Determination of Bacmecillinam, an Amdinocillin Prodrug, in Human and Canine Whole Blood by Reversed-Phase Liquid Chromatography

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Abstract □ Bacmecillinam is an amdinocillin prodrug designed to be easily hydrolyzed in biological materials, so special procedures were developed for the collection of blood specimens. Whole blood was collected in tubes containing bacampicillin as an adsorption inhibitor and kept at −70°; the extracting solvent, hexane–methylene chloride (9:1, v/v), was added to the cold tubes, and the extraction was performed during the thawing of the samples. The organic phase was partially evaporated before a reextraction to a small volume of acidic aqueous phase was made. The separation was performed on a microparticulate C_{18}-alkyl bonded silica packed in glass-lined stainless steel columns. Mobile phase was a buffer (pH 6)–acetonitrile mixture containing *N*-hexyl-*N*-methylamine as an adsorption inhibitor. The detection limit was 600 pg/ml of whole blood, and the within-run precision ($s_{rel\%}$) was ~8% at the 5-ng/ml level.

Keyphrases □ Bacmecillinam—determination in biological fluids by reversed-phase liquid chromatography □ Biological fluids—determination of bacmecillinam by reversed-phase liquid chromatography □ Reversed-phase liquid chromatography—determination of bacmecillinam in biological fluids

Amdinocillin, a 6β-amidinopenicillanic acid, is a relatively new antibiotic agent for which a synergistic effect *in vitro* and *in vivo* has been demonstrated, in combination with β-lactam antibiotics, against many strains of Gram-negative bacteria. Since amdinocillin is poorly absorbed after oral administration, prodrugs such as amdinocillin pivoxil and bacmecillinam have been developed in order to enhance the oral drug absorption efficiency. Bacmecillinam, the 1′-ethoxycarbonyloxyethyl ester, is rapidly hydrolyzed by endogenous esterases in blood and tissues releasing ethanol, acetaldehyde, and carbon dioxide. Recently (1), the superior pharmacokinetic properties of bacmecillinam compared with amdinocillin pivoxil were demonstrated in studies on healthy volunteers, resulting in statistically significant differences regarding areas under the curve, urinary excretion, and peak plasma concentrations.

During the evaluation of the toxicological and clinical properties of the drug, a need for the determination of the intact prodrug, bacmecillinam, arose. This report presents such a method for the quantitative determination of the compound in whole blood and bile. Since the compound is very unstable in the presence of biological fluids, special precautions in sample handling had to be developed. At the very low concentration levels encountered, severe adsorption tendencies of the compound had to be overcome by careful handling of the sample. An extraction procedure comprising a base extraction and a reextraction to an acidic aqueous phase precedes the chromatographic step which relies on reversed-phase liquid chromatography and UV detection. Amdinocillin pivoxil was utilized as the internal standard.

EXPERIMENTAL

Apparatus—Chemicals were weighed on analytical[1] or microanalytical[2] balances; a shaker[3] was used for extraction, speed control 6.5; and the extraction tubes were centrifuged at 300×g[4] (Step 1 in the extraction) or at 650×g[5] (Step 2). The equipment for liquid chromatography was comprised of a dual reciprocating piston pump[6], an injection valve[7] equipped with a 20- or 200-μl loop, an injection syringe[8] with a total volume of 1 ml, a variable UV detector[9] monitored at 230 nm, and a recorder[10] with an input of 10 mV and a paperspeed of 40 cm/hr.

Chemicals—Bacampicillin[11], bacmecillinam[11], and amdinocillin pivoxil[12] were from released batches. *N*-Hexyl-*N*-methylamine[13] of pure quality, phosphoric acid (85%)[14] of analytical quality, and acetonitrile[13] of HPLC quality were used for preparation of the mobile phases. Methylene chloride[13] of HPLC quality and *n*-hexane[14] of spectroscopic quality were used in the extractions. The water was deionized.

Standard Solutions—Solution S consisted of 5 mg of bacmecillinam/250 ml of water in a polypropylene flask[15]; Solution I: amdinocillin pivoxil, internal standard, 1.25 mg/250 ml of water in a polypropylene flask; Solution A: bacampicillin, adsorption inhibitor, 100 mg/100 ml of water in a polypropylene flask. Solutions S, I, and A were divided into 1-ml portions and stored at −70° in tubes[16]. Each day of analysis, appropriate amounts were thawed, and out of Solution S, a series of seven twofold dilutions (S_1–S_7), containing 10–0.156 μg/ml, were made in tubes[16].

