Journal of Chromatography, 430 (1988) 81–92
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4219

DETERMINATION OF CEFETAMET AND ITS ORALLY ACTIVE ESTER, CEFETAMET PIVOXYL, IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. WYSS* and F. BUCHELI

Pharmaceutical Research, Preclinical Development, Bau 68/121A, F. Hoffmann-La Roche & Co., Ltd., CH-4002 Basle (Switzerland)

(First received January 11th, 1988; revised manuscript received March 24th, 1988)

SUMMARY

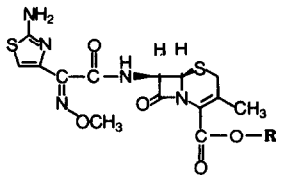
Two different, simple and rapid high-performance liquid chromatographic methods with ultraviolet detection, using a common sample work-up procedure, were developed for the determination of cefetamet, an in vitro active cephalosporin, and its orally absorbed pivaloyloxymethyl ester, cefetamet pivoxyl. After protein precipitation with perchloric acid, plasma samples were analysed on C_{18} reversed-phase columns with 4 m*M* perchloric acid–acetonitrile (83:17, v/v) and 0.1 *M* phosphate buffer (pH 6.5)–acetonitrile (60:40, v/v) as mobile phases for the determination of cefetamet and cefetamet pivoxyl, respectively. Urine samples were diluted with water and analysed in the same manner, using 4 m*M* perchloric acid–acetonitrile (85:15, v/v). The limits of quantification were 0.2, 0.5 and 20 μg/ml for the determination of cefetamet and cefetamet pivoxyl in plasma and cefetamet in urine, respectively. The intra-assay precision was ≤1.5% for cefetamet and ≤2.3% for cefetamet pivoxyl. The inter-assay precision for cefetamet was ≤2.4%. Cefetamet was stable in human plasma when stored at −20°C for three months or at 22°C for 24 h. For the determination of cefetamet pivoxyl, which was extremely unstable in plasma (>70% degradation in 1 h), samples were drawn into vacutainers containing citric acid and immediately added to sodium fluoride. The method for cefetamet was successfully applied to several thousand plasma and urine samples from humans, dogs and rats. No unchanged drug could be detected in human or dog plasma after the administration of cefetamet pivoxyl.

INTRODUCTION

Cefetamet (I, Fig. 1) is the main metabolite [1] and active form of the oral cephalosporin antibiotic cefetamet pivoxyl (II, Fig. 1). The latter, which is presently under clinical development, is a pivaloyloxymethyl ester of cefetamet and is inactive in vitro. However, following oral administration, the ester is rapidly absorbed and hydrolysed after first pass through the gut wall and/or the liver.

Cefetamet is active in vitro against a wide range of Gram-negative and Gram-

0378-4347/88/$03.50



I: Cefetamet R = H

II: Cefetamet pivoxyl R = $CH_2-O-C(=O)-C(CH_3)_2-CH_3$

Fig. 1. Chemical structures of cefetamet (I) and cefetamet pivoxyl (II).

positive aerobes and shows important advantages over existing oral β-lactams, such as amoxicillin, cephalexin and cefaclor, with respect to its activity against Gram-negative pathogens [2–4].

The original objective was to develop a single method for the simultaneous determination of both cefetamet and cefetamet pivoxyl. However, preliminary studies showed that this could not be achieved for biological samples with an isocratic method. Ion-pair reversed-phase high-performance liquid chromatography (HPLC) has been tried, creating an ion-pair between the acid and the ion-pairing reagent [5]. Although the injection of aqueous solutions produced acceptable chromatograms, analysis of plasma resulted in negative peaks. At that time, we observed that the ester is so unstable in plasma that the need to analyse this compound in biological samples would probably not arise. As a consequence, we decided to develop two different HPLC methods, without an ion-pairing technique, using a common sample work-up. This paper describes two simple and rapid HPLC methods for the determination of cefetamet and cefetamet pivoxyl in plasma, and another for the determination of cefetamet in urine.

