio of carumonam to internal standard or, if an internal standard was not used, of the peak height of spiked samples versus the concentrations of carumonam added to plasma. All data processing and calculating was done using a special BASIC program [14].

RESULTS AND DISCUSSION

General considerations

Carumonam is extremely soluble in water (50 g per 100 ml) and much less soluble in organic solvents (e.g. 0.1 g per 100 ml of methanol). It was therefore

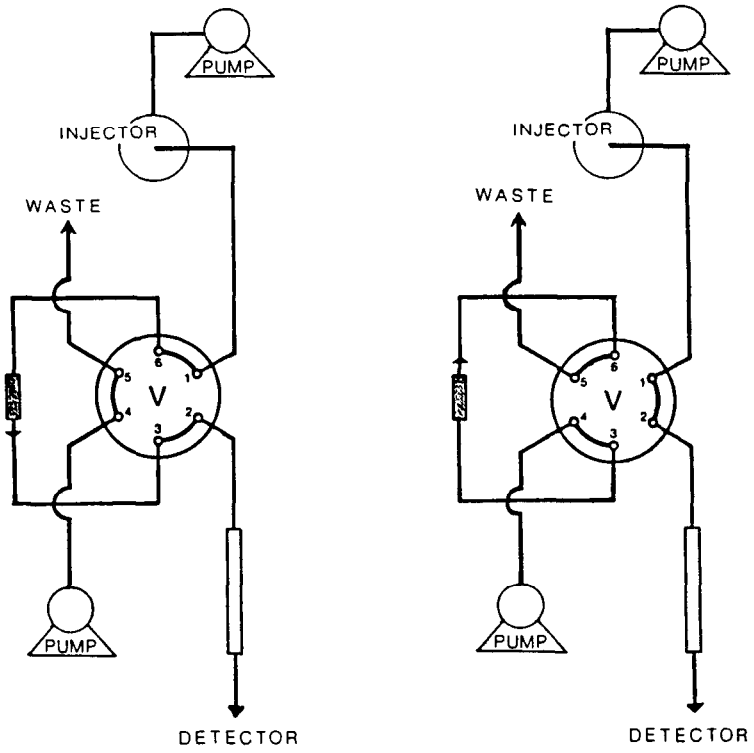

Fig. 2. Precolumn backflush to cut off late-eluting peaks.

considered unlikely that this drug would be amenable to conventional extraction procedures. On-column enrichment techniques as described for cephalosporins by other authors [15–17], failed since carumonam was readily eluted from a C_{18} reversed-phase column by water following injection of a plasma sample. Another hindrance to the development of a sensitive method was the low UV absorbance of this substance, which is less than one quarter of that of most other β-lactam antibiotics. Protein precipitation steps resulting in further dilution of the sample should therefore be avoided. A further potential problem was lack of stability, since most β-lactams are liable to undergo degradation even in weak alkaline solutions and/or in some organic solvents.

Any method, therefore, should include a protein removal step, and an additional clean-up and concentration step to obtain the required sensitivity. Methods for the determination of β-lactam antibiotics that meet the requirements of a clean-up *and* a concentration step have been published by Carlqvist and Westerlund [18], Rudrik and Bawdon [19], and by Dell et al. [20], and have been later modified by several authors [21–23].

Final sample preparation

The method of Rudrik and Bawdon [19] was adopted, with some modifications. The dilution of plasma with phosphate buffer prior to precipitation of proteins was introduced as an essential step to reduce protein binding and hence to