Solution IA was made by mixing 1.0 ml of I and 0.5 ml of A in a tube[16] and Solution ID was made by mixing 1.0 ml of I and 0.5 ml of water in a tube[16]. Solvent for extraction (Solvent E): methylene chloride (50.0 ml) was added by pipet into a 500-ml flask and *n*-hexane was added to the mark. Solution S_4 (200 μl), 300 μl of Solution IA, and 3500 μl of 0.01 *M* H_3PO_4 were mixed to make the chromatographic test solution.

Control of Standards—Two independent weighings for the preparation of standard solutions were made. The solutions were diluted with an equal volume of deionized water and the absorbances at 220 nm were measured on a spectrophotometer. If the absorbances agreed within 2%, one of the standard solutions was prepared and stored according to the described procedure. If the absorbances deviated, new sets of dilutions were prepared and the control performed once more. If disagreement still persisted, new weighings had to be made.

Instrument Control—The performance of the HPLC instrumentation was controlled each day of operation by the injection of two standard solutions (S_2 and S_5). The slope of this standard curve was calculated, and the baseline noise level was observed. If data were in accordance with previously reported data (results within ±10% of established data) the instrument performance was approved.

Sample Collection—*Whole Blood*—Whole blood (0.25–2 ml) was collected by means of a cannula[17] in 5-ml tubes[18], containing 10–100 μl

[1] Mettler 2002 MP 1, Mettler Instrumente AG, Greifensee, Switzerland.
[2] Mettler M5 SA, Mettler Instrumente AG, Greifensee, Switzerland.
[3] Universal Shaking Machine, SM BI, Edmund Bühler, Tübingen, West Germany.
[4] Wifug X-1, AB Winkelcentrifug, Stockholm, Sweden.
[5] Labsystem OY CF 510 A, Helsinki, Finland.
[6] Constametric I, Laboratory Data Control, Riviera Beach, Fla.
[7] Rheodyne 7120, Rheodyne, Berkeley, Calif.
[8] Gilette Scimitar, Gilette Surgical, Isleworth, Middlesex, U.K.
[9] SpectroMonitor III, Laboratory Data Control, Riviera Beach, Fla.
[10] Linear 255, Linear Instruments Corp., Irvine, Calif.
[11] Astra Läkemedel AB, Södertälje, Sweden.
[12] Løvens Kemiske Fabrik, Ballerup, Denmark.
[13] Fluka AG, Buchs, Switzerland.
[14] Merck, Darmstadt, West Germany.
[15] Kartell, Binasco, Italy.
[16] Ellerman tubes Cerbo, Trollhättan, Sweden.
[17] Vacutainer Needle, Becton & Dickinson, Rutherford, N.J.
[18] Venoject, Terumo Corp., Tokyo, Japan.

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of Solution A, diluted 10 times, with each dilution containing 10 μg of bacampicillin. Care was taken to avoid blood coming into contact with the stopper. The content was then rapidly poured into preweighed centrifuge glass tubes, which were placed in a carbon dioxide–ethanol bath in order to rapidly freeze the content. The whole procedure had to be performed within 10 sec.

Bile—Bile from dogs was collected directly in preweighed centrifuge tubes, containing ~10 μg of bacampicillin as an adsorption inhibitor, and kept in a carbon dioxide–ethanol bath, which was isolated[19] to prevent the freezing of the bile in the tube coming from the dog.

Sample Preparation—For preparation of standards, 2.00 ml of human whole blood or 1.00 ml of canine whole blood or bile was added by pipet into 14 centrifuge tubes and the tubes were chilled in an ice bath. A 20 μl volume of Solutions S_1–S_7 was added to the standard tubes, each solution to two tubes leaving two tubes as blanks; the tubes were mixed for 5 sec and put in a carbon dioxide–ethanol bath (~−70°) in a fast and reproducible manner. All sample handling from then on was performed by keeping the samples cold in the carbon dioxide–ethanol bath. The sample tubes were weighed on an analytical balance and put in the freezing mixture. Solution IA (30 μl) was added to all standard tubes, and 30 μl of Solution ID was added to all sample tubes.