EXPERIMENTAL

Materials and reagents

Methanol (puriss. p.a.) was obtained from Fluka (Buchs, Switzerland) and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Citric acid monohydrate, sodium fluoride, perchloric acid (70%), disodium hydrogenphosphate dihydrate and sodium dihydrogenphosphate monohydrate (all p.a.) were purchased from E. Merck (Darmstadt, F.R.G.). Water was distilled twice from an all-glass apparatus. Helium was obtained from PanGas (Lucerne, Switzerland). Compounds I and II were provided by F. Hoffmann-La Roche (Basle, Switzerland).

Plasma standards of cefetamet were prepared using fresh frozen plasma from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland). Plasma standards of cefetamet pivoxyl were prepared from citrated plasma, the pH of which was adjusted to 6.7 with 1 *M* citric acid (ca. 3.6 μl/ml), and to which 5 mg/ml sodium fluoride had been added.

Blood samples for the determination of cefetamet were drawn into vacutainers filled with sodium citrate or potassium oxalate–sodium fluoride. Blood samples (5 ml) for the determination of cefetamet pivoxyl were drawn into vacutainers containing 100 μl of 0.7 *M* citric acid (yielding a plasma pH of 6.7) and immediately transferred to a centrifuge tube containing 25 mg of sodium fluoride. In both cases the plasma was immediately separated by centrifugation.

Solutions and standards

Plasma standards of cefetamet. A stock solution was prepared by dissolving 52.76 mg of cefetamet (sodium salt, MW = 419.4) in 10 ml of bidistilled water (= 5 mg/ml free acid). Appropriate amounts of the stock solution were diluted with water to give solutions in the range 5–0.02 mg/ml. These solutions were used as plasma standards by diluting 0.1 ml with blank plasma to 10 ml, yielding concentrations of 50, 10, 2, 0.5 and 0.2 μg of cefetamet per ml of plasma. The plasma standards were stored at −20°C (for no more than six weeks). The aqueous solutions were freshly prepared prior to use.

Urine standards of cefetamet. The urine standards were prepared in the same manner as the plasma standards, starting with a stock solution of 105.52 mg of cefetamet in 10 ml of bidistilled water (= 10 mg/ml free acid), and aqueous dilutions in the range 10–0.4 mg/ml. The resulting urine standards were made by diluting 0.5 ml with urine to 10 ml, yielding concentrations of 500, 200, 100, 50 and 20 μg of cefetamet per ml of urine. The urine standards were stored at −20°C (for no more than six weeks). The aqueous solutions were freshly prepared prior to use.

Plasma standards of cefetamet pivoxyl. A 20-mg amount of cefetamet pivoxyl was dissolved in 10 ml of methanol to give the stock solution. Appropriate amounts of the stock solution were diluted with methanol to give solutions in the range 2–0.05 mg/ml, and 0.05 ml of these solutions were further diluted to 5 ml with citrated plasma at pH 6.7 containing sodium fluoride, yielding concentrations of 20, 10, 5, 2 and 0.5 μg/ml of plasma. The stock solution and the plasma standards were freshly prepared immediately before use.

Chromatographic system

A modular HPLC system was used, consisting of: a Kontron LC pump 410 with pulse damper (Kontron, Zurich, Switzerland); a Wisp 710 B automatic sample injector with limited volume inserts (Waters, Milford, MA, U.S.A.; run-time 15 min, needle purge before each injection) or a Rheodyne injector Model 7125 with 30-μl (laboratory-made) or 50-μl loop (Rheodyne, Cotati, CA, U.S.A.); a Spectroflow 773 UV detector (Kratos, Westwood, NJ, U.S.A.; wavelength 265 nm, rise time 1 s, range 0.02 a.u.f.s. for the analysis of cefetamet and 0.01 a.u.f.s. for the analysis of cefetamet pivoxyl); an integrator SP 4200 with Kerr minifile 4100 D (Spectra-Physics, San José, CA, U.S.A.) and a special BASIC program (an improved version to that described earlier for the integrator SP 4100 [6]; sensitivity 8 mV, chart speed 0.5 cm/min) or a recorder W + W Model 320 (Kontron; sensitivity 10 mV, chart speed 0.5 cm/min).