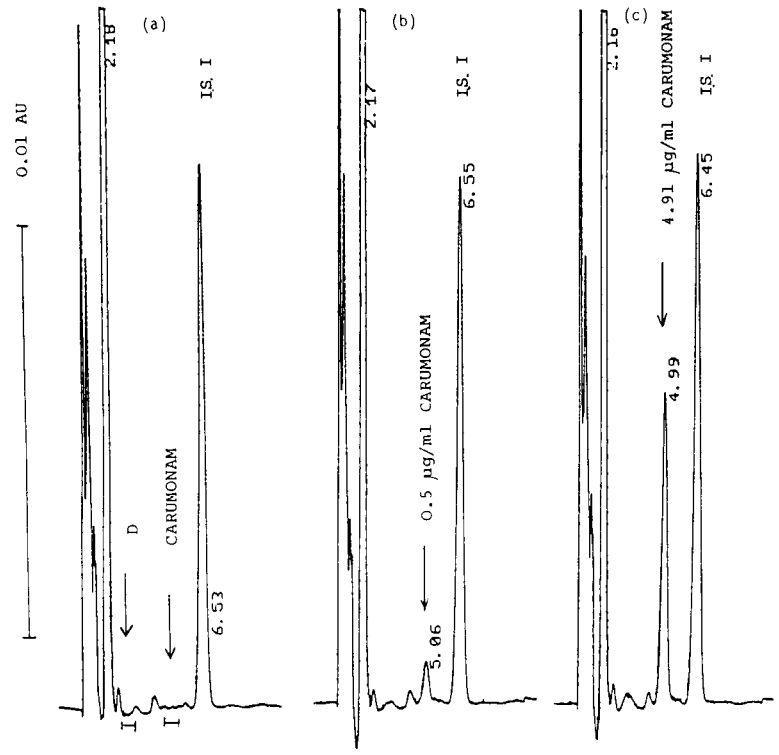

Fig. 3. (a) Chromatogram of a blank plasma sample, spiked with I.S. I. (b) Chromatogram of a blank plasma sample, spiked with 0.5 µg/ml carumonam, and I.S. I. (c) Chromatogram of a plasma sample from a human subject after i.v. infusion; calculated concentration: 4.91 µg/ml. HPLC system I; detector, Kratos Spectroflow at 293 nm and 0.02 a.u.f.s.

avoid co-precipitation. Furthermore, possible degradation of the monobactam at ambient temperature, during work-up and analysis in an autosampler, was prevented.

Acetonitrile was chosen as precipitating agent. The subsequent extraction of the transferred supernatant with dichloromethane conferred the advantages of concentrating carumonam in the aqueous phase (acetonitrile partitions into the dichloromethane layer) and complete removal of proteins, together with extraction of less polar material.

To avoid baseline distortion in the chromatograms, the dichloromethane remaining in the aqueous phase was evaporated under reduced pressure. As a result, predominantly aqueous mobile phases (without ion-pairing reagents) could be used routinely (Fig. 3a–c).

Chromatographic systems

Ion-pair reversed-phase chromatography is the method of choice for analysing the very polar and often amphoteric β-lactam antibiotics, since it has the advan-

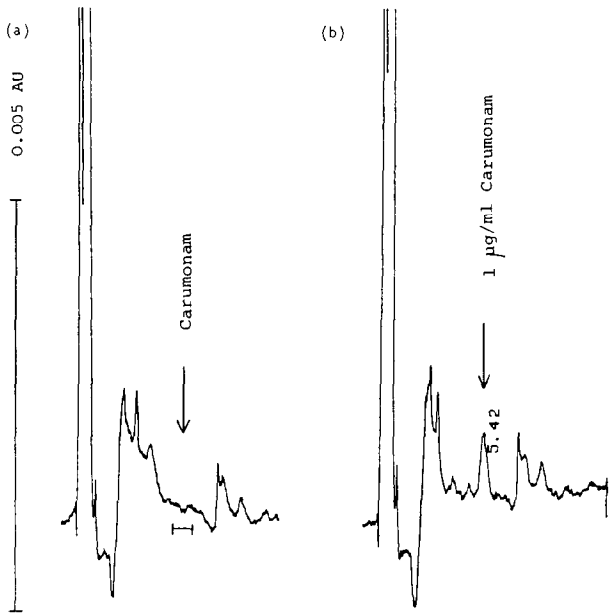

Fig. 4. (a) Chromatogram of a human blank plasma. (b) Chromatogram of a human blank plasma, spiked with 1 µg/ml carumonam. HPLC system II; detector, Kontron LCD 725 at 293 nm and 0.01 a.u.f.s.

tage of conferring selective retention on the analyte whilst not retaining other components that do not form ion-pairs. However, ion-pair chromatography has some practical drawbacks, such as being deleterious to apparatus and columns. Methods based on ion-suppression [24,25] or specific retention mechanisms, e.g. amino columns [26,27], offer several advantages and are used increasingly. Therefore, a method based on ion-suppression [13] was developed further for the determination of carumonam in plasma and nearly all urine samples (system I, Fig. 3a–c), in addition to an ion-pair method (system II, Figs. 4 and 5), which could be used for both plasma and urine.

