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High-performance liquid chromatographic determination of loracarbef, a potential metabolite, cefaclor and cephalexin in human plasma, serum and urine

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method is reported for the determination of a new carbacephem antibiotic, loracarbef, a hydroxylated analogue, and two cephalosporins, cefaclor and cephalexin, in plasma, serum, and urine. The antibiotics are extracted from plasma by means of C_{18} solid-phase cartridges. Urine samples are diluted with water and directly injected on the HPLC system. The HPLC system utilizes a Supelcosil LC-18-DB (250 mm × 4.6 mm I.D.) reversed-phase column and ultraviolet detection at 265 nm. The limit of quantitation is 0.5 μg/ml for each compound. Excellent correlation of plasma concentrations is shown between results determined by HPLC and those obtained by microbiological agar-well diffusion assays. Stability studies of loracarbef in human plasma show the antibiotic to be stable for at least 24 h at room temperature, and for at least twelve months at −20°C.

INTRODUCTION

Loracarbef (I, Lorbid®) represents the new class of synthetic, orally administered carbacephem antibiotics. The carbacephems are similar in structure and activity to the cephalosporins. Loracarbef differs structurally from cefaclor only by the substitution of a carbon atom for the sulfur atom in the 1-position of the dihydrothiazine ring (Fig. 1). The *in vitro* activity of loracarbef is similar to that of cefaclor, with good activity against staphylococci, streptococci, *Haemophilus influenzae, Branhamella catarrhalis, Escherichia coli, Klesiella sp.*, and *Proteus mirabilis*. Methicillin-resistant staphylococci, enterococci, *Enterobacter, Pseudomonas,* and *Seratia* are resistant [1–4]. The antibacterial action of loracarbef results from the interaction of the drug with penicillin-binding proteins of the organism, with subsequent interruption of cell-wall synthesis.

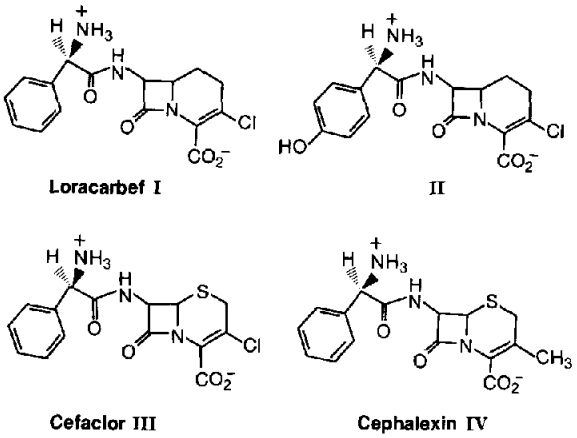

Fig. 1. Structures of loracarbef (I), the *p*-hydroxy analogue of loracarbef (II), cefaclor (III), and cephalexin (IV).

We report the development of a rapid and rugged high-performance liquid chromatographic (HPLC) assay for two new carbacephems and two commonly administered cephalosporins. The assay permits the simultaneous determination of these antibiotics in plasma, serum, and urine without interference from endogenous substances or commonly co-administered drugs, such as acetaminophen and caffeine. The assay may be adapted to the analysis of other carbacephems or cephalosporins. In addition, data are reported on the stability of the antibiotics in plasma as a function of storage parameters.

EXPERIMENTAL

Chromatography

The chromatography system consisted of a Beckman 112 pump (Beckman Instruments, Irvine, CA, U.S.A.), a Waters WISP Model 710B autoinjector (Waters Assoc., Millipore, Milford, MA, U.S.A.), an ABI Model 783 programmable absorbance detector (Applied Biosystems, Foster City, CA, U.S.A.), and a BAS Model LC-23A column heater (Bioanalytical Systems, West Lafayette, IN, U.S.A.). A 250 mm × 4.6 mm I.D. Supelcosil LC-18-DB column with 5-μm packing (Supelco, Rohm and Haas, Bellefonte, PA, U.S.A.) was used with a 2-μm in-line filter (Upchurch Scientific, Oak Harbor, WA, U.S.A.). A 150 mm × 4.6 mm I.D. Supelcosil LC-18-DB column with 5-μm packing was used for the analysis of urine samples to reduce the separation time; this may be substituted for the 25-cm column without any loss of resolution.