The analytical column, 125 mm × 4 mm I.D., Hibar type (E. Merck), was packed

in the laboratory. The mobile phase was degassed with helium prior to use and utilized without recycling. The flow-rate was 1.0 ml/min.

System I for cefetamet in plasma: Spherisorb ODS 1, 5 μm (Phase Separations, Queensferry, U.K.), as stationary phase, and 4 m*M* perchloric acid–acetonitrile (83:17, v/v) as mobile phase.

System II for cefetamet in urine: Spherisorb ODS 1, 5 μm, as stationary phase, and 4 m*M* perchloric acid–acetonitrile (85:15, v/v) as mobile phase.

System III for cefetamet pivoxyl in plasma: Nucleosil 5 C_{18} (Macherey-Nagel, Düren, F.R.G.) as stationary phase and 0.1 *M* phosphate buffer (pH 6.5)–acetonitrile (60:40, v/v) as mobile phase.

Sample preparation

Plasma. To 0.5 ml of plasma, 0.5 ml of 0.5 *M* perchloric acid was added and vortexed for 5 s. After 15 min standing at room temperature, the vial was centrifuged for 5 min at 1500 *g*. The clear supernatant was transferred to the automatic sample injector vial and 30 μl were injected into chromatographic system I for the analysis of cefetamet. If cefetamet pivoxyl had to be analysed simultaneously, the supernatant was divided, and 50 μl were injected into chromatographic system III for the determination of cefetamet pivoxyl.

Urine. Urine (0.5 ml) was diluted with bidistilled water to 25 ml. If a concentration of cefetamet greater than 500 μg/ml was expected, then a greater dilution was used. The samples were vortexed for 5 s, and 30 μl were injected into chromatographic system II.

Calculations

Together with the unknown and quality control samples, five plasma or urine standards, distributed over the whole set of samples, were processed as described above. The calibration curve ($y=a+bx$) was obtained by a weighted linear least-squares regression (weighting factor $1/y$) of the peak height y versus the concentration x. The calibration curve was used to interpolate unknown concentrations in the biological samples from measured peak heights. All data processing and calculations were carried out by the computing integrator.

RESULTS AND DISCUSSION

Sample preparation and chromatographic system

Polar cephalosporins cannot usually be extracted with organic solvents. In the case of cefetamet, the extraction would have been possible with ethyl acetate, but with a poor yield (ca. 50%). Thus, a simple protein precipitation was performed, which has the advantage that no evaporation step is necessary. Addition of perchloric acid to the plasma was optimal for protein precipitation and gave the clearest supernatant. The amount and concentration of perchloric acid had to be optimized to minimize the dilution factor.

On the other hand, cefetamet pivoxyl is relatively non-polar and can be easily extracted with most organic solvents, e.g. dichloromethane. This approach was used at first, but, because of the poor reproducibility and the need for as simple a

