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# Determination of Apalcillin and its Metabolites in Human Body Fluids by High-Pressure Liquid Chromatography

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We describe two methods for the quantitative analysis of apalcillin and its metabolites in serum and urine by reverse-phase high-pressure liquid chromatography (HPLC), a fast isocratic method for the parent drug, and a gradient method that allows the simultaneous assay of two metabolites. Serum was deproteinized with acetonitrile, and urine was diluted with buffer solution. The detection limit was about 0.5 μg/ml at a detection wavelength of 254 nm and 1.5 μg/ml at 310 nm. Within-batch precision (coefficient of variation) varied from 10.2 to 1.1% for concentrations of 7.8 and 185.3 μg/ml of serum, respectively. Recovery rates of 95.1 and 97.7% were found in spiked sera. Results obtained by HPLC correlated well with those from a standard microbiological assay (agar diffusion test); the resulting bivariate regression equation for serum was $y_{bioassay} = 2.5$ μg/ml + $0.992 \cdot x_{HPLC}$, and that for urine was $y_{bioassay} = 12.0$ μg/ml + $1.009 \cdot x_{HPLC}$. At a detection wavelength of 315 nm, no interferences were observed in 10 healthy volunteers. Healthy subjects who were given 2 g of apalcillin intravenously excreted 18% of the parent drug within 24 h in the urine. Two inactive compounds were furthermore identified in urine as the isomeric forms of the penicilloic acids. Their excretion within 24 h amounted to 6.9 and 11.2% of the dose.

Apalcillin (sodium(2*S*, 5*R*, 6*R*)-6-[(*R*)-2-(4-hydroxy-1,5-naphthyridine-3-carboxamido-)-2-phenylacetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylate) was developed by N. Noguchi et al. (3). It has a high in vitro antimicrobial activity against *Pseudomonas aeruginosa* and other gram-negative bacteria. This property is attributed to the naphthyridine-carboxamido side chain (2). Preliminary studies on its pharmacokinetics show poor intestinal absorption, a high serum protein binding rate (98%), and a high biliary excretion in rats (2). For our studies on the pharmacokinetics of apalcillin in humans, we developed a liquid chromatographic method for the quantitative analysis of apalcillin and its potential metabolites in serum and urine.

## MATERIALS AND METHODS

**Chemicals.** Reference material of apalcillin (lot 10417; potency, 860 μg/mg) and of several degradation products (A1, B, C) were kindly supplied by Thomae GmbH, Biberach, Germany. Unless otherwise stated, all reagents and solvents were of reagent grade purity and were obtained from E. Merck AG, Darmstadt, Germany.

**Microbiological assay of apalcillin.** The microbiological assay of apalcillin was performed with a standard agar plate diffusion technique (4). The test organism was *Bacillus subtilis* ATCC 6633. For concentrations of less than 10 μg/ml, *Sarcina lutea* ATCC 9341 was used.

**HPLC. (i) Preparation of buffers.** For high-pressure liquid chromatography (HPLC), a stock solution of buffer 3.15 was prepared in the following manner. A total of 8.203 g of sodium acetate was dissolved in 800 ml of distilled water. A total of 165 ml of concentrated acetic acid was added, the pH was adjusted to 3.15, and the solution was brought up to 1 liter. The working buffer was prepared by a 10-fold dilution of this stock solution.

Buffer 5.0 was prepared in the following manner. A total of 8.203 g of sodium acetate was dissolved in 800 ml of distilled water. The pH was adjusted to 5.0 with approximately 1.5 ml of concentrated acetic acid, and the solution was diluted 10-fold for use.

**(ii) Sample preparation.** One volume of serum or aqueous standard was diluted with one volume of acetonitrile. After centrifugation, one volume of the supernatant was mixed with one volume of buffer (pH 3.15). To measure the extraction rate, one volume of blank serum was spiked with one volume of a standard solution (200 μg/ml) and treated as described above. Urines were diluted with buffer (pH 3.15 or 5.0) to 1:20 or 1:2. Samples diluted with buffer 3.15 should be analyzed as soon as possible or at least within 4 h after preparation.