Solid-phase extraction apparatus

Solid-phase extraction was performed on Sep-Pak® C_{18} (No. 51910, Waters

Assoc.), Bond-Elut® C_{18}/OH (low hydrocarbon, No. 1210-2020, Analytichem International, Harbor City, CA, U.S.A.) and Bond-Elut® C_{18} (No. 1210-2001) cartridges. The extractions were performed with the aid of a vacuum manifold box (Vac-Elut®, Analytichem International) at a manifold vacuum of 10–15 mmHg.

Chemicals and reagents

Loracarbef {I, 7-[D-(aminophenylacetyl)amino]-3-chloro-8-oxo-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid}, the *p*-hydroxy analogue of loracarbef {II, 7-[D-(amino-(4-hydroxyphenyl)acetyl)amino]-3-chloro-8-oxo-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid}, cefaclor (III), and cephalexin (IV) were obtained from Lilly Research Laboratories (Eli Lilly and Company, Indianapolis, IN, U.S.A.). Methanol and tetrahydrofuran (THF) were HPLC-grade solvents (Burdick and Jackson, Division of Baxter Healthcare, Muskegon, MI, U.S.A.). All other reagents were analytical grade and used without further purification. The ion-pairing reagent, 1-heptanesulfonic acid, sodium salt, was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Purified water (Milli-Q System, Millipore) was used in all aqueous solutions. Fresh human plasma, serum, and urine, shown to be free of interferences, were obtained from healthy volunteers.

Mobile phase

The mobile phase consisted of methanol, THF, and an aqueous component at a proportion of 16:4:80, respectively. The aqueous component was prepared by addition of 1 g of 1-heptanesulfonic acid, sodium salt, and 15 ml of triethylamine to 1000 ml of purified water. The solution was adjusted to pH 2.3 with concentrated phosphoric acid. The aqueous component was filtered through a 0.2-μm Nylon-66 Millipore® filter and degassed before the addition of methanol and THF. The pH of the aqueous phase is critical to the proper resolution of the compounds. The HPLC system was operated at a flow-rate of 1.4 ml/min at a column temperature of 30°C. A total chromatography time of 15 min is required per sample.

Preparation of standard solutions

A standard stock solution containing loracarbef, II, and cefaclor was prepared in purified water in the same volumetric flask. Plasma and serum standards were prepared to contain 1, 2, 5, 10, 20, or 30 μg/ml of each antibiotic compound. The internal standard was prepared by dissolution of 1 mg of cephalexin in 10 ml of water. The method may be adapted for the determination of cephalexin by the use of an alternative internal standard, such as another cephalosporin.

A standard stock solution for the analysis of urine samples was prepared with blank urine. The urine standards were in the same concentration range as above, except that dilutions to the final concentrations were made with purified water. An internal standard was not used for the analysis of urine samples since they were analyzed without extraction.

Preparation of samples

Bond-Elut C_{18}, Bond-Elut C_{18}/OH and Sep-Pak C_{18} solid-phase extraction cartridges were tested. Each extraction cartridge was pre-equilibrated by sequential aspiration of 2 ml of methanol and 2 ml of purified water on a vacuum manifold box. A small amount of purified water was left in the barrel of the cartridge to prevent formation of air voids in the cartridge prior to sample application. Human plasma or serum samples (500 μl) were transferred to small test tubes, 100 μl of internal standard were added, and then the samples were acidified by addition of 50 μl of 25% acetic acid solution. Samples were mixed and transferred to the extraction catridge. The mixture was aspirated onto the cartridge and washed with two aliquots of 1 ml purified water. Compounds were eluted from the cartridge with 3 ml of methanol and collected in small tubes. The eluates were evaporated under nitrogen, and the residues were reconstituted by addition of 200 μl of mobile phase to each collection tube followed by vortexing. Extracts were transferred to autosampler vials and 25 μl of each were injected on the HPLC system.