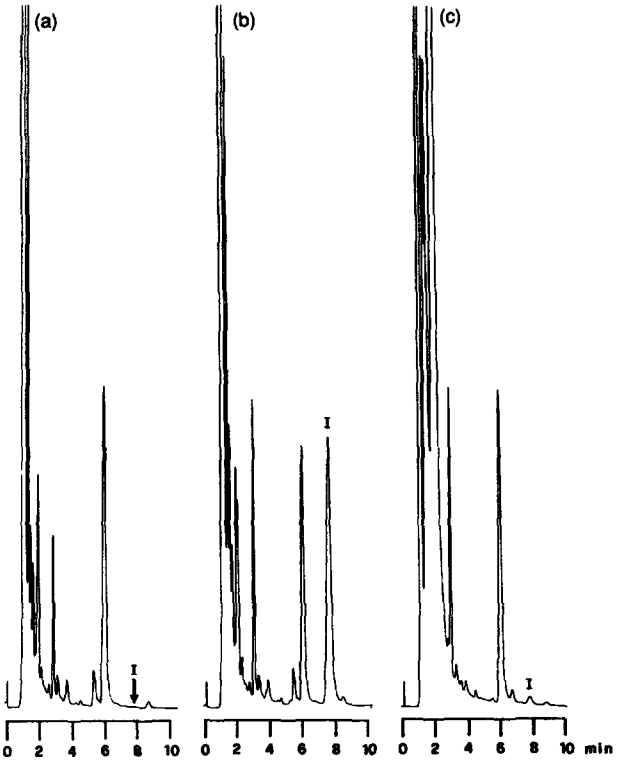


Fig. 2. Chromatograms of cefetamet (I) in human plasma. (a) Blank plasma sample, the arrow indicates the retention time of cefetamet. (b) Volunteer plasma sample, collected 2 h 45 min after a single oral dose of 1 g of cefetamet pivoxyl as a suspension in 100 ml of water; measured concentration, 5.91 μg/ml. (c) Blank plasma sample, spiked with 0.2 μg/ml.

work-up procedure as possible, was later abandoned in favour of the same sample preparation method as for cefetamet, in spite of the low recovery. In this way biological samples could be subjected to a common work-up procedure and then analysed on two different HPLC systems for the determination of the ester and the acid.

Several stationary phases have been tried for the determination of cefetamet in plasma. Hypersil ODS and LiChrosorb RP 18 were found to be generally unsuitable, whereas Spherisorb ODS 2 had only one disadvantage, namely too long retention times, and, therefore, broad peaks and poor sensitivity. An appropriate system proved to be a Nucleosil C_{18} column with 2–4 m*M* perchloric acid–acetonitrile (84:16, v/v) as mobile phase. These were the conditions that were validated and used to analyse human and dog plasma samples. This system was later abandoned, however, owing to an interfering peak in rat plasma, which could not be separated from cefetamet; in addition, small but disturbing interferences were observed in human plasma when new batches of the Nucleosil material were used. Nucleosil C_{18} could be adequately replaced by Spherisorb ODS 1 as stationary phase, with 4 m*M* perchloric acid–acetonitrile (83:17, v/v) as mobile phase. Perchloric acid was preferred to phosphate or citrate buffers in the mobile phase,

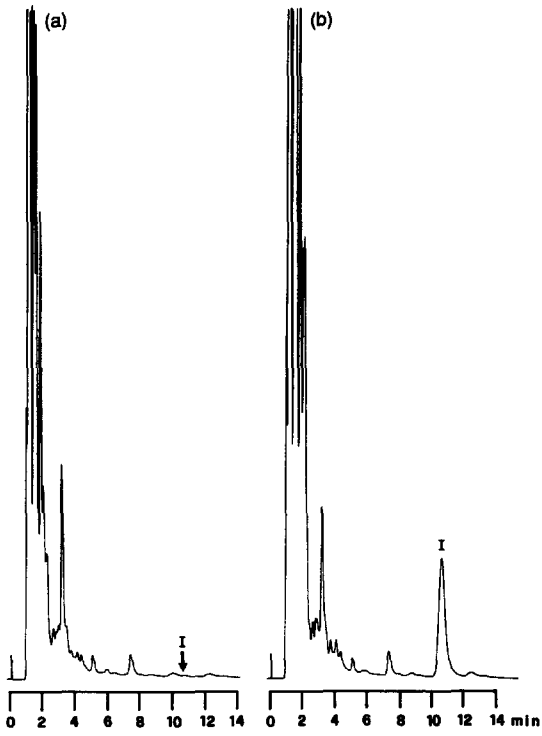


Fig. 3. Chromatograms of cefetamet (I) in human urine. (a) Blank urine sample from a volunteer. (b) Blank urine sample, spiked with 100 μg/ml.