For the analysis of plasma samples from patients with impaired renal function, a different mobile phase had to be developed because, owing to severe interference, both systems I and II could only be applied to samples from patients with moderated renal clearance. A series of mobile phases, based on tetraalkylammonium and trimethylalkylammonium salts, was tried; a modification of mobile phase II proved to be best (system III), and all the plasma samples from this clinical trial (even those of patients with no creatinine clearance) could be analysed without further change of the eluent. In order to minimize the analysis time, precolumn backflush (cf. Fig. 2) was used to cut off late eluting peaks. Typical chromatograms are shown in Fig. 6.

Characteristics of the method

The limit of quantification was ca. 0.5 µg/ml plasma, using 0.5-ml specimens, and 25 µg/ml urine, using 50-µl specimens. The relative standard deviation

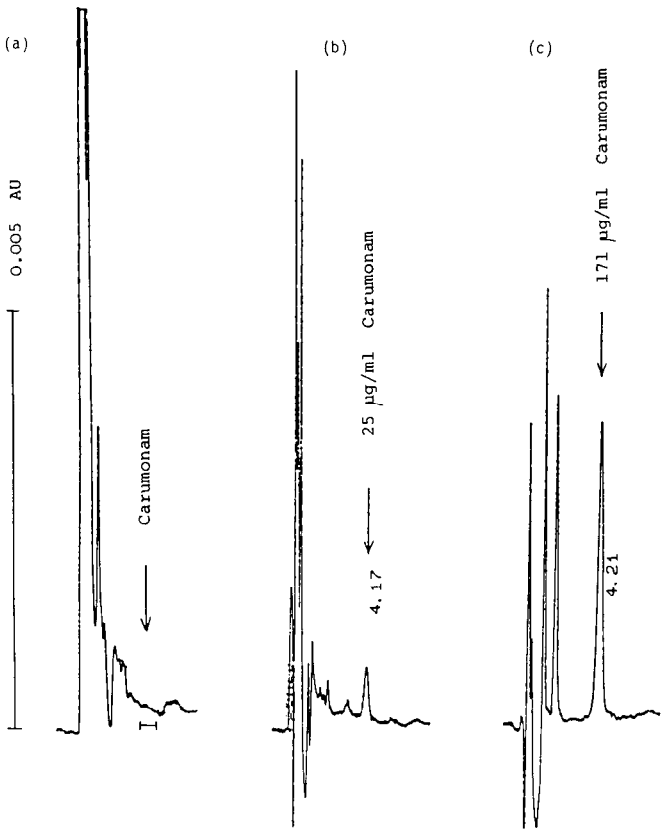

Fig. 5. (a) Chromatogram of human blank urine. (b) Chromatogram of human blank urine, spiked with 25 µg/ml carumonam. (c) Chromatogram of a urine sample from a human subject; calculated concentration: 171 µg/ml. HPLC system II; detector, Kontron LCD 725 at 293 nm and 0.01 a.u.f.s.

(R.S.D.) and the relative error for plasma at this level were 3.9% and −2.5%, using an internal standard, and 6.1% and −3.7% without an internal standard, respectively.

The limit stated was achieved with a less sensitive UV detector (cf. Fig. 5); the limit could certainly be reduced by using columns with smaller internal diameter, 3-µm packing materials or a more sensitive detector.

The correlation between peak height and concentration of carumonam was found to be linear in the range 0.5–200 µg/ml plasma, and 25–8000 µg/ml urine. The coefficient of determination (r^2) was generally 0.999, using the weighting factor $1/y^2$.

The mean recovery was 101% and 97.0% from plasma and plasma ultrafiltrate, respectively, using blank plasma extracts spiked with the corresponding amount of carumonam as the 100% values (Table II). The data obtained indicate that no coprecipitation occurred during the protein precipitation step. The additional treatment with acetonitrile–dichloromethane did not alter the volume of the buffered plasma.