Urine samples were prepared by dilution with purified water and injection of 50 μl onto the HPLC system. The dilution of the urine samples was dependent on the dose and time after drug administration. A typical dilution for samples from a 70 kg male human who was administered 200 mg loracarbef in a capsule may be in the range of 200:1 for peak times and 100:1 for later times after administration.

Determination of recovery, precision and accuracy

The extraction efficiency of the solid-phase sample preparation procedure was tested by comparing the recovery of pooled plasma samples spiked with the drugs relative to standards of absolute concentrations. The absolute standards were prepared to account for any dilution or concentration effects in the sample preparation procedure.

The precision and accuracy of the method were determined by performing replicate analyses of five pools of plasma spiked with known concentrations of the compounds. The concentrations were selected to span the range of the standard curve and to include the limit of quantification. The results were evaluated for variance and for accuracy of the determination. The limit of quantification was chosen to be the smallest quantity that could be determined within acceptable limits of variance (R.S.D. $\leq$ 15%).

Samples for the determination of the stability of the carbacephems and cephems were prepared in pooled, control human plasma that was shown to be free of endogenous interferences. Known amounts of antibiotic were spiked into each pool and the pools were stored for given periods at the appropriate temperature.

Microbiological assay procedure

Microbiological agar-well diffusion assays were performed as outlined below according to accepted guidelines [5], using *Micrococcus luteas* (ATCC 9341) as

the test organism. Plasma samples were placed in wells on agar slants inoculated with a 0.1% standardized suspension of the microorganism. The plates were incubated at 30°C for 16–18 h. After incubation, the diameters of the zones of inhibition were measured with the Lilly-Fisher zone reader. All standards were prepared in matrices similar to that of the test samples. The standard curve was constructed in a semi-logarithmic manner with diameter on the linear axis. The concentration of the antibiotic was plotted *versus* the relative inhibition of growth of the organism on the agar slants. The concentration of antibiotic in test samples was calculated against the standard curve and corrected for dilution and for the relative response of standards on each slant.

RESULTS AND DISCUSSION

Chromatography

A chromatogram of a plasma extract shows good resolution between the four compounds and no interference from endogenous substances (Fig. 2). Chromatograms of plasma or serum extracts did not differ, and those of diluted urine samples are similar.

Reproducible separations were attained under the given mobile phase conditions on columns from several different lots of packing material. It was noted during development of the assay that proper adjustment of the mobile phase pH was necessary to obtain the desired separation. This is possibly related to the pK_a values of the carbacephem and cephem compounds. The lower pK_a of these compounds is approximately 2.2. Slight changes towards more basic pH increased their retention time, which may occur due to the interaction between the compounds' carboxylate functionality and triethylamine in the mobile phase.

The separation was examined for interference from drugs which could be coadministered with the antibiotics. Acetaminophen, which eluted at approximate-

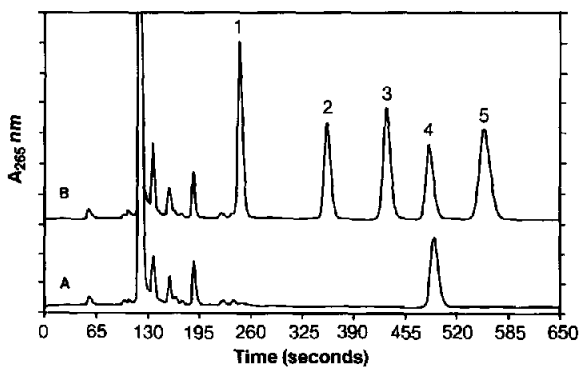

Fig. 2. Chromatograms of human plasma blank (A) and 9.5 μg/ml standards in plasma (B). Peaks: 1 = II; 2 = cefaclor; 3 = loracarbef; 4 = plasma constituent; 5 = cephalexin (internal standard).

ly 185 s (0.42 relative to loracarbef), and caffeine (0.34 relative to loracarbef) did not interfere.