because the injection solution already contained perchloric acid, and, therefore, no disturbance of the chromatographic system occurred. Minor modification of the mobile phase (2–4 m*M* perchloric acid, ± 1% of the acetonitrile content) was necessary, owing to alterations in the stationary phase after some days of analysis, or variability between new batches. Owing to the distinct sensitivity of the cefetamet k' value to this change of perchloric acid content, interfering peaks could always be separated easily.

The Spherisorb ODS 1 column with 4 m*M* perchloric acid–acetonitrile (85:15, v/v) as mobile phase was also used to analyse urine samples, which only needed to be diluted 1:50 with water before injection. If a cefetamet concentration of more than 500 μg/ml is expected, the urine samples should be diluted accordingly.

After protein precipitation with perchloric acid had been chosen for use in the determination of cefetamet pivoxyl, a chromatographic system had to be found that would not be disturbed when an acid injection solution was injected onto the column. Reproducible results were achieved with a mobile phase of phosphate buffer (pH 6.5)–acetonitrile (60:40, v/v), although some solvent tailing had to be tolerated. The assay for cefetamet pivoxyl is not optimal, and probably could be improved if necessary. However, since the ester has not yet been detected in biological samples, little time has been invested in such improvements.

No internal standards were used (i) because other cephalosporins investigated

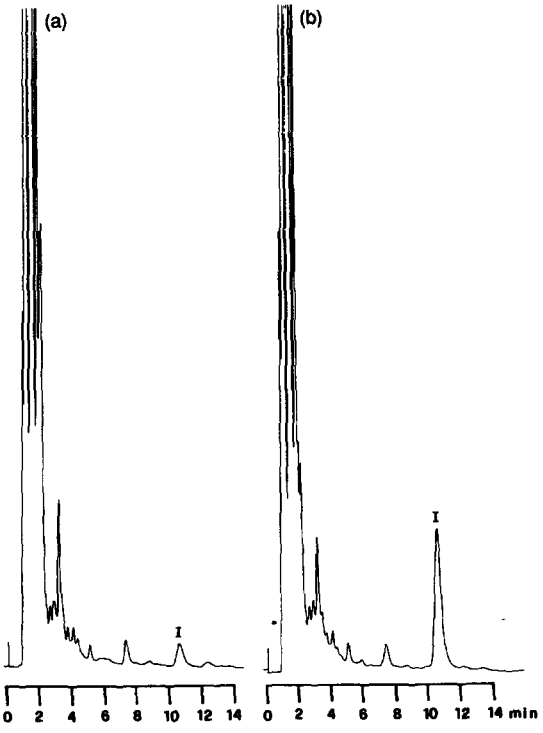


Fig. 4. Chromatograms of cefetamet (I) in human urine. (a) Blank urine sample, spiked with 20 μg/ml. (b) Volunteer urine sample, collected 10–12 h after a single oral dose of 1 g of cefetamet pivoxyl as a suspension in 100 ml of water; measured concentration, 240.6 μg/ml (dilution 1:100 instead 1:50).

for this purpose were less stable than cefetamet or cefetamet pivoxyl, (ii) because good precision was obtained with the external standard method and (iii) because of the need to keep sample handling as simple as possible.

The retention time of cefetamet in the chromatographic systems I and II was ca. 8 and 10 min, respectively; in system III, the retention time for cefetamet pivoxyl was 5.6 min. Typical chromatograms are shown in Figs. 2–5.

