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Note

High-performance liquid chromatographic assay of ampicillin and its prodrug lenampicillin

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Ampicillin (AP), D(−)-α-aminobenzylpenicillin, is a widely used semisynthetic penicillin-like drug (Fig. 1). A series of AP derivatives, produced by esterification of the carboxyl group at C-3, have been developed to improve its oral bioavailability. These include pivampicillin, talampicillin, bacampicillin (BAP) and the novel prodrug lenampicillin (LAP), obtained by esterification of AP with an oxodioxolone (acetoin) group. In previous investigations, this prodrug has yielded a systemic bioavailability higher than that after administering AP as such[1,2]

In this work, two methods were standardized in order to investigate the *in vitro* stability of LAP and the comparative bioavailability of AP after the oral administration of LAP and BAP to twelve healthy volunteers, according to the cross-over design.

EXPERIMENTAL

Materials

Solvents and chemicals, all of analytical-reagent of high-performance liquid chromatographic (HPLC) grade, were supplied by Merck (Bracco, Milan, Italy). Ampicillin and its two esters investigated were supplied by Sigma Tau (Pomezia, Rome, Italy). A Varian Model 5020 liquid chromatograph, a Model 2050 variable-wavelength detector UV (Varian, Sunnyvale, CA, U.S.A.) and a 50-μl fixed loop (Rheodyne, Cotati, CA, U.S.A.) were used for all analyses. The column was a μBondapak C_{18}, 5 μm (300 × 3.9 mm I.D.) supplied by Waters Assoc. (Milford, MA, U.S.A.). The statistical computations were performed on a Macintosh Plus personal computer (Apple Computer, Cupertino, CA, U.S.A.).

Methods

A 0.5-ml sample of plasma was deproteinized with 1 ml of methanol. After stirring for 5 min and centrifuging at 2400 *g* for 10 min, 1 ml of the supernatant was separated and 2 μg of cefazolin as internal standard (in the pharmacokinetic method)

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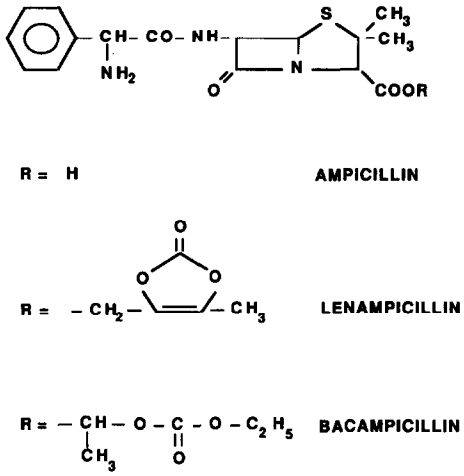

Fig. 1. Structures of ampicillin and its two prodrugs studied.

was added. The mobile phase used consisted of methanol–0.067 *M* KH_2PO_4 in ratios of 35:65 in the stability investigation and 20:80 in the bioavailability study. The flow-rate was 1.5 ml/min and absorbance was monitored at 225 nm in both methods. As internal standards, (I.S.) *o*-tolylpiperazine and cefazolin, respectively, were used. Figs. 2 and 3 show typical chromatograms of the analytical substances under the HPLC conditions validated for the stability and bioavailability investigation, respectively.

