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Note

High-performance liquid chromatographic determination of apalcillin in plasma and urine

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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, is a new semisynthetic derivative of the ureidopenicillin group with broad-spectrum activity against Gram-positive cocci and Gram-negative bacilli [1, 2]. The high antimicrobial activity against *Pseudomonas aeruginosa* is due to the presence of a naphthyridine-carboxamido side-chain in its chemical structure (Fig. 1).

Two types of methods for the determination of apalcillin in human body fluids have been published: a microbiological assay [3] and a reversed-phase high-performance liquid chromatographic (HPLC) procedure [4]. The bioassay reported by Borner et al. [3] involves two different strains of microorganisms depending on the concentration of apalcillin in the biological fluid. In comparison, the HPLC method allows the determination of apalcillin at a wide range of concentrations under the same conditions, and thereby permits

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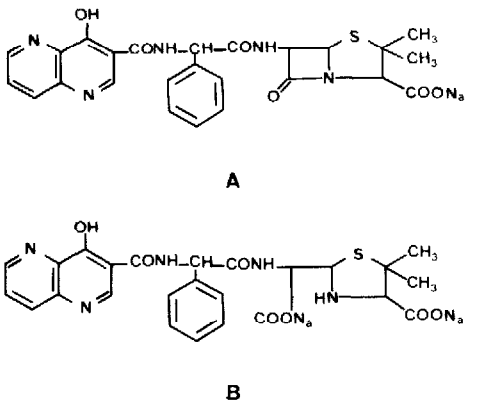

Fig. 1. Structures of apalcillin (A) and its penicilloic acid metabolite (B).

a more rapid analysis, which is an important advantage for pharmacokinetic studies.

This paper describes a micro-scale method involving the use of an internal standard, cefalotine. Under our reversed-phase HPLC conditions, a single protein precipitation by acetonitrile (the sample pre-treatment used by Borner et al. [3] is not satisfactory as endogenous substances prevent the determination of apalcillin). We chose a sample pre-treatment including another purification step: first a protein precipitation by 2-propanol, followed by removal of endogenous compounds with an organic solvent (chloroform), providing a good sample for HPLC. Precision and accuracy were studied and the coefficients of variation were lower than 4.3% for all the concentrations investigated. This isocratic reversed-phase method allows the separation of apalcillin from one of its metabolites, penicilloic acid (Fig. 1).

EXPERIMENTAL

Materials

Apalcillin and its penicilloic acid metabolite were supplied by Boehringer (Mannheim, F.R.G.) and cefalotine by Eli Lilly (Saint-Cloud, France).

The chromatographic solvents (acetonitrile and methanol) were of HPLC grade and were purchased locally. Water was doubly glass-distilled and deionized. All other chemicals (2-propanol, chloroform, isoamyl alcohol, ammonium acetate, acetic acid, orthophosphoric acid and triethylamine) were of analytical-reagent grade (Merck, Darmstadt, F.R.G.).

Chromatographic procedure

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph was used. The instrument was equipped with a Model 45 pump, a Lambda-Max Model 480 ultraviolet detector operated at 258 nm, a WISP Model 710B automatic injector and a radial compression Z module. The column was a Radial—Pak μBondapak C_{18} (Waters Assoc.) (100 × 8 mm I.D.; 10 μm particle size).

The data obtained were recorded on a Model 730 data module integrator (Waters Assoc.). The mobile phase was water—acetonitrile—methanol—tri-

ethylamine (600:340:150:4), the pH being adjusted to 3.40 with ortho-phosphoric acid. The working pressure of the pump was 55 bar at a flow-rate of 4 ml/min.

Standard solutions

Stock solutions of apalcillin (1 mg/ml) and cefalotine (internal standard) (1 mg/ml) were prepared in doubly distilled water and stored at −20°C.

Plasma standards. Appropriate dilutions of the apalcillin stock solution were prepared in drug-free human plasma to provide plasma concentrations ranging from 2 to 25 μg/ml (internal standard at 25 μg/ml) and from 20 to 200 μg/ml (internal standard at 250 μg/ml). Dilutions of cefalotine were made in 0.1 *M* acetate buffer (pH 5).