During the analysis of plasma and urine samples from different clinical trials,

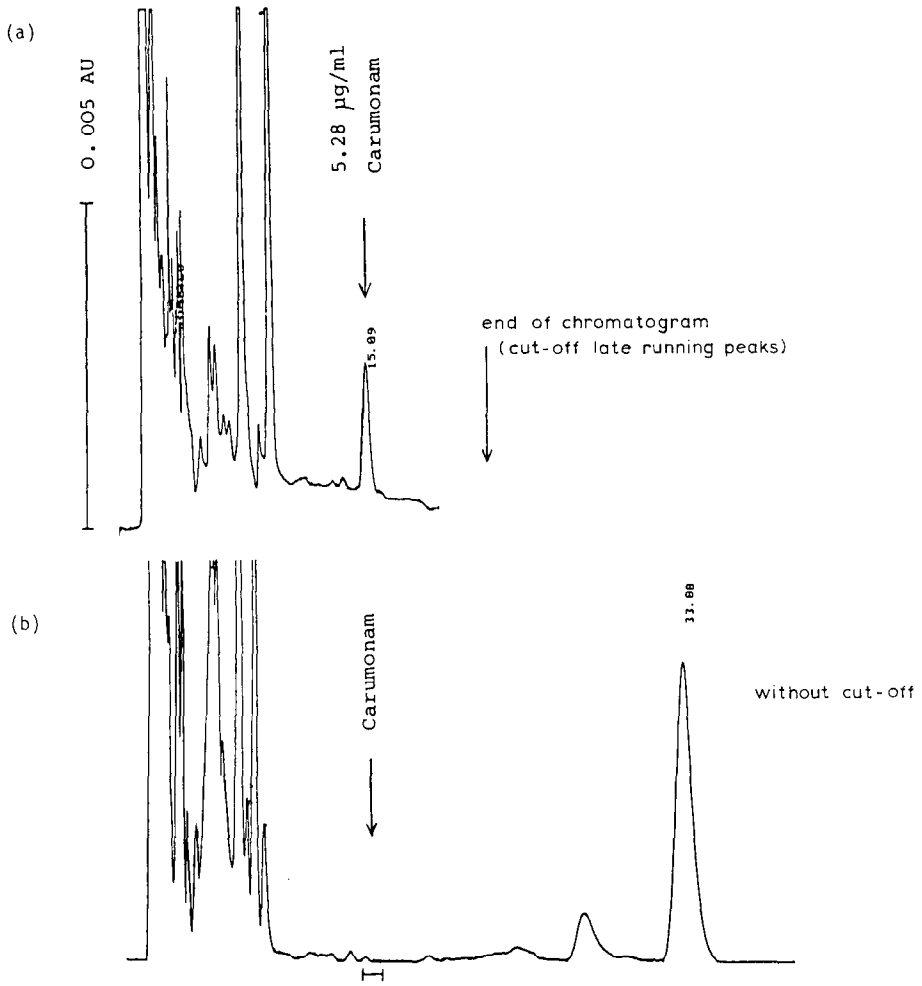

Fig. 6. (a) Chromatogram of a plasma sample from a patient suffering from severe renal insufficiency, sample collected 48 h after i.v. infusion of 1000 mg of carumonam; calculated concentration: 5.28 µg/ml. (b) Chromatogram of a plasma sample of a patient collected before infusion. HPLC system III; detector, Kratos Spectroflow 773 at 293 nm an 0.01 a.u.f.s.

no interferences from endogenous compounds were observed. Typical chromatograms are shown in Figs. 3–6.

The accuracy of the method (defined as the percentage difference between added and found concentrations) and the precision (defined as the R.S.D. of replicate analyses of the same sample) were evaluated over the concentration range 0.5–80 µg/ml. The inter-assay reproducibility was evaluated by analysing spiked plasma samples over several consecutive days, and the intra-assay reproducibility by analysing five specimens of spiked plasma on the same day (Table III). A mean precision of ca. 3.5% and 5.2% was found for the concentration range of 0.5–80 µg/ml plasma, calculated with and without an internal standard, respectively. In the same range, the accuracy was 2.3% and 2.7%, respectively.