Limit of quantification

The limit of quantification of cefetamet was 0.2 μg/ml in plasma samples, using a 0.5-ml specimen, and 20 μg/ml in urine samples (diluted 1:50). The intra-assay ($n=5$) coefficients of variation (C.V.) at these concentrations were 6.2 and 2.3%, with deviations from the nominal values of −5.0 and 1.6% for plasma and urine, respectively. The limit of quantification of cefetamet pivoxyl in plasma samples was 0.5 μg/ml, using a 0.5-ml specimen. The intra-assay C.V. ($n=5$) at this concentration was 3.5%, with deviation from the nominal value of 4.0%. The limit of detection of cefetamet pivoxyl, defined by a signal-to-noise ratio of ca. 3:1, was 0.2–0.4 μg/ml.

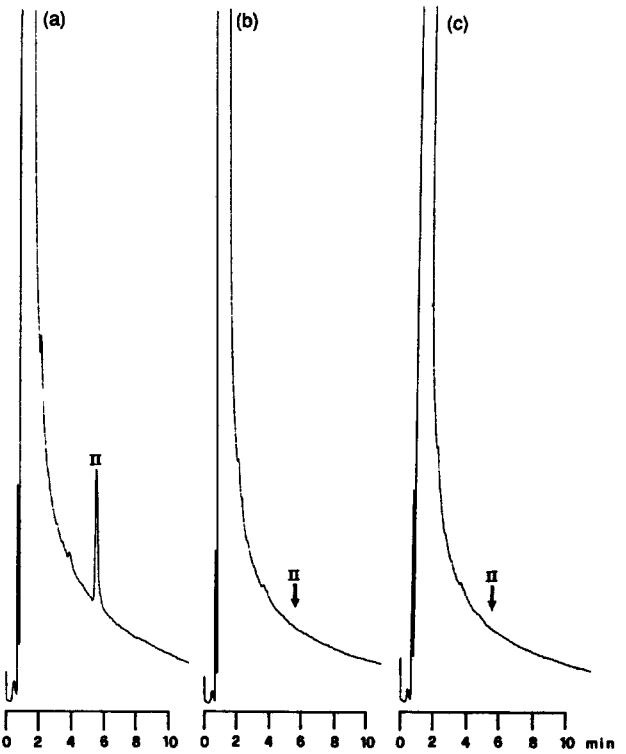


Fig. 5. Chromatograms of cefetamet pivoxyl (II) in human plasma. (a) Blank plasma, spiked with 2 μg/ml. (b) Pre-dose plasma sample from a volunteer; the arrow indicates the retention time of cefetamet pivoxyl. (c) Volunteer plasma sample, collected 0.5 h after a single oral dose of 1 g of cefetamet pivoxyl as a suspension in 100 ml of water.

Linearity

The correlation between peak height and concentration of cefetamet was linear in the range 0.2–50 μg/ml for plasma and 20–500 μg/ml for urine. A linear correlation between peak height and concentration of cefetamet pivoxyl in plasma was found from at least 0.5 to 20 μg/ml. The coefficients of determination (r^2) were better than 0.999, using the weighting factor $1/y$.

Recovery

The mean recovery of cefetamet from plasma, using an aqueous solution of the same concentration as 100% and analysing in quintuplicate at three different concentrations, was 87.4% (Table I). The mean recovery of cefetamet pivoxyl, established in the same way, was 37.9% (Table II). The reason for this low, but reproducible, value may be the poor solubility of the ester in the aqueous solution. Use of a 0.5 *M* perchloric acid solution in 40% methanol for protein precipitation, instead of an aqueous perchloric acid solution, increased the recovery of cefetamet pivoxyl to 60%. In a liquid–liquid extraction, using dichloromethane and Extrelut columns, the recovery was 89%. As previously mentioned, however, pro-

TABLE I

RECOVERY OF CEFETAMET FROM HUMAN PLASMA ($n=5$)

Concentration (μg/ml)	Recovery (%)	C.V. (%)
1	86.5	1.5
5	88.0	0.4
20	87.6	0.7

TABLE II

RECOVERY OF CEFETAMET PIVOXYL FROM HUMAN PLASMA ($n=5$)

Concentration (μg/ml)	Recovery (%)	C.V. (%)
0.5	37.7	2.6
2	38.4	1.9
10	37.6	1.1

tein precipitation with perchloric acid was preferred because of the rapid and simple handling.