Extraction recovery

Three types of solid-phase extraction cartridges were evaluated for the extraction of the carbacephem and cephem antibiotics from plasma. Bond-Elut C_{18}/OH and Sep-Pak C_{18} cartridges provided the highest absolute recoveries (Table I), with essentially complete recovery of loracarbef from plasma. The Bond-Elut C_{18}/OH cartridge provided significantly better recovery of II than did the Bond-Elut C_{18}. This is likely due to the lower carbon loading of the C_{18}/OH material which exposes silyl hydroxyls to hydrogen bonding with II. Cefaclor had the lowest extraction efficiencies with an absolute recovery of approximately 80% on all three types of stationary phase. The lower recovery of cefaclor is apparently related to the dihydrothiazine ring of the cephem structure. Consistent recovery of the internal standard is obtained on all of the cartridges. There was minimal retention of endogenous substances on any of the cartridges. This extraction method avoids the use of strong solvent conditions that are commonly associated with methods that precipitate plasma proteins. These harsh conditions can degrade the chromatographic separation and shorten column lifetime.

Precision and accuracy

The analysis of the carbacephem and cephem antibiotics was evaluated for precision and accuracy by replicate determinations of plasma pools spiked with the drugs at various concentrations. Overall, the precision and accuracy of determination were very good on all C_{18} materials with a relative error of approximately 5% and relative standard deviations typically less than 5% (Table II). At concentrations of the drugs near the quantification limit of 0.5 μg/ml, precision and accuracy approach the maximum acceptance limit of 15%.

The data indicate that the C_{18} cartridges provide consistent and accurate determination of loracarbef, II, and cefaclor. Despite the lower recovery of the

TABLE I

ABSOLUTE RECOVERY OF CARBACEPHEM AND CEPHEM ANTIBIOTICS FROM HUMAN PLASMA BY SOLID-PHASE EXTRACTION

Compound	Recovery (%)		
	Bond-Elut C_{18}/OH	Sep-Pak C_{18}	Bond-Elut C_{18}
Loracarbef	100	99	95
II	100	84	<25
Cefaclor	79	78	82
Cephalexin	93	100	93

TABLE II

SUMMARY OF PRECISION AND ACCURACY DATA OF HPLC ASSAY FOR CARBACEPHEM AND CEPHEM ANTIBIOTICS IN HUMAN PLASMA

Number of replicate samples was 5 in all cases; R.S.D. = relative standard deviation of replicate determinations; relative error = percentage difference from nominal concentration.

Nominal concentration (μg/ml)	Bond-Elut C_{18}/OH			Sep-Pak C_{18}			Bond-Elut C_{18}		
	Mean (μg/ml)	R.S.D. (%)	Relative error (%)	Mean (μg/ml)	R.S.D. (%)	Relative error (%)	Mean (μg/ml)	R.S.D. (%)	Relative error (%)
Loracarbef									
0.5	0.6	4.3	13.9	0.5	3.0	3.1	0.4	14.7	−12.1
5.0	4.8	5.8	− 3.6	5.1	2.3	1.5	5.0	4.7	− 0.6
20.0	19.0	2.9	− 5.0	19.8	1.0	− 1.2	19.8	3.3	− 0.8
30.0	28.0	1.8	− 6.7	29.8	3.4	− 0.5	29.4	4.2	− 1.9
Compound II									
0.5	0.6	11.8	22.4	0.6	7.6	17.0	0.6	17.3	21.7
5.0	4.9	4.7	− 1.9	5.1	5.0	1.9	5.1	5.8	1.7
20.0	19.5	3.0	− 2.4	19.9	0.9	− 0.8	21.3	9.0	6.6
30.0	28.7	2.9	− 4.5	29.7	6.7	− 0.9	33.4	8.7	11.3
Cefaclor									
0.5	0.7	3.2	36.0	0.5	3.4	7.3	0.5	11.2	− 1.2
5.0	4.8	5.4	− 4.3	4.9	2.2	− 1.9	5.0	3.1	0.9
20.0	18.8	3.6	− 6.2	19.5	1.1	− 2.5	20.0	2.5	− 0.1
30.0	28.1	1.9	− 6.5	29.7	3.3	− 0.9	29.9	3.0	0.2

more polar analogue, good precision and accuracy can be obtained. The C_{18}/OH material appears more applicable for extraction of low concentrations of more polar species, such as II. This material produced the highest overall recovery of all of the drugs of interest, but more overall variance was observed.