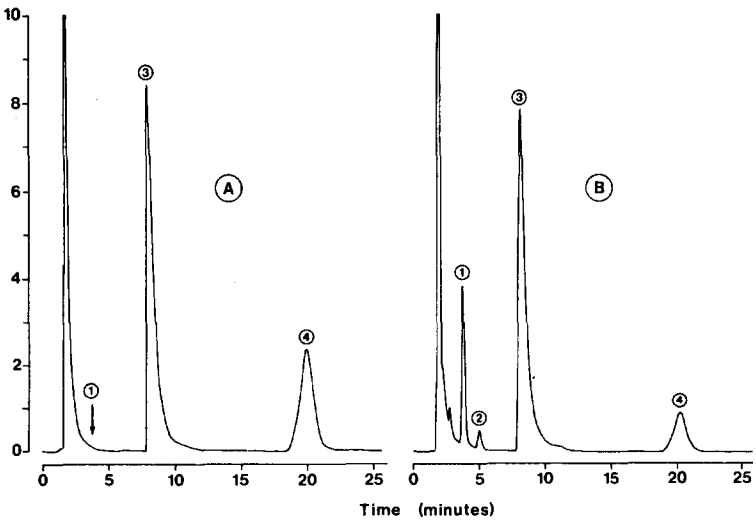

Fig. 2. Chromatograms of (1) ampicillin, (2) unknown, (3) *o*-tolylpiperazine (I.S.) and (4) lenampicillin. Column, μBondapak C_{18}, 5 μm (300 × 3.9 mm I.D.); mobile phase, methanol–0.067 *M* KH_2PO_4 (35:65); flow-rate, 1.5 ml/min; detection, UV, 225 nm. (A) Incubation with phosphate buffer (pH 7.4) at time 0; (B) after 4 h of incubation.

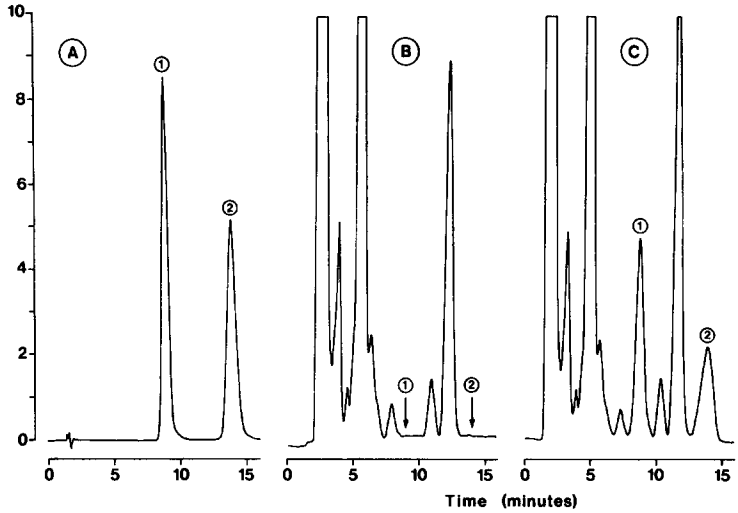

Fig. 3 Chromatograms of (1) ampicillin and (2) cefazolin (I.S.). Column as in Fig. 2; mobile phase, methanol–0.067 *M* KH_2PO_4 (20:80); flow-rate, 1.5 ml/min; detection, UV, 225 nm. (A) Authentic standards; (B) blank plasma; (C) plasma from a volunteer treated with lenampicillin (18.7 μg/ml plasma).

The bioavailability investigation of the two AP prodrugs was carried out on twelve healthy volunteers of both sexes, ranging in age from 18 to 40 years, to whom LAP and BAP (800-mg tablets in both instances) were administered by the oral route according to a randomized cross-over design. The AP concentrations were evaluated in serial plasma samples and in urine excreted over 0–3, 3–6, 6–12 and 12–24-h periods following administration.

RESULTS AND DISCUSSION

Both methods were validated in terms of linearity, reproducibility, specificity and sensitivity. The method used in the stability trial proved to be linear in the range 0.5–10 μg with an inter-assay coefficient of variation (%C.V.) of 0.43 and a detection limit of 1.5 μg/ml.