TABLE II

RECOVERY OF CARUMONAM FROM HUMAN PLASMA AND HUMAN PLASMA ULTRAFILTRATE

Concentration (μg/ml)	Human plasma			Human plasma ultrafiltrate		
	Recovery (%)	R.S.D. (%)	*n*	Recovery (%)	R.S.D. (%)	*n*
100	102.6	1.1	3	96.3	1.0	3
10	99.6	0.7	3	97.1	1.8	2
2	100.9	1.7	3	97.9	2.1	3
Mean	101.1	1.7	9	97.0	1.7	8

These data indicate that the use of an internal standard is not absolutely necessary, as previously reported by Haefelfinger [28]. The use of an internal standard is nevertheless recommended, since it compensates for handling errors that can occur during sample preparation and/or for errors as a result of instrumental malfunction.

Stability

The instability of many β-lactams above pH 7.5 has been reported by several workers [22,29–31]. The physiological pH levels of plasma and urine are in the range of 7.2–7.5 and 4.6–8.2, respectively [32]. During storage, however, the pH of biological fluids will increase to levels above 8.0, owing to loss of carbon dioxide

TABLE III

REPRODUCIBILITY OF THE ASSAY FOR PLASMA

Concentration added (μg/ml)	Concentration found (μg/ml)	R.S.D. (%)	Relative error (%)	90% Confidence interval (%)
Intra-assay reproducibility (n=5) without internal standard				
2.50	2.48	1.6	−0.6	−1.9 to +0.7
50.0	51.2	2.5	+2.4	+0.3 to +4.5
Day-to-day reproducibility (5 consecutive days) without internal standard				
0.50	0.48	6.1	−3.7	−8.6 to +1.3
5.00	4.90	2.2	−2.1	−3.8 to −0.3
80.0	76.9	3.3	−4.0	−6.5 to −1.3
Day-to-day reproducibility (5 consecutive days) with internal standard				
0.50	0.49	3.9	−2.5	−5.6 to +0.7
5.00	4.97	2.8	−0.6	−2.9 to +1.8
80.0	81.0	1.2	+1.3	+0.3 to +2.3
Long-term reproducibility (quality control samples), determined over a period of 39 days				
5.00	5.10	4.2	+2.0	+0.7 to +3.2
40.0	40.3	4.2	+0.8	−0.6 to +1.7

TABLE IV

STABILITY OF CARUMONAM IN 0.129 *M* CITRATE BUFFER AT +20°C FOR 24 H

100% value (mean ± % S.D.) = 50 ± 1.0 μg/ml.

pH	Value after storage (μg/ml ± % S.D.)	*n*	Mean difference (%)	90% Confidence interval (%)
6.0	49.5 ± 1.3	3	−1	+1.7 to −3.6
7.0	48.6 ± 0.4	2	−2.9	−1.9 to −3.8
7.5	47.1 ± 1.6	4	−5.8	−4.1 to −7.4
8.0	44.9 ± 0.5	2	−10.3	−9.3 to −11.3
8.5	34.2 ± 0.1	2	−31.5	−31.3 to −31.7

[33]. Thus, the pH levels of biological fluids have to be carefully controlled during storage and analysis to prevent serious degradation of alkaline-labile β-lactam antibiotics. Exceptionally, a few members from this class of compounds, ceftriaxone for example, exhibit a stability maximum around pH 7.5 [34].

Detailed investigations of the stability of carumonam in aqueous solutions, plasma, and urine were carried out as a part of the development of the assay. A set of five freshly prepared samples was processed and analysed, together with the stored samples, and the stability data were calculated according to our established method (for definitions of the terms "significant", "non-relevant", and "acceptable degradation" see ref. 35). The stability data are listed in Tables IV–VII. The degradation of carumonam in aqueous solutions (0.129 *M* citrate buffer) was strongly pH-dependent. In the pH range 5.0–7.0, carumonam was stable at +20°C for ca. 24 h. At a pH of 7.5, significant, but non-relevant degradation (−5.8%) occurred. From pH 8.0 to 8.5, the degradation of carumonam increased rapidly (−10% to −31%). The stability of carumonam in urine was determined at 37°C for 16 h to estimate the possible degradation in the bladder during long sampling periods. Again, carumonam was stable in the pH range 5.0–6.0, whereas relevant degradation occurs above 7.0 (Table V).