Extraction—While keeping the tubes at −70°, 5.00 ml of Solvent E/ml of sample was added to each tube. The stoppers were sealed by rubber bands and the tubes were put in the shaker horizontally for 20 min. After loosening the stoppers, the tubes were centrifuged[4] for 10 min. The organic phase was carefully transferred to new glass centrifuge tubes (10-ml volume) and evaporated to 4 ml under a gentle stream of air or nitrogen at room temperature, 400 μl of 0.01 *M* H_3PO_4 was added, the stoppers were sealed by rubber bands, and the tubes were put in the shaker horizontally for 15 min. After centrifugation[5] for 5 min the organic phase was removed carefully by a Pasteur pipet and remaining traces were evaporated by compressed air. The aqueous phase (200 μl) was injected into the chromatographic column.

Chromatography—The support[20] was packed in glass-lined stainless steel columns[21] (100 × 4-mm, i.d.) by an upwards slurry packing technique (2) with methylene chloride as solvent. Mobile phase was pH 6 phosphate buffer (ionic strength = 0.05)–acetonitrile (6:4, v/v) containing *N*-hexyl-*N*-methylamine (10^{-4} *M*); the mixture was degassed for 2 hr by magnetic stirring before use. The chromatographic procedure was as follows. The column was equilibrated with the mobile phase for ~1 hr before starting the analysis. Samples (200 μl) were injected in the following order: (*a*) 0.01 *M* H_3PO_4 for control of chromatographic purity; (*b*) chromatographic test solution; (*c*) extract from a whole blood blank sample; (*d*) Standard S_1, (*e*) sample; (*f*) Standard S_2 and so on from there. (This sequence of injections is not critical to the outcome of the analysis but is recommended in order to get as reliable results as possible from the quantitations.) The standard injecting frequency depended on the number of samples and was distributed as evenly as possible among the samples. Finally, the injections were concluded by another sample of the chromatographic test solution, and its chromatographic appearance was compared with the first sample injected in order to confirm the column retention throughout the study.

For quantitation a standard curve was constructed by plotting the peak height ratio of sample–internal standard against the concentration of bacmecillinam in the standards.

RESULTS AND DISCUSSION

Sample Handling—Bacmecillinam as a prodrug is designed to be unstable in biological material, and the compound is rapidly hydrolyzed in whole blood as demonstrated in Fig. 1 (~30% of the compound is lost within 1 min at 37°). The enzyme systems responsible for the degradation are not identified, but similar studies on other prodrugs, amdinocillin pivoxil, talampicillin, and bacampicillin (3), have revealed that blood, plasma, and serum, as well as homogenates from liver, gastric, and duodenal mucosa from humans, dogs, and rats, are capable of efficiently hydrolyzing these types of prodrugs. A fast procedure for collection of samples, involving an immediate freezing of the sample to −70° and subsequent storage at this temperature, therefore, is an essential step in the method.

In bile the compound was more stable: after 10 min at 23° and 37°, ~4.0 and 6.5%, respectively, was lost.

Table I—Stability of Bacmecillinam in Canine Biological Fluids [a]

Day of Analysis	Mean, ng/ml	*s*, ng/ml	Confidence Interval[b]	*n*
Whole Blood				
1	7480	—	—	2
42	7310	134	7090–7520	4
1	89	—	—	2
42	83	6.2	73–92	4
Bile				
1	105	4.8	93–117	3
42	100	1.7	97–103	4

[a] At −70°. [b] $p = 0.05$.

Bacmecillinam is stable both in canine whole blood and bile (Table I) at −70° for at least 40 days; although, the mean values of the analyses indicate a small decrease in concentration after this time, it is not statistically significant.

Extraction—The extraction was optimized by mixing a nonpolar organic solvent, *n*-hexane, with a more polar one, methylene chloride. Preliminary experiments gave log $k_d \times K_s' = 6.3$ and 2, respectively, for the two solvents (k_d is the partition coefficient, K_s' is the acid dissociation constant = $10^{-6.83}$ at 21° and ionic strength = 0.1[22]). The extraction yield was optimized at pH 7.4, ionic strength = 0.5 (equal to the plasma pH) to eliminate the need of buffer addition to the whole blood. Under these conditions the percent extraction from buffer increased from 92 to 97% (Fig. 2) by adding 10% methylene chloride. It can be calculated that with a phase ratio (V_{org}/V_{aq}) equal to three, a quantitative extraction (99%) is achieved with such an organic phase, compared to $V_{org}/V_{aq} = 9$ required with pure *n*-hexane. In extractions from biological fluids, a phase ratio of five was used in order to compensate for the influence of biological material on the extraction.