**(iii) Isocratic method.** The chromatograph consisted of the following modules: an LC 420 automatic sampler (injection volume, 10 μl; Perkin-Elmer Corp., Norwalk, Conn.); a 2/1 pump (flow rate, 2 ml/min; Perkin-Elmer); an LC 15 fixed-wavelength detector,

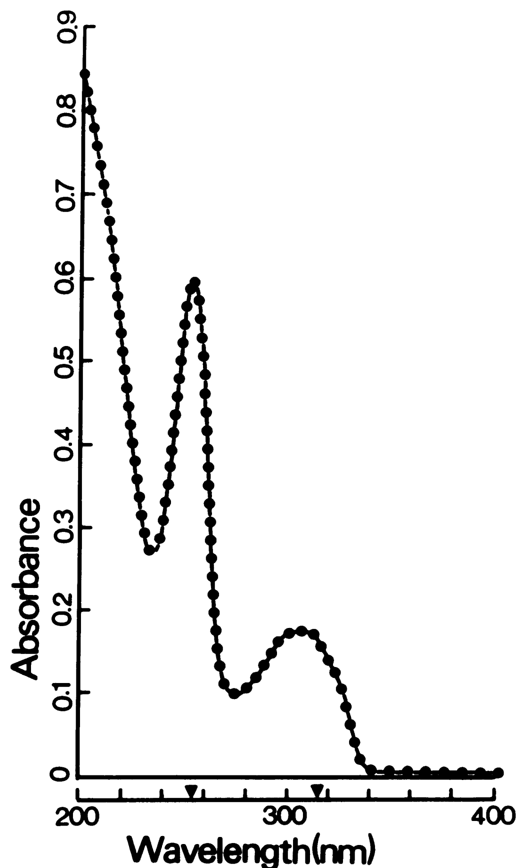

FIG. 1. Absorption spectrum of apalcillin.

lambda 254 nm, or an LC 55 variable-wavelength detector, lambda 315 nm (Perkin-Elmer); and an SP 4000 integrator (Spectra-Physics Inc., Santa Clara, Calif.). Lichrosorb RP 18 columns (5-μm particle diameter, 125 by 4 mm; E. Merck) were used at ambient temperature. The mobile phase consisted of sodium acetate buffer (10 mmol/liter, pH 3.15) mixed with acetonitrile in a ratio of 80/20 (vol/vol).

**(iv) Gradient method.** The chromatograph consisted of the following modules: a 2/2 pump (flow rate, 2 ml; Perkin-Elmer); a 7125 injector (injection volume, 10 μl; Rheodyne Inc., Berkeley, Calif.); a Lichrosorb RP 18 column (5-μm particle diameter, 125 by 4 mm; E. Merck); and a variable-wavelength detector, lambda 315 nm (Perkin-Elmer). (For technical reasons, measurements were performed at 315 instead of 310 nm.) A model 3390A integrator (Hewlett-Packard Co., Palo Alto, Calif.) was also used. Solvent A consisted of sodium acetate buffer (10 mmol/liter, pH 5.0) plus acetonitrile (50/50, vol/vol). Solvent B consisted of sodium acetate buffer (10 mmol/liter, pH 5.0). The starting solution consisted of 10% solution A plus 90% solution B, with a gradient of 4% solution A per min. The run time was 16 min, and the reset time was 10 min. All calculations were based on peak areas and external calibration. Serum results were corrected for extraction rates.

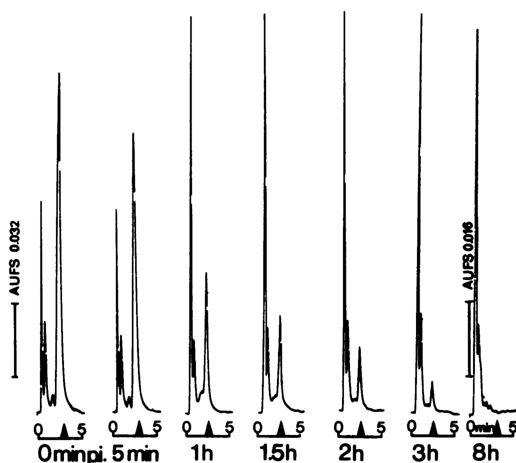

FIG. 2. Example chromatograms. Serum of a normal volunteer after a single intravenous dose of 2 g of apalcillin. The isocratic method was used. Lambda = 254 nm.

**Storage of samples.** Human sera and urine were stored at −20°C immediately after collection and analyzed within a few days.

**Statistical calculations.** Statistical calculations were performed according to standard methods (5). Bivariate regression analysis was performed as described previously (1).