The other method, used in the bioavailability investigation of the two ampicillin prodrugs, allowed AP to be evaluated with a more favourable signal-to-noise ratio and hence greater sensitivity, the detection limit being 0.5 μg/ml. The linearity of this method was verified in the range 0.1–10 μg of AP and cefazolin as I.S., injected at drug-to-internal standard ratios of 1:1 (C.V. = 0.80%) and 1:4 to 4:1 (C.V. = 0.48%), respectively. The recovery of AP from plasma was 97.6%, linear in the range of 0.5–25 μg/ml, with a correlation coefficient $r = 0.9997$ (Table I).

As ampicillin is a hydrophilic molecule, it could not be extracted by solvent partition. The plasma deproteinization with methanol did not completely clean the sample, as shown in the blank plasma chromatogram (Fig. 3). In any case, a good signal-to-noise ratio was achieved when the ampicillin peak was at 9.00 min and cefazolin was selected as the I.S. The latter is eluted at 14.00 min, where no interfering base peaks are present, and showed a detection limit of 0.5 μg/ml. This method can

TABLE I

RECOVERY OF AMPICILLIN FROM PLASMA IN QUADRUPLICATE TRIALS

The linear regression method gave the correlation $y = 0.062 + 0.959x$; $r = 0.9997$.

Ampicillin added (x) (μg/ml)	*Ampicillin recovered (y)*		*Recovery (%)*
	Mean (μg/μl) (n = 4)	*C.V. (%)*	
–			
0.5	0.5	2.52	100.0
1	1	0.62	100.0
2	1.93	2.88	96.5
5	4.85	1.43	17.0
7.5	7.15	2.96	95.3
10	10.1	4.21	101.0
15	14.25	5.01	95.0
25	24.02	1.49	96.1
			Mean: 97.6 ± 2.36
			%C.V. 2.42

therefore be used in pharmacokinetic and bioavailability investigations as an alternative to the microbiological assay[3] for either ampicillin or cefazolin.

When stored at 37°C for 30 min in blood samples, ampicillin proved to be stable and lose about 2–3% of its titre in 2 h. Hence serum can be used instead of blood.

Ampicillin proved to have poor stability in aqueous solution, mainly in the presence of perchloroacetic acid. For this reason, methanol was used instead for deproteinizing plasma, with injection into the column just after the extraction, thus achieving good stability. When stored in a freezer at −20°C, ampicillin in plasma samples proved to be stable for *ca.* 10–15 days.

When administered to healthy volunteers, both LAP and BAP proved to be

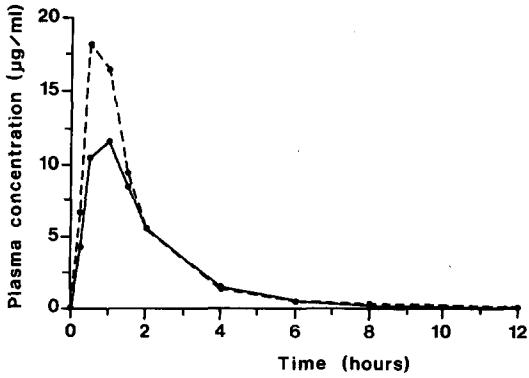

Fig. 4. Plasma concentration of ampicillin *vs.* time after oral administration of lenampicillin (dashed line) and bacampicillin (solid line) tablets (800 mg per subject in both instances) to healthy volunteers. Values lower than the detection limit (0.5 μg/ml) were taken as zero with respect to the mean; other values are means of twelve results.

well absorbed through the intestine, giving plasma concentrations of AP measurable over a 6-h period with BAP and an 8-h-period with LAP (Fig. 4). The cumulative urinary excretion of AP in the 0–24-h period was 561 mg (39%) after administration of LAP and 203 mg (36%) after administration of BAP.

When evaluated from the area under the curve, the systemic bioavailability of ampicillin in healthy volunteers after administration of LAP proved to be 26% higher than after administration of BAP (Fig. 4), the greatest difference being observed in the absorption phase and demonstrated by the C_{max} (peak concentration) values 18.14 and 11.65 μg/ml for LAP and BAP administration, respectively (Table I).

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