For a quantitative reextraction of bacmecillinam to an aqueous phase, 0.01 *M* H_3PO_4 was used. It was found empirically that even with $V_{org}/V_{aq} = 20$, the compound is completely reextracted onto the aqueous phase. In the analytical procedure, the initial organic extract is partially evaporated to about half the volume prior to extraction resulting in $V_{org}/V_{aq} = \sim 12$.

In extractions from bile using a mechanical shaker, a heavy emulsion was obtained. It could be decreased by mixing the phases more gently in a rotating device[23], but to break up the emulsion completely, it was necessary to freeze the samples to −25° after a preliminary centrifugation

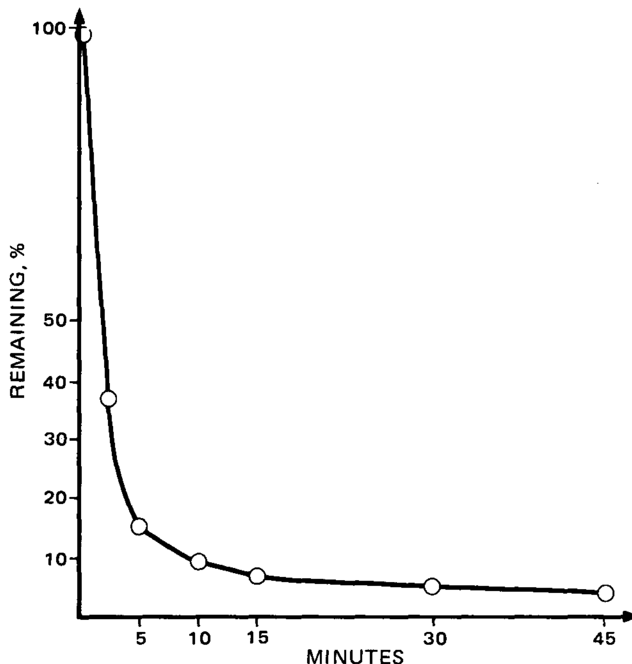

Figure 1—*Stability of bacmecillinam in whole blood at 37°.*

[19] Frigolit, Plastolit, Täby, Sweden.
[20] Nucleosil C_{18} (5 μm), Macherey-Nagel & Co., Düren, West Germany.
[21] Scientific Glass Engineering, Australia.
[22] J. Lindqvist, personnal communication.
[23] Test Tube Rotator Rotamix RK 20 VS, Heto Lab Equipment A.S., Birkerød, Denmark.

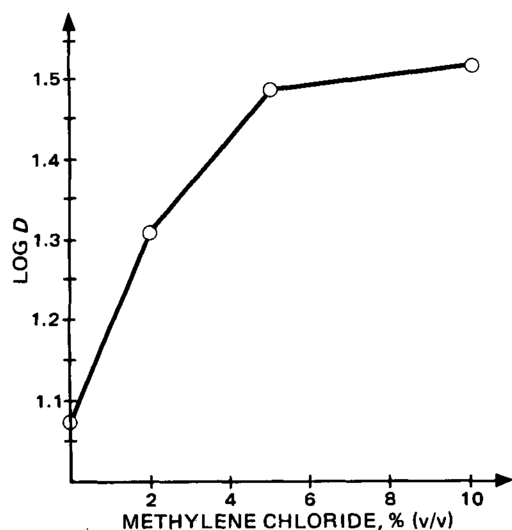

Figure 2—*Extraction of bacmecillinam: Aqueous phase: phosphate buffer pH 7.4 (ionic strength = 0.1); organic phase: n-hexane–methylene chloride; D = distribution ratio = total concentration in organic phase–total concentration in aqueous phase.*

(900×g for 5 min), followed by another identical centrifugation. Bacmecillinam also has a tendency to adsorb at certain interfaces. This was first observed in studies on extraction properties of the compound during method development when nonreproducible results initially were obtained. Silanization of glassware or use of plastic (polypropylene) tubes did not improve the results; only by the addition of a comparatively large excess (100–5000 times) of a related prodrug, bacampicillin, the results became reproducible. The adsorption of bacmecillinam to glass surfaces was further illustrated in an experiment (Table II) where whole blood containing bacmecillinam was transferred by Pasteur pipets. The recovery in the transferred samples was ~57% (median value), and with a large variation, the difference was statistically significant compared with controls.

Considering the facts on hydrolysis and absorption, the blood is collected in sampling tubes which contain an aqueous solution of the adsorption inhibitor, bacampicillin. The content is then rapidly poured into preweighed centrifuge tubes of glass placed in a freezing bath. The whole procedure must be performed within 10 sec to avoid significant hydrolysis of the compound.