## RESULTS

**Development of method.** The absorption spectrum of apalcillin showed two distinct maxima at 254 and 310 nm, with a ratio of absorbances of approximately 3/1 (Fig. 1). The in vitro degradation products A1 and 4-hydroxy-1,5-naphthyridine-3-carboxylic acid yielded quantitatively identical spectra. Both maxima can be used for quantitative determination. Apalcillin has three dissociable groups with $pK_a$ values of 8.39, 2.97, and 2.28. Elution of apalcillin from the reverse-phase column RP 18 is accordingly influenced by the pH of the mobile phase. Retention times at pH 5 or 6 were shorter than those at pH 3.15. Unfortunately, considerable tailing was observed at pH values higher than 3.15 in the isocratic mode. Therefore, in this mode, we chose a rather low pH and a higher amount of acetonitrile in the mobile phase. A typical series of chromatograms obtained from sera is shown in Fig. 2. The isocratic method is simple, fast, and suitable for assay of the parent drug in both serum and urine. The separation of metabolites that contain additional ionic groups (Fig. 3) requires the application of a gradient method as described in Materials and Methods. Typical separation patterns of urine specimens are shown in Fig. 4. The total run time for the gradient method was 26 min, whereas the isocra-

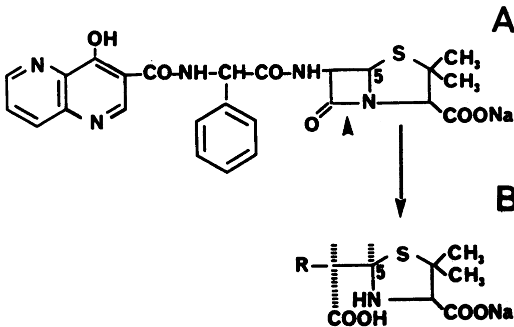


FIG. 3. Chemical structure of apalcillin (A) and its penicilloic acid metabolite A1 (B).

tic method required only 6 min. The average lifetime of the column packings varied between 150 and 600 runs and could be prolonged somewhat by filling up the column with additional sorbent.

**Linearity and sensitivity.** Peak areas of standard curves of apalcillin were linear proportional to at least 600 μg/ml. Variation of the injection volume yielded linear proportional peak heights up to 15 μl. At 254 and 315 nm, the detection limits were approximately 0.5 and 1.5 μg/ml, respectively.

**Precision, accuracy, and identification of peaks.** Within-batch precision was measured by using pooled sera in three concentrations (7.8, 32.8, and 185.3 μg/ml), each in 10 replicates. Coefficients of variation were 10.2, 5.2, and 1.1%. Recovery rates for the drug added to serum pools were 95.1% (100 μg/ml) and 97.7% (200 μg/ml). The average percent recovery of drug added to blank sera of 10 healthy subjects was 99.0%.

The presence of apalcillin in serum and urine was confirmed by enzymatic degradation with beta-lactamase type II from *Bacillus cereus* (EC.3.5.2.8), which resulted in a complete disappearance of the apalcillin peak. Concomitantly, the peak of metabolite A1 in urine increased.

In another experiment, a large urine specimen (100 μl) from a 0- to 3-h period was chromatographed by the gradient method at a reduced flow rate. One-milliliter fractions of the eluate were collected. UV spectra of the fractions (cf. Fig. 4C) revealed three peaks, the spectrum of the naphthyridine group being typical. Both apalcillin and the metabolite A1 yielded identical retention times when pure reference material was chromatographed (Fig. 4A and B). The peak AX is most likely the isomer A2 of metabolite A1 which, according to the internal data of the manufacturer, is formed spontaneously from A1 in an aqueous solution at 40°C.