Urine standard. Blank urine was spiked with apalcillin stock solution to yield concentrations ranging from 20 to 500 μg/ml. The concentration of the internal standard in 0.1 *M* acetate buffer (pH 5) was 250 μg/ml. Standard solutions were prepared daily.

The calibration graphs were constructed by plotting peak height ratios of apalcillin to internal standard against concentrations of apalcillin in plasma or urine.

Sample preparation

Plasma and urine samples were prepared in the same way. Volumes of 100 μl of plasma or urine and 100 μl of 0.1 *M* ammonium acetate buffer (pH 5), containing internal standard, were transferred into a 1.5-ml conical centrifuge tube and 500 μl of 2-propanol were added. The mixture was vortexed for 30 sec and centrifuged for 2 min at 8700 *g* (Beckman Microfuge centrifuge). In another conical centrifuge tube (1.5 ml), the clear supernatant was mixed with 500 μl of chloroform—isoamyl alcohol (100:4). The tube was vortexed for 30 sec and centrifuged at 8700 *g* for 2 min. An appropriate aliquot of the upper aqueous phase was injected into the chromatograph.

RESULTS AND DISCUSSION

Selecitivity

The first step in the development of the HPLC procedure was to establish the selectivity. The retention times of apalcillin and cefalotine were 3.40 and 2.60 min, respectively. No interfering peak was observed with drug-free plasma and urine (Fig. 2). Potential interferences of some other compounds were investigated (Table I).

Repeatability and reproducibility

The reproducibility (within-day study, $n = 10$) and repeatability (between-day study, $n = 10$) were determined on plasma and urine samples from a subject treated with apalcillin. The coefficients of variation were less than 4.3% (Table II).

Accuracy and precision

Accuracy and precision were measured on blank human plasma and urine

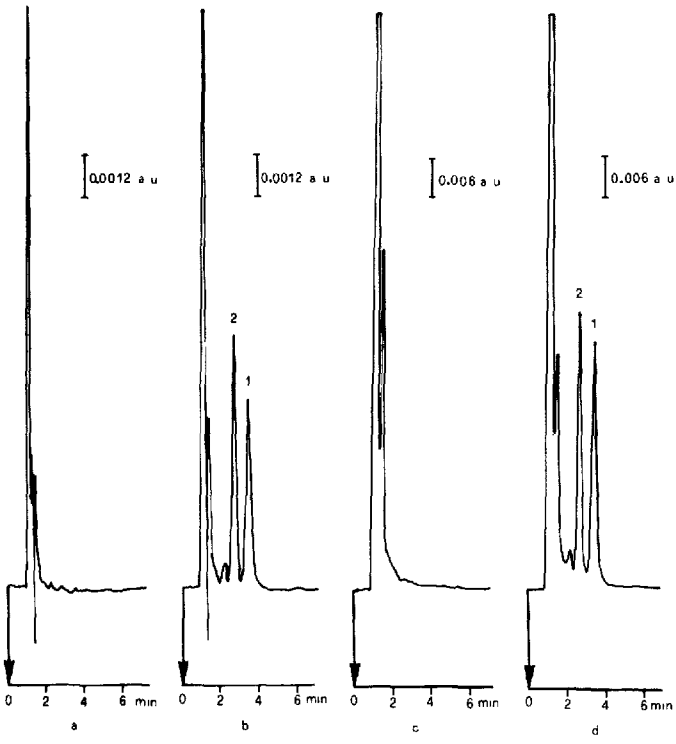

Fig. 2. Chromatograms of (a) human drug-free plasma; (b) human plasma spiked with 10 μg/ml apalcillin; (c) human drug-free urine; (d) human urine spiked with 125 μg/ml apalcillin. Peaks: 1 = apalcillin; 2 = cefalotine.