Chromatography—Nucleosil C_{18} (5 μm) has been found suitable for the chromatography of hydrophobic amines (4) and was chosen as the support in this study. Initial experiments indicated that bacmecillinam gave unacceptable asymmetric peaks ($As_{10\%}$ = 2.4–3.0) when the support was packed in conventional stainless steel columns and run with phosphate buffer (pH 7; ionic strength = 0.05)–acetonitrile (3.5–4.0—6.5–6.0, v/v) as mobile phase. With glass-lined stainless steel columns, however, the peaks were symmetrical ($As_{10\%}$ = 1.0–1.2) and had equally good efficiencies (h ~ 5: h = reduced plate height; HETP/dp; dp = support particle diameter). With this system, however, nonreproducible peak heights were obtained, although other chromatographic parameters were unaffected. The phenomenon was interpreted as being due to adsorption of the compound to the glass walls, similar to the problems encountered during the development of the described extraction procedure, and the effect could be avoided by the addition of a secondary amine (*N*-hexyl-

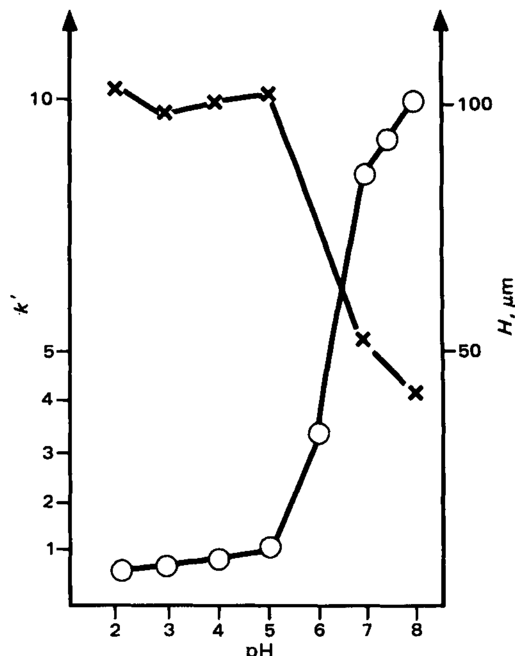

Figure 3—*Relation of pH in mobile phase with capacity ratio and efficiency. Mobile phase: phosphate or citrate buffer (ionic strength = 0.1)–acetonitrile (4:6, v/v). Key: X = H and O = k'.*

Table II—Adsorption of Bacmecillinam onto Pasteur Pipets [a]

Treatment	Range Found, ng/g	Median, ng/g	Median Recovery, %
Not transferred	20.7–32.0	27.6	99.3
Transferred	0.5–16.8	15.8	56.8

[a] Procedure: From five centrifuge tubes with 4 ml of whole blood, each containing bacmecillinam (27.8 ng/g), ~2 ml was transferred to new centrifuge tubes by Pasteur pipets during 30 sec maximally. The tubes were then immediately frozen to −70° and later analyzed by HPLC.

Table III—Adsorption of Bacmecillinam onto Glass-Lined Stainless Steel Columns [a]

Mobile Phase	Amount Injected, ng	Peak Height Range, mm	Peak Height Median, mm	*n*
Without *N*-hexyl-*N*-methylamine	23	30–68	61	7
With *N*-hexyl-*N*-methylamine	20	78–80	79	6

[a] Procedure: Bacmecillinam was dissolved in water and analyzed by HPLC with and without the addition of *N*-hexyl-*N*-methylamine (10^{-4} *M*) to the mobile phase.

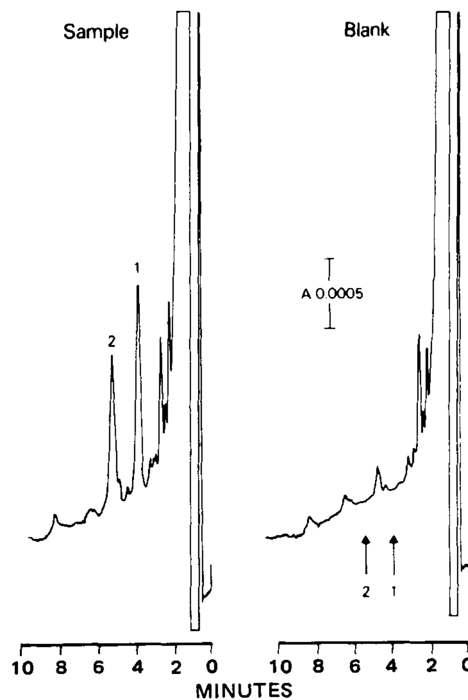

Figure 4—*Blank and sample chromatograms of (1) bacmecillinam (21 ng) and (2) amdinocillin pivoxil (20 ng). Analysis was performed as described in the text.*