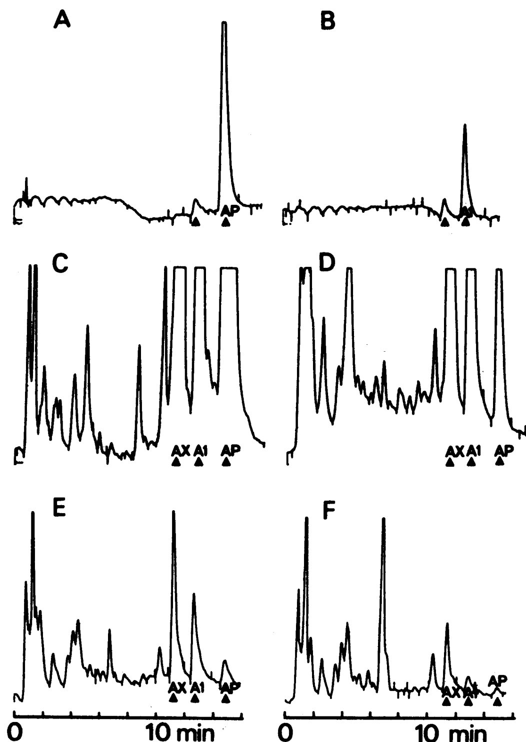


FIG. 4. Example chromatograms. Urine of a normal volunteer after a single intravenous dose of 2 g of apalcillin (AP). The gradient method was used. Lambda = 310 nm. (A) Apalcillin; (B) degradation product A1. Collection intervals: (C) 0 to 3 h; (D) 3 to 6 h; (E) 6 to 12 h; (F) 12 to 24 h.

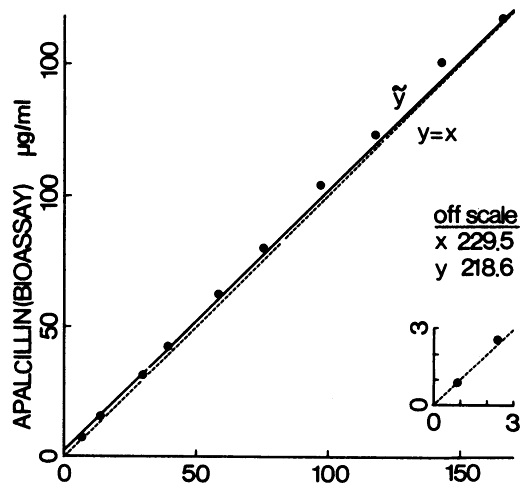


FIG. 5. Method comparison. Apalcillin in serum. Each point represents the mean of 10 samples. The following formulae were used: $\bar{x}_{HPLC}$ = 65.1 μg/ml; $\bar{y}_{bioassay}$ = 67.1 μg/ml. $n$ = 15. Sign test: $P$ = 0.05. Bivariate regression analysis: $y_{bioassay}$ = 2.5 μg/ml + 0.992 · $x_{HPLC}$. $r$ = 0.998. Lambda = 1.016.

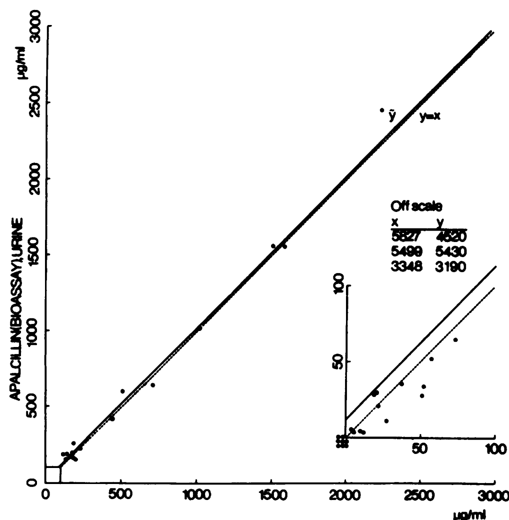


FIG. 6. Method comparison. Apalcillin in urine. The following formulae were used: $\bar{x}_{HPLC}$ = 593.2 μg/ml; $\bar{y}_{bioassay}$ = 610.8 μg/ml. $n$ = 39. Sign test: $P$ = 0.10. Bivariate regression analysis: $y_{bioassay}$ = 12.0 μg/ml + 1.009 · $x_{HPLC}$. $r$ = 0.995. Lambda = 0.982.

**Method comparison and interferences.** Bioassay and HPLC showed good correlations for serum (Fig. 5) and urine (Fig. 6). It should be noted, however, that in using a 254-nm wavelength for detection, one may encounter interferences from caffeine and its metabolites at low concentrations of apalcillin. Therefore, the use of a 310-nm wavelength for detection is preferred, with which interferences from other substances are less likely to occur.

The chromatographic behavior of several endogenous substances and other antibiotics and drugs is shown in Table 1.

**Renal excretion of apalcillin and its metabolites.** Ten healthy subjects were given a single intravenous dose of 2 g of apalcillin. The average recovery from 24-h urine specimens was 18.3% for apalcillin, 6.9% for metabolite A1, and 11.2% for metabolite A2.