Reproducibility

The precision (defined as the C.V. of replicate analyses) and the accuracy (defined as the deviation between found and added concentration) of the method for cefetamet were evaluated over the concentration range 1–20 μg/ml in plasma. The intra-assay reproducibility was determined by analysing five specimens of spiked plasma samples on the same day. The inter-assay reproducibility was obtained by analysing one specimen of a spiked plasma sample on five days over a period of two weeks. The intra-assay reproducibility for cefetamet pivoxyl was determined over the concentration range 0.5–10 μg/ml in plasma. The results are compiled in Table III.

Stability

Cefetamet was added to human blank plasma at three different concentrations and stored for three months at −20°C and for 24 h at 22°C.

A set of five freshly prepared blank plasma samples was analysed* together with five stored samples at the same concentration. The results of these stability tests, which were carried out according to our established method [7], are presented in Table IV. The data indicate that cefetamet is stable in human plasma. This substance is also stable in the acidic injection solution, since no change in cefetamet peak heights was observed when such a solution was reinjected 22 h

*Chromatographic system: stationary phase, Nucleosil 5 C_{18}; mobile phase, 4 m*M* perchloric acid–acetonitrile (84:16, v/v); injection volume, 20 μl.

TABLE III

REPRODUCIBILITY OF THE ASSAYS IN PLASMA ($n=5$)

	Concentration (μg/ml)		C.V. (%)	Difference between found and added (%)
	Added	Found		
Cefetamet,	1	1.00	1.5	±0
intra-assay	5	5.07	0.4	+1.4
	20	20.15	0.7	+0.7
Cefetamet,	1	1.00	2.4	±0
inter-assay	5	4.91	1.6	−1.8
	20	20.20	1.4	+1.0
Cefetamet pivoxyl,	0.5	0.52	2.3	+4.0
intra-assay	2	2.03	1.0	+1.5
	10	9.95	0.4	−0.5

TABLE IV

STABILITY OF CEFETAMET IN PLASMA ($n=5$)

Sample	Concentration (μg/ml)	Peak height ± C.V. (%)	Change of concentration after storage (%)	90% confidence interval (%)
Freshly prepared	2	1776 ± 1.0		
24 h at 22°C	2	1756 ± 1.1	−1.1	−2.3 to +0.1
3 months at −20°C	2	1648 ± 0.9	−7.2	−8.2 to −6.1
Freshly prepared	20	16667 ± 0.8		
24 h at 22°C	20	16561 ± 0.5	−0.6	−1.4 to +0.1
3 months at −20°C	20	16136 ± 0.8	−3.2	−4.1 to −2.3
Freshly prepared	100	83213 ± 0.6		
24 h at 22°C	100	82425 ± 0.8	−0.9	−1.8 to −0.1
3 months at −20°C	100	77769 ± 0.9	−6.5	−7.4 to −5.7

after standing at room temperature. This is in direct contrast to some other cephalosporins [8,9] or monocyclic β-lactams [10].

On the other hand, cefetamet pivoxyl is so unstable in spiked plasma under normal conditions, that it was difficult to effect any recovery at all. Detailed investigations showed the dependence of the stability on the plasma pH and on the addition of an esterase inhibitor (see Fig. 6). A plasma pH of 6.7 was chosen instead of pH 5.0 for the final conditions since this gave a better yield of plasma after centrifugation of blood. The addition of 5 mg of sodium fluoride per ml of plasma improved the stability of cefetamet pivoxyl, allowing its determination several hours after spiking the plasma. After the protein precipitation, cefetamet pivoxyl was stable in the injection solution during a 20-h period.