From these data it was clear that serious degradation of carumonam was to be

TABLE V

STABILITY OF CARUMONAM IN HUMAN URINE AT +37°C FOR 16 h

100% value (mean ± % S.D.) = 1000 ± 1.9 μg/ml.

pH	Value after storage (μg/ml ± % S.D.)	Mean difference (%)	90% Confidence interval (%)
5.0	996 ± 2.8	−0.4	+2.6 to −3.3
6.0	981 ± 2.0	−1.9	+0.5 to −4.3
7.0	911 ± 4.1	−8.9	−5.3 to −12.3
7.5	875 ± 3.9	−12.5	−9.3 to −15.6
8.0	818 ± 3.0	−18.2	−15.6 to −20.6

TABLE VI

STABILITY OF CARUMONAM IN HUMAN PLASMA STORED AT ROOM TEMPERATURE

Sample	Storage conditions	100% value (μg/ml ± % S.D.)	Value after storage (μg/ml ± % S.D.)	Mean difference (%)	90% Confidence interval (%)
Citrated plasma, initial pH 7.4, 5 samples	+22°C 3 h	2.5 ± 2.0	2.42 ± 0.8	−3.0	−1.2 to −4.8
		50 ± 1.9*	47.3 ± 0.8*	−5.4	−3.4 to −7.3
		500 ± 1.6	477 ± 0.4*	−4.6	−3.1 to −6.1
As above	+22°C 24 h	2.5 ± 3.3	1.90 ± 2.7	−24	−21 to −26
		50 ± 1.4	35.1 ± 1.7	−30	−29 to −31
		500 ± 1.1	379 ± 1.1	−24	−23 to −25
As above, but buffered to pH 6.5 final pH ca. 7.0; 5 subjects, 2 samples of each	+22°C 24 h	23.8 ± 3.4	24.6 ± 4.0	+3.6	+6.6 to +0.7

*$n = 4$.

expected during storage of plasma samples unless precautions were taken. Preliminary results confirmed this: when plasma (initial pH 7.4) was stored for less than 3 h at room temperature, the degradation was significant, but still acceptable (up to −8%, Table VI). When stored for 24 h, the decomposition was unacceptable (−24% to −30%). However, no significant degradation was observed when plasma was buffered with citric acid to an initial pH of 6.5.

For long-term storage at −20°C (Table VII), buffering of the plasma to an initial pH of 6.5 is a prerequisite to avoid serious degradation. Even under the

TABLE VII

LONG-TERM STABILITY OF CARUMONAM IN HUMAN PLASMA

Storage conditions	100% value (μg/ml ± S.D.)	Value after storage (μg/ml ± % S.D.)	Mean difference (%)	90% Confidence interval (%)
Citrated plasma buffered to pH 6.5, 5 weeks at −20°C	2.51 ± 0.5 ($n = 3$)	2.34 ± 4.4 ($n = 5$)	−6.9	−2.1 to +11.6
	49.9 ± 0.2 ($n = 3$)	45.6 ± 1.6 ($n = 5$)	−8.6	−6.8 to −10.0
Citrated plasma buffered to pH 6.5, 18 weeks at −20°C	2.50 ± 1.5 ($n = 5$)	1.96 ± 3.6 ($n = 4$)	−21.5	−18.9 to −24.1
	50.4 ± 2.3 ($n = 5$)	40.8 ± 2.4 ($n = 5$)	−19.1	−16.7 to −21.4
Citrated plasma (pH 7.4)-0.1 *M* phosphate buffer (pH 3.0) (2:1, v/v), 18 weeks at −20°C	As above	2.44 ± 1.8 ($n = 5$)	−2.2	−0.3 to −4.1
		49.4 ± 3.5 ($n = 5$)	−1.9	+1.6 to −5.3
Citrated plasma buffered to pH 6.5, 18 weeks at −70°C	2.46 ± 1.0 ($n = 5$)	2.56 ± 1.9 ($n = 5$)	+4.1	+5.9 to +2.3
	50.4 ± 2.3 ($n = 5$)	50.8 ± 2.2 ($n = 5$)	+0.8	+3.4 to −1.8
Citrated plasma (pH 7.4), 18 weeks at −70°C	As above	2.62 ± 3.2 ($n = 5$)	+6.6	+9.6 to +3.7
		49.4 ± 2.8 ($n = 5$)	−2.0	+1.0 to −4.9

above conditions, the plasma concentrations of carumonam decreased significantly (ca. −8%) within five weeks, but the degradation was within acceptable limits. After 4.5 months, the degradation was no longer acceptable (ca. −20%). No decomposition was found, on the other hand, when plasma was diluted with phosphate buffer (2:1, v/v) to pH 6.0 and stored for 4.5 months at −20°C. When plasma was stored at −70°C, no significant decomposition of carumonam could be detected in either plasma (initial pH 7.4 or 6.5).