TABLE I

COMPOUNDS STUDIED FOR POTENTIAL INTERFERENCE

Compound (2 μg injected)	Relative retention time*	Compound (2 μg injected)	Relative retention time*
Uric acid	0.35	Cefalotine	0.76
Cefotaxime	0.36	Salicylic acid	0.84**
Cephalexin	0 40	Apalcillin	1.00
Acetaminophen	0.43	Ketoprofen	2.49
Latamoxef	0.44	Niflumic acid	3.88
Theophylline	0.44	Metiazinic acid	5.23
Cefoperazone	0.46	Indomethacin	7.44
Caffeine	0.49	Flufenamic acid	No response
Cephaloridine	0.49	Mefenamic acid	No response
Cefazolin	0.50		

*Relative to apalcillin = 3.40 min
**Negligible peak at 258 nm.

samples spiked with apalcillin at different concentrations (n = 10). Accuracy, defined as (amount found/amount added) · 100, was around 100% for all concentrations investigated (Table III).

TABLE II

REPRODUCIBILITY AND REPEATABILITY OF THE HPLC ASSAY OF PLASMA AND URINE SAMPLES FROM A SUBJECT ADMINISTERED APALCILLIN

Sample		Within-day determinations (n = 10)		Between-day determinations (n = 10)	
		Mean (μg/ml)	Coefficient of variation (%)	Mean (μg/ml)	Coefficient of variation (%)
Plasma	1	6.4	2 7	6.3	4.3
	2	16.1	1.6	15.9	3.2
	3	49.0	2.1	49.5	3.7
	4	98.7	2.5	99.8	3.9
Urine	I	31.1	4.2	30.7	3.6
	II	415 5	1.0	413.7	1.4

TABLE III

ACCURACY AND PRECISION RESULTS FOR PLASMA AND URINE SAMPLES SPIKED WITH APALCILLIN

Sample	Spiked concentration (μg/ml)	Within-day determination (n=10)			Between-day determinations (n=10)		
		Mean (μg/ml)	Coefficient of variation (%)	Accuracy (%)	Mean (μg/ml)	Coefficient of variation (%)	Accuracy (%)
Plasma	7.5	7.4	1.4	98.6	7.4	2.1	98.6
	15	15 4	1.1	102.6	15.0	3.2	100
	75	74.1	1.4	98.8	73.4	3.1	97.8
	125	123.5	1.8	98.8	123.8	2 6	99.0
Urine	75	75 5	2.6	100 6	73.4	2.8	97.8
	375	376.2	1.5	100.3	377.5	1.9	100.6

Linearity

Using the internal standard method, the HPLC response was found to be linear over the ranges 2—200 μg/ml for plasma and 20—500 μg/ml for urine. A good correlation was obtained between concentrations and peak height ratios (apalcillin/cefalotine): for plasma, r = 0.995 (low concentrations, 2—25 μg/ml) and r = 0.998 (high concentrations, 20—200 μg/ml), and for urine r = 0.998.

Plasma and urine calibration lines were chosen from results of pharmacokinetic studies.

Plasma and urine levels of apalcilline

In ten healthy volunteers, mean plasma concentrations of apalcillin 5 min after intravenous infusion of 2 g were 218 ± 39.6 μg/ml and after 8 h 0.9 ± 0.7 μg/ml [5]. In six healthy volunteers receiving 2 g of apalcillin by a 2-h infusion, the mean concentration was 58.58 ± 9.14 μg/ml at the end of perfusion. The 24-h urine recovery rate of unchanged apalcillin was around 18% of the administered dose [3—5].

Sensitivity

The detection limit (signal-to-noise ratio $\geqslant$2) was 0.2 μg/ml for both plasma and urine.

Stability

The stability of apalcillin during sample conservation and automatic injection was studied. Two samples (plasma and urine) were kept for one month at −20°C and no degradation of apalcillin was noted (Table IV).

