Journal of Chromatography, 277 (1983) 251—259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1791

QUANTITATIVE HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF AMPICILLIN IN HUMAN URINE

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(First received February 15th, 1983; revised manuscript received May 6th, 1983)

SUMMARY

A quantitative high-performance thin-layer chromatographic (HPTLC) method was developed for the analysis of ampicillin in urine. A dioxane—water—*n*-butanol—formic acid mobile phase system and HPTLC Silica gel 60 F254 as stationary phase were used. Quantitation was realized on a Zeiss PMQ 2 densitometer connected to a Varian A-25 recorder and an Apple II microcomputer or alternatively with an HP 9830A computer and HP 9862A plotter. A good linear range of detection (0.05—1.00 μg) at 480 nm was obtained. Standard statistical methods demonstrated good reproducibility (coefficient of variation is not greater than 3%). The method is appropriate for ampicillin quality control and pharmacokinetic studies.

INTRODUCTION

Ampicillin (D-(—)-α-aminobenzylpenicillin, anhydrous or as trihydrate or sodium salt), derived from 6-aminopenicillanic acid, is an antibiotic used therapeutically on a wide scale because of its low toxicity and its biological activity against a broad spectrum of Gram-positive and several Gram-negative pathogens. Its pharmacokinetic properties (relatively rapid systemic absorption, small extent of biotransformation and nearly complete elimination in the urine) ensure its high applicability in the treatment of systemic and urinary infections [1—3]. The assay of ampicillin currently consists of the following

0378-4347/83/$03.00

official and non-official procedures: microbiological agar diffusion [4], iodine titration [5], spectrophotometry [6, 7], non-aqueous acid and base titration [8], fluorometry [9], high-performance liquid chromatography [10—14], and thin-layer chromatography [15]. The microbiological assay proved to be slow and tedious and to lack precision and specificity. The chemical and spectrophotometric assay require available functional groups and as such are limited by the fact that they depend on reactions that may also occur with other components of the sample. High-performance liquid chromatography (HPLC), because of its effective separation, selective detection and appropriate sensitivity, reproducibility, accuracy and precision, offers the best potential for quantitating the intact drug and/or its degradation products or metabolites in stability, pharmacokinetic and quality control studies. Thin-layer chromatography and especially high-performance thin-layer chromatography (HPTLC) are becoming, due to the great evolution of microelectronics (densitometers connected to microcomputers) and appropriate equipment (chromatographic plates, spotting, developing and visualization equipment), methods equivalent to HPLC in terms of all the mentioned criteria and superior in terms of flexibility [16, 17].

Because none of the mentioned references provides a rapid and flexible analytical method applicable to pharmacokinetics, when a large number of blood and urine samples must be analysed, this study was undertaken to develop a selective and reliable method for ampicillin analysis in human urine using HPTLC. The second reason for the present work arises from the intention to ascertain the applicability of HPTLC among the other analytical methods used in pharmacokinetics.

EXPERIMENTAL

Apparatus

Investigations were carried out at various times with two different systems: (1) a Zeiss PMQ 2 densitometer (Opton, 7082 Oberkachen, F.R.G.) connected to an A-2 recorder (Varian, Palo Alto, CA, U.S.A.) and an Apple II microcomputer (Apple Computer Inc., CA, U.S.A.); and (2) a Zeiss PMQ 2 densitometer connected with an HP 9830 A calculator and an HP 9862 A plotter (Hewlett-Packard, CA, U.S.A.).

Chromatographic plates, spotting and developing equipment

The satisfactory plates were HPTLC plates of silica gel 60 F254 for nano-TLC, 0.25 mm thick, 10 × 10 cm (E. Merck, Darmstadt, F.R.G.). By means of Drummond microcaps (1 μl), 1-μl aliquots of the samples were spotted onto the chromatographic plates. The spots were dried and developed at room temperature with the solvent system (mobile phase) in a Camag twin-trough chamber, 20 × 10 cm, previously saturated with solvent vapours. Development was continued until the distance between the origin and the solvent front was 7 cm. The plates were air-dried, sprayed with ninhydrin solution and treated for 5 min at 110°C to visualize the spots. The R_F of ampicillin was 0.65.

Chemicals and reagents

Ampicillin trihydrate, 860 μg/mg activity, was obtained from the Institute for Pharmacy and Drug Quality Control, Ljubljana, Yugoslavia.

Ninhydrin reagent (0.3 g of ninhydrin, 100 ml of *n*-butanol and 3 ml of glacial acetic acid) was prepared according to the method of Stahl [18].

Ammonium sulphate, p.a., chloroform, p.a., benzalkonium chloride, p.a., dioxane, p.a. and formic acid, p.a., were also used.

Preparation of solutions and chromatographic plates

Solvent for standard solution. This was a mixture of dioxane—water (4:1) with formic acid to give pH 5.

Mobile phase. This was a mixture of dioxane—water—*n*-butanol—formic acid (70:15:15:1.25).

Standard solution. An amount of the working ampicillin standard equivalent to 290.697 mg was weighted on a Mettler H542 balance and dissolved in 250 ml of the solvent for standard solution. The solution was shaken and ultrasonicated until it became totally clear.

Chromatographic plates for the calibration curve and ampicillin extraction ratio from urine. Solutions with 0.1, 0.15, 0.20, 0.25, 0.35, 0.40, 0.50 and 0.86 μg/ml were prepared by diluting the standard solution, and spotted onto the chromatographic plates parallely without and with 1 μl of urine extract. As we wanted to obtain the ampicillin extraction ratio from urine, a known amount of ampicillin was added to urine which was further treated in the same manner as that with ampicillin after oral administration.