Table IV—Repeatability in Quantitative Determinations of Bacmecillinam [a]

Added, ng/ml	Found, %	CV, %
4.7	100.4	8.3
18.6	95.7	4.1

[a] Four samples were analyzed as described in *Experimental* relative to five standards in the range of 3.3–53 ng/ml.

N-methylamine) as an adsorption inhibitor (Table III). The chromatographic parameters (efficiency, peak symmetry, and capacity ratio) were virtually unaffected by the amine addition. Later experiments with stainless steel columns indicate that peak asymmetries observed initially, and described above, were also eliminated by the presence of N-hexyl-N-methylamine in the mobile phase.

The capacity factor of bacmecillinam was optimized by varying the pH of the mobile phase while keeping the volume ratio between buffer and acetonitrile constant (4:6, v/v) (Fig. 3). At pH 6 the retention time is suitable and the chromatographic efficiency is acceptable, while at pH 5 the capacity ratios are too low and the efficiencies are about half of those at higher pH. The compound used as the adsorption inhibitor for the extraction, bacampicillin, is almost nonretained at the chosen conditions and elutes near the front of the chromatogram. Amdinocillin, the antibiotically active compound, also elutes near the front in this chromatographic system, and the compound, furthermore, is not extracted to a significant amount in the extraction step. The quantitative determination of amdinocillin in biological fluids still requires the methodology described earlier (5).

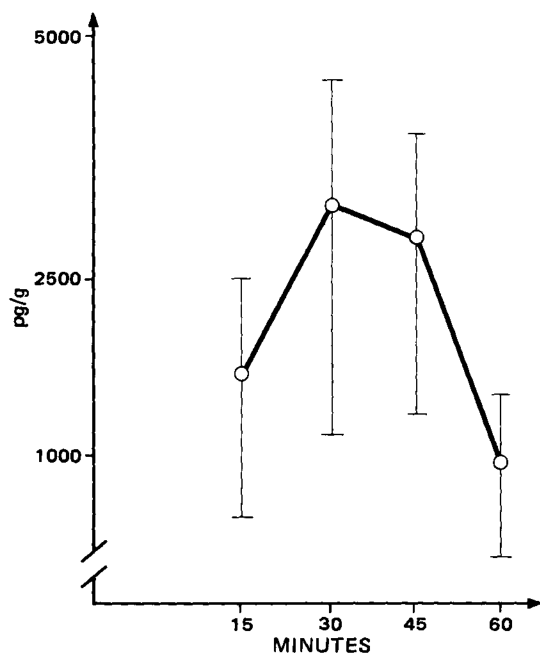

Figure 5—*Mean levels of bacmecillinam from three volunteers. Dose: 244 mg of bacmecillinam hydrochloride orally.*

Representative blank and sample chromatograms from whole blood (Fig. 4) illustrate that the chosen conditions are suitable for the analysis of bacmecillinam, and that the internal standard, amdinocillin pivoxil, elutes largely unaffected by blank disturbances.

The injected sample differs in composition from the mobile phase, and some of the small peaks that appear in the chromatogram originate from the resulting composition disturbance and not from the biological sample. Late in the chromatogram, after ~2 hr, an additional broad composition disturbance peak elutes and has to be taken into account in routine determinations in order to avoid interferences. This kind of disturbance can be reduced by the injection of smaller sample volumes. Some preliminary experiments have indicated the possibility of reextracting and injecting a volume 10 times smaller (*i.e.*, 20 μl instead of 200 μl).

Quantitative Determinations—The limit of detection, defined as a signal equal to twice the background noise level of the baseline, is ~600 pg/ml of whole blood. The repeatability of the method (Table IV) is ~8% (CV) at the level of 5 ng/ml, and it is estimated that at 1–2 ng/ml, the precision still is acceptable (*i.e.*, 15–20%, CV) for quantitative determinations.

Mean levels from three volunteers receiving 244 mg of bacmecillinam as a single dose are in the range of 1–3 ng/g of whole blood (Fig. 5).

The method has also been applied to determination of the compound in canine portal and peripheral whole blood as well as bile.

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