Stability

The stability of loracarbef, II, and cefaclor was determined in human plasma at room temperature (25°C) and at −20°C. Data at both temperatures indicate that loracarbef and II are stable. Cefaclor shows significant degradation under both conditions; however, the stability of cefaclor can be improved significantly by acidification of the plasma prior to storage.

Storage at room temperature showed loracarbef and II to be stable for at least 24 h. Cefaclor showed a loss of approximately 30% of the parent compound after 8 h at room temperature and an average loss of more than 50% at 24 h (Fig. 3). Acidification of the samples to pH < 4.5 with concentrated acetic acid significantly improved the stability of cefaclor at room temperature, with no effects on the stability of loracarbef or II.

Loracarbef and II, when stored in plasma at −20°C, were stable for at least twelve months (Fig. 4). Cefaclor showed a loss of more than 5% at one month, and approximately 55% at one year. Acidification of the plasma retards the degradation of cefaclor in frozen plasma for at least four months.

These studies indicate that plasma samples that contain loracarbef and its analogues may be handled with normal laboratory precautions without bias to the determination of the drug. These results agree well with stability studies performed on loracarbef and cefaclor in a variety of media by microbiological assay [4]. Plasma that contains cefaclor must be handled with precaution to minimize degradation of the antibiotic. Typical treatment in this laboratory is acidification of the biological fluid to pH ≤ 4.5 with acetic acid and storage at −20°C as rapidly as possible.

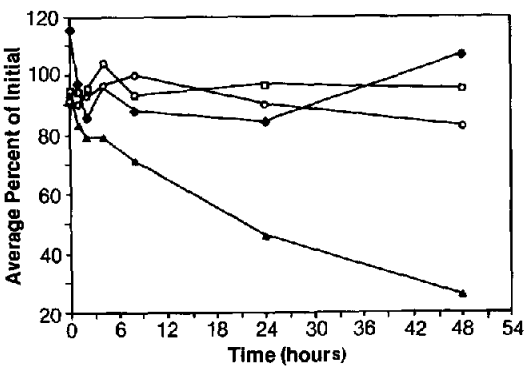

Fig. 3. Stability of carbacephem and cephem antibiotics in human plasma at room temperature. Key: (□) loracarbef; (◆) II; (▲) cefaclor; (○) cefaclor with acetic acid to pH < 4.5.

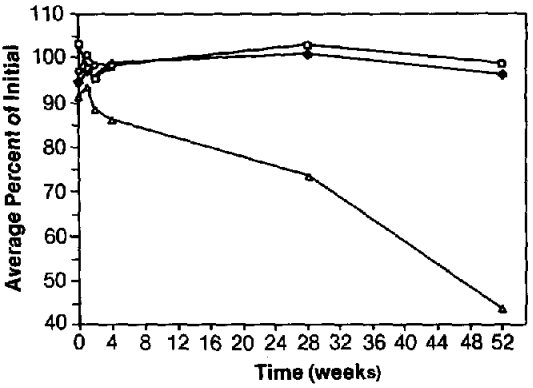

Fig. 4. Stability of carbacephem and cephem antibiotics in human plasma stored at −20°C. Key: (□) loracarbef; (▲) II; (△) cefaclor; (○) cefaclor with acetic acid to pH<4.5.

Comparison of HPLC versus microbiological assay

Eight adult, male volunteers were administered a 200-mg capsule of loracarbef; plasma samples were obtained at predetermined times up to 12 h. The samples were assayed by the HPLC method described herein and compared to microbiological agar-well diffusion assay. Compound II, which is a possible metabolite of loracarbef, was not observed in any of the samples analyzed by the HPLC method (Fig. 5). The mean peak plasma concentration by HPLC from this study was 7.4 μg/ml loracarbef at 60 min after administration (Fig. 6). Previous studies have reported a half-life of 1.1 h and linear pharmacokinetics up to 500 mg administered [6,7]. The predominant route of excretion was via the kidney, with approximately 90% of the drug recovered unchanged in the urine, within 24 h of