TABLE 1. Retention times of apalcillin and other compounds by the isocratic method

| Compound[a] | Retention time (min) | | | |
|---|---|---|---|---|
| | Actual | Relative[b] | Detected at (nm): | |
| | | | 254 | 310 |
| Uric acid | 0.8 | 0.21 | + | − |
| Xanthine | 0.8 | 0.21 | + | − |
| Hypoxanthine | 0.8 | 0.21 | + | − |
| Allopurinol | 0.8 | 0.21 | + | − |
| Ceftazidime | 0.9 | 0.24 | + | (+)[c] |
| Paraxanthine | 1.0 | 0.26 | + | − |
| Theophylline | 1.0 | 0.26 | + | − |
| Moxalactam | 1.0 | 0.26 | + | − |
| Desacetyl-cefotaxime | 1.0 | 0.26 | + | + |
| Cefotiam | 1.1 | 0.29 | + | − |
| Cefotaxime | 1.1 | 0.29 | + | + |
| Ceftizoxime | 1.1 | 0.29 | + | + |
| Paracetamol | 1.1 | 0.29 | + | − |
| Cefotetan | 1.2 | 0.32 | + | + |
| Cefmenoxime | 1.2 | 0.32 | + | + |
| Caffeine | 1.3 | 0.34 | + | − |
| Metronidazole | 1.3 | 0.34 | + | + |
| Amoxicillin | 1.5 | 0.39 | (+) | − |
| Cefoperazone | 1.7 | 0.45 | + | − |
| Ticarcillin | 1.8 | 0.47 | (+) | − |
| Salizylic acid | 2.6 | 0.68 | (+) | + |
| Sulfamethoxazole | 3.7 | 0.97 | + | − |
| Apalcillin | 3.8 | 1.00 | + | + |
| Trimethoprim | 4.3 | 1.13 | + | − |
| Piperacillin | 6.2 | 1.63 | + | − |
| Nafcillin | 28.0 | 7.73 | + | + |

[a] Compounds not detectable by the present method were aminoglycoside antibiotics, ampicillin, azlocillin, floxacillin, and penicillin G.

[b] Relative retention times are defined relative to apalcillin.

[c] (+), Very small peak at a concentration of 100 μg/ml.

## DISCUSSION

Only 18% of intravenously administered apalcillin could be recovered from 24-h urine specimens of healthy volunteers when a conventional bioassay was used. This result prompted us to develop an alternative assay method that might confirm this result and give additional quantitative information on the excretion of metabolites.

In developing a suitable HPLC method, we had to take into consideration the UV spectra of the substances to be analyzed (Fig. 1), the influence of pH on their polarity and stability during chromatography and in a prior deproteinization step, and possible interferences by endogenous or exogenous compounds. The absorbance at 254 nm was about three times higher than that at 310 nm. Nevertheless, measurement at 310 nm is to be preferred, because none of the always-present methylxanthines (caffeine and theophylline) and their metabolites or other endogenous compounds absorb at this wavelength under the given conditions (cf. Table 1). If the use of an internal standard appears to be desirable, piperacillin may be suitable for a wavelength of 254 nm after evaluation by the present method. Many beta-lactam antibiotics show maximal stability at around pH 5 to 7. Unfortunately, considerable peak tailing was observed at these pH values. Thus, we had to lower the pH value of the eluant to 3.15 for the isocratic method, with a concomitant increase of the concentration of the organic phase. This version of the method is very rapid and convenient for the analysis of the parent drug in both serum and urine. Deproteinization of serum with acetoni-

trile avoids any extreme pH value and does not require an evaporation of the extraction solvent. Since a high volume of injected organic phase disturbs the separation, an additional dilution of the supernatant with aqueous phase is required. The addition of the acidic buffer 3.15 reduces the stability of the analyte. Therefore, the final dilution step should be followed by chromatography as soon as possible. The packing of the column showed fair but variable stability, requiring frequent replacements when large numbers of samples were to be analyzed. The gradient method also separated the more polar metabolites (Fig. 4). Its main disadvantage was a threefold-longer run time as compared with the isocratic method.

The presence of the parent drug in serum and urine chromatograms was confirmed by treatment with beta-lactamase and by the UV spectrum of the drug isolated from urine. The good correlation of results from bioassay and from HPLC also supports this identification. Another two peaks were observed in the urine of 10 volunteers after a single dose of the drug. They were tentatively called A1 and AX. Both compounds yield, upon isolation from urine, qualitatively identical UV spectra as compared with apalcillin. A1 has the same retention time as a reference substance which also contains a minor impurity appearing at the retention time of AX. From physical degradation studies (unpublished data), it is likely that AX is the isomer A2 which differs from A1 only by the position of the hydrogen atom at position 5S (cf. Fig. 1).

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