Application of the methods to biological samples

The method for cefetamet was successfully applied to the analysis of several thousand plasma and urine samples from humans, dogs and rats in several phar-

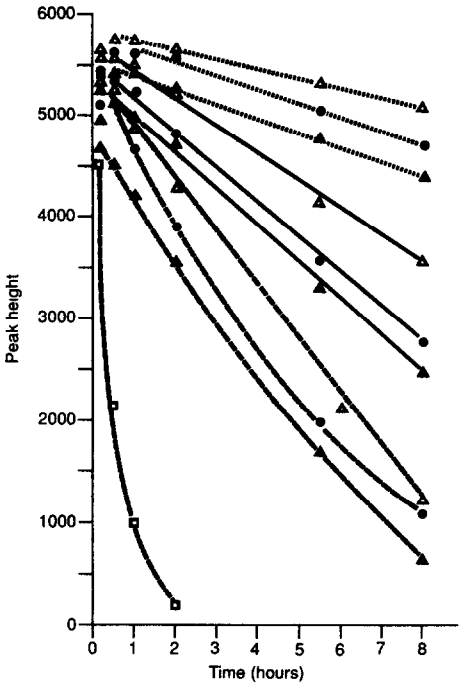


Fig. 6. Stability of cefetamet pivoxyl in human plasma at different pH values. (····) pH 5.0; (——) pH 6.7; (- - -) pH 7.4; (△) citrated plasma with 2.5 mg/ml sodium fluoride; (●) potassium oxalate-sodium fluoride plasma with an additional 2.5 mg/ml sodium fluoride; (▲) potassium oxalate-sodium fluoride plasma; (□) citrated plasma.

macokinetic studies [11]. Following oral administration of cefetamet pivoxyl, plasma samples from humans (see Fig. 5) and dogs [12] were also analysed for the unchanged compound. No cefetamet pivoxyl could be detected, which is consistent with the rapid and quantitative hydrolysis of the ester to the microbiologically active acid cefetamet.

CONCLUSION

Two different, simple and rapid methods, using a common sample work-up, were developed for the determination of cefetamet and cefetamet pivoxyl in biological fluids. Since cefetamet pivoxyl could not be found in any biological sample, this approach proved to be successful and at the present state-of-the-art, superior to a more complicated method for both substances analysed in the same run.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. H.-J. Egger for preliminary investigations on ion-pair chromatography, Dr. K. Stoeckel and Dr. E. Eschenhof for supplying biological samples, Dr. P. Hohl for helpful advice, Mr. H. Suter for the drawings and Dr. D. Dell for correcting and Mrs. Th. Itin for typing the manuscript.

REFERENCES

1 E. Eschenhof, W. Vetter and W. Arnold, data on file, F. Hoffmann-La Roche & Co. Ltd., Basle.
2 R. Wise, J.M. Andrews and L.J.V. Piddock, Antimicrob. Agents Chemother., 29 (1986) 1067.
3 H.C. Neu, N.X. Chin and P. Labthavikul, Antimicrob. Agents Chemother., 30 (1986) 423.
4 R.N. Jones, P.C. Fuchs, A.L. Barry, L.W. Ayers, E.H. Gerlach and T.L. Gavan, Antimicrob. Agents Chemother., 30 (1986) 961.
5 H.-J. Egger and G. Fischer, J. Chromatogr., submitted for publication.
6 U. Timm, D. Dell and D.A. Ord, Instrum. Comput., 3 (1985) 29.
7 U. Timm, M. Wall and D. Dell, J. Pharm. Sci., 74 (1985) 972.
8 K.-H. Trautmann and P. Haefelfinger, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 54.
9 R. Wyss, unpublished results.
10 H.-J. Egger and G. Fischer, J. Chromatogr., 420 (1987) 357.
11 J.-R. Koup, U.C. Dubach, R. Brandt, R. Wyss and K. Stoeckel, Antimicrob. Agents Chemother., 32 (1988) 573.
12 E. Eschenhof and R. Wyss, unpublished results.