TABLE IV

STABILITY OF APALCILLIN IN PLASMA AND URINE FROZEN AT −20°C FOR 30 DAYS

Day	Plasma (125 μg/ml)	Urine (375 μg/ml)
0	125	370
1	126	373
2	122	380
3	127	380
10	127	370
15	124	370
30	125	376

The sample pre-treatment left the drug in the aqueous phase, which had to be of a suitable pH to prevent degradation of the antibiotic. Previous studies [3] have shown that maximal stability of apalcillin solution occurred in the pH range 4.5—7. Under the described conditions, the pH of the upper aqueous layer was 6.5, which is suitable to prevent degradation. Stability was demonstrated on a human plasma sample spiked with 50 μg/ml apalcillin. This plasma sample, treated as described above (internal standard at 250 μg/ml), was kept at 25°C for 6 h. Every hour, an aliquot of the aqueous phase was injected and the peak heights of apalcillin and cefalotine were measured; the coefficients of variation (n = 8) were 2.4% for apalcillin and 1.6% for the internal standard. This result confirmed the stability of these compounds in the final aqueous layer and allowed us to use automatic injection for several hours.

In the same way, we studied the potential degradation of apalcillin in the mobile phase (pH 3.4, chosen to prevent peak tailing). The coefficient of variation was 1.2% (apalcillin at 10 μg/ml in the eluent, eight injections during 2 h at 25°C).

Penicilloic acid metabolite of apalcillin

In plasma and urine samples of a subject who had received apalcillin, we always noted the presence of an unknown peak (Fig. 3). This compound was isolated by HPLC of large urine samples prior to mass and infrared spectral examination.

Mass spectrometry was attempted using a Micromass Type 16F mass spectrometer, but the low volatility of apalcillin and penicilloic acid did not permit molecular peaks to be observed either with an ionization energy of 70 eV or with chemical ionization (NH_3). Even methylation of these compounds with diazomethane did not enable mass spectra to be obtained.

Infrared spectra of apalcillin, penicilloic acid and this unknown compound were recorded on a Beckman Acculab 1 spectrometer (KBr pellet). In apalcillin, the β-lactam CO absorption occurred at 1765 cm^{-1} [6]; in penicilloic acid and

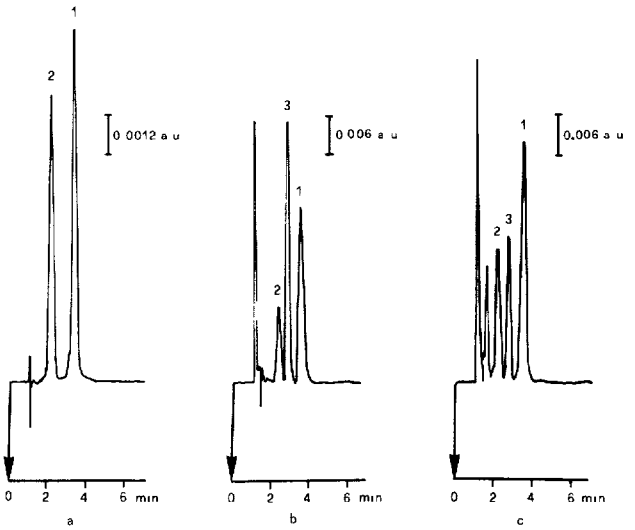

Fig. 3. (a) Chromatogram of an aqueous solution of apalcillin and penicilloic acid (25 μg/ml). (b) Chromatogram of human plasma after intravenous infusion of 3 g of apalcillin for 2 h. Plasma sample was collected at the end of perfusion (concentration found: 95 μg/ml). (c) Chromatogram of urine sample from the same subject treated with apalcillin. Urine was collected during 12 h (concentration found· 215 μg/ml). Peaks· 1 = apalcillin; 2 = penicilloic acid; 3 = internal standard.

the unknown compound, this band disappeared, which corresponded to opening of the ring [7]. In addition, on comparison of an aqueous solution of penicilloic acid with treated samples, the same retention time was found (2.02 min) (Fig. 3). These results indicate that the isolated unknown compound may be penicilloic acid, the main metabolite of apalcillin [3].

CONCLUSION

The rapidity (for each sample the total time analysis is about 10 min) and the sensitivity of this selective assay (detection limit 0.2 μg/ml) make it suitable for pharmacokinetic studies and monitoring. This method will also be useful in any case where only very small volumes of sample are available, as in the determination of apalcillin levels in paediatric subjects.

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