The degradation of carumonam at 37°C in plasma and plasma ultrafiltrates was pronounced and depended on the age of the plasma used (up to −20% in 3 h). Since protein-binding studies (dialysis and ultrafiltration) are carried out at this temperature, only fresh plasma was used. Control plasma samples were precessed and analysed with the samples from the protein-binding studies under nearly identical conditions. This allowed calculation of the protein binding, taking into account any degradation under these conditions [7].

More detailed investigations concerning the stability of carumonam in aqueous solutions confirmed the supposition of non-linear degradation kinetics: decomposition products reduce the pH of the solution, which results in reduced decomposition [36].

Recommendations for sample collection, storage and handling

Various procedures have been reported for the stabilization of β-lactams during storage in solution. Some workers acidify or buffer the specimens before freezing [15,21,31,37], while others add stabilizers such as ethyleneglycol [38], acetonitrile [39], sodium dodecyl sulphate [30,37], EDTA [22] or 2-(N-morpholino)ethanesulphonic acid [40]. Storage at −70°C is recommended by several authors [15,41–44] and repeated thawing and freezing should be avoided [29,45–47]. For analysis, precipitation of the proteins with acid or buffering of the sample before precipitation down to a pH below 6.5 has been recommended [31,48,49]. Amazingly, many reports mention neither any addition of stabilizers nor specific storage conditions.

However, many of these procedures, such as the addition of preservatives or buffers to the specimens and/or freezing immediately after collection until assay, are somewhat impractical and cumbersome. For these reasons, special citric acid-containing vacutainers [12] were used to adjust the blood pH to ca. 6.5 immediately when the sample was drawn. To avoid any subsequent increase in the pH of plasma, centrifugation at room temperature rather than at 4°C is recommended [50]. If these vacutainers are not available, 1% (v/v) 0.5 *M* citric acid should be added to the fresh plasma before freezing.

Urine samples should be adjusted with citric acid crystals to pH 5.0–6.0 fi necessary.

Multiple thawing and freezing must be avoided. Using the procedure recommended, degradation of carumonam can be minimized.

To prevent degradation during sample work-up and analysis, plasma was buffered as a preventive step in early method development [31]: one volume of 0.1 *M* phosphate buffer (pH 3.0) was added to two volumes of plasma to adjust the

plasma pH to ca. 6.0. Under these conditions the extract was found to be stable during analysis with the autosampler.

The degradation of carumonam, either during storage or sample work-up, could be monitored from the appearance of a peak in a chromatogram corresponding to the ring-opened product D.

Application of the assay to biological samples

This assay has been successfully applied to the analysis of samples from several pharmacokinetic studies, following intravenous, intramuscular and subcutaneous administration in humans, including a study involving patients with kidney failure [7,8].

With the additional clean-up step for the removal of lipids, and other non-polar substances and proteins, the lifetime of the column (system I) averaged ca. 1.5 months, or 1500 injections. Removing the material from the first few millimetres at the top of the column and exchanging the frits was generally sufficient to restore the initial resolution power and sensitivity.

Generally, no interferences were observed; however, the advantage of having a choice of more than one mobile phase is clear, since interferences encountered with one system can probably be avoided by using the other system.

Data obtained from the microbiological method [9] and the HPLC method agreed well: correlation coefficients of 0.977 and 0.985 were calculated for the plasma and urine assay, respectively.

CONCLUSION

A sensitive and selective HPLC method for the determination of the monocyclic β-lactam antibiotic carumonam is described. Citric acid-containing vacutainers have proved a simple means of preventing the degradation of alkaline-labile substances in biological fluids.

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