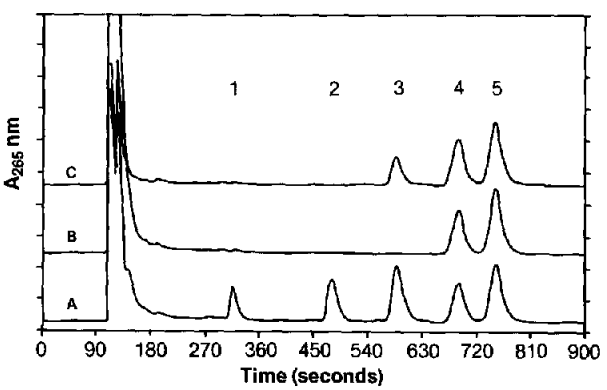

Fig. 5. Chromatograms from a patient administered 200 mg of loracarbef. (A) Human plasma standard (9.5 μg/ml); (B) patient sample at 0 min; (C) patient sample at 45 min. Peaks: 1 = II; 2 = cefaclor; 3 = loracarbef; 4 = plasma constituent; 5 = cephalexin.

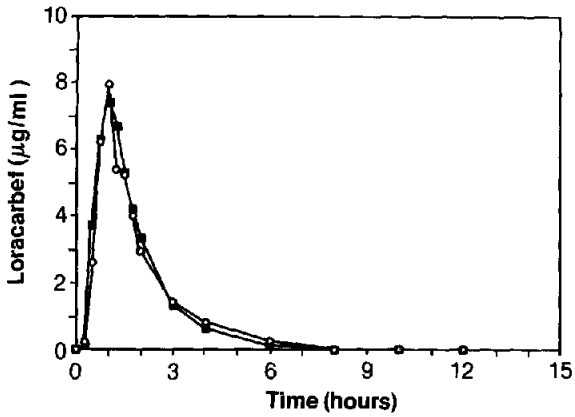

Fig. 6. Profile of the average plasma concentrations of loracarbef in eight normal male volunteers determined by HPLC (■) and microbiological assay (○).

administration [6]. The pharmacokinetics of loracarbef has also been studied in infants and children [8].

The concentration of loracarbef as determined by HPLC assay shows an excellent correlation to the microbiological determination, with an inter-assay slope that is essentially unity (Fig. 7). The y-intercept shows a deviation of 0.2 μg/ml which is within the error of experimental measurement.

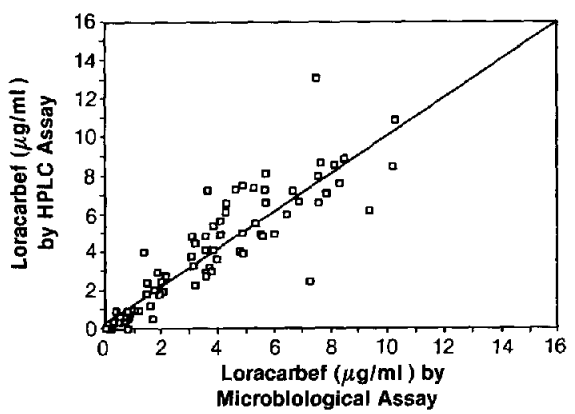

Fig. 7. Correlation of HPLC and microbiological determination of loracarbef in human plasma. Slope, 0.9988; y-intercept, 0.211; correlation coefficient, 0.9072.

CONCLUSIONS

The procedures reported here provide a rapid and convenient method for the determination of carbacephem and cephem antibiotics in physiological fluids. The HPLC method permits simultaneous determination of these compounds and, possibly, metabolites in a single assay. Data reported herein show that the carbacephem compounds have significantly better stability in plasma than the cephems, so that sample handling variations should cause few problems for the carbacephems. This permits greater flexibility in the assay of samples. Very good correlation is obtained between the HPLC method and a common microbiological assay for antibiotic activity. The HPLC method has the advantages of rapid sample throughput and antibiotic specificity in the sample. The procedure reported in this paper has been successfully employed by several different laboratories, indicating its ruggedness.

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