Chromatographic plates for the statistical evaluation of the method. Ten 1-μl aliquots containing 0.5 μg of ampicillin in dioxane—water mixture were spotted on plate 1; another ten aliquots containing 0.5 μg of ampicillin in urine extract were spotted on plate 2; plates 3 and 4 were arranged in the same way but contained 1.5 μg of ampicillin. Plates 1 and 2 were evaluated on the recorder without subtracting the base line and the line of the blank sample, while plates 3 and 4 were evaluated on the plotter after subtracting the base line and the line of the blank sample.

Conditions for quantitation

Densitograms were obtained using the densitometer in the remission mode [16, 17] at 480 nm, slit 3.5 mm wide and 0.1 mm high, table 200 mm/min *y* axis; *x* axis was adjusted manually, white background.

Protocol of in vivo pharmacokinetic study and preparation of urine samples

The pharmacokinetic study was carried out with three male volunteers (VI, VII, VIII). Each of them swallowed in the evening a capsule of Ampicillin® (Lek, Ljubljana) containing 500 mg of ampicillin trihydrate and urine was collected during the night (8 h). VIII repeated the trial three times. Next morning, urine volumes were measured and 10-ml aliquots were transferred into separating funnels together with 3 g of ammonium sulphate. Separately, we prepared 30 ml of chloroform with 500 mg of benzalkonium chloride. It was added in three aliquots to each separating funnel and ampicillin was extracted from the aqueous to the organic phase. The extracts were collected

in round-bottomed flasks and chloroform evaporated at 40°C to give a volume of less than 10 ml. The solutions were transferred into 10-ml volumetric flasks which were filled to volume with chloroform. Aliquots (1 μl) of the samples were spotted onto the chromatographic plates.

Assay and calculations

Ampicillin concentrations in urine samples were determined using statistically treated relation between ampicillin quantity on the chromatogram and area under the Gaussian curve (AUC), resulting from the densitometric measurements. Eqns. 1 and 2 were selected to obtain the calibration curve and ampicillin concentrations in the urine samples:

$$\mathrm{AUC} = A \cdot h \tag{1}$$

$$\mathrm{AUC} = (b \pm e_b) + (a \pm e_a) \cdot Q \tag{2}$$

where AUC = area under the Gaussian curve, A = width of the peak at half height, h = height of the peak from the base line, Q = ampicillin quantity in the spot, a and b = linear regression coefficients for the calibration curve — the slope of the calibration curve and intercept with AUC axis when Q is zero, and e_a and e_b are the errors of a and b. The data from the chromatograms were transferred to computer memory. The base line and the line of the blank sample were subtracted and the corrected Gaussian curve reflected the amount of ampicillin in the sample. The reproducibility of the method was tested using standard statistical parameters: arithmetic mean, variance, standard deviation, relative standard deviation (coefficient of variation), standard error, relative standard error and 95% confidence interval of the mean.

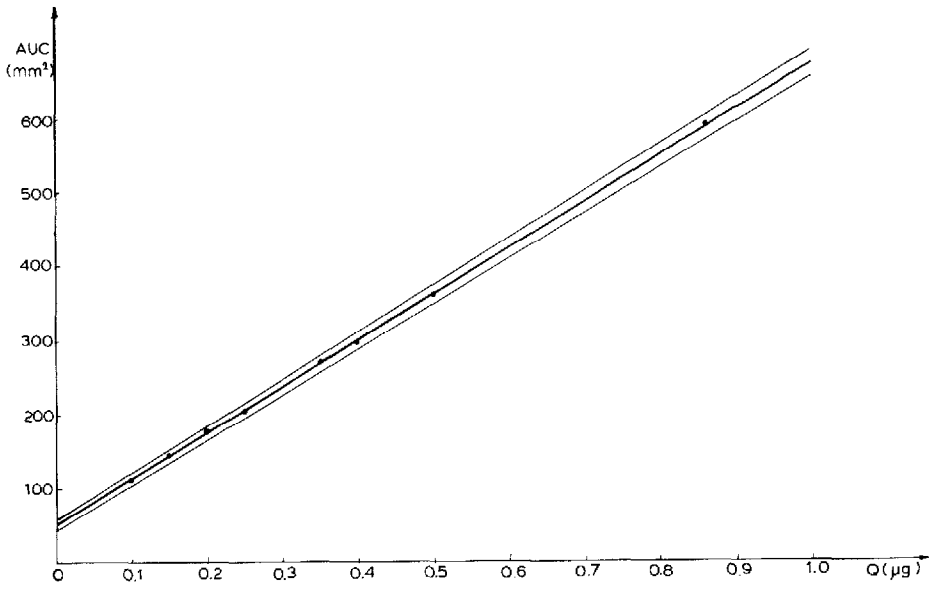


Fig. 1. Calibration curve for densitometric determination of ampicillin in water and urine. AUC = (51.21 ± 6.65) + (621.17 ± 8.06)Q. r = 0.9998, n = 16, P = 0.05, each point represents the mean of two measurements.

RESULTS

Ampicillin trihydrate was dissolved in dioxane–water using formic acid as pH moderator. The solution was easily spotted on the chromatographic plate, rapidly dried and after development gave spots of suitable quality. It is well known that ampicillin is unstable in aqueous solutions and the pH of its maximal stability coincides with the pH of its minimal solubility (pH 5) [19]. The mentioned fact did not represent the limitation, because our working concentrations at pH 5 were greater than the ampicillin intrinsic solubility ($C_0 = 0.0223\ M$).

Calibration

Fig. 1 shows the calibration curve for densitometric determination of ampicillin in water and urine using eqns. 1 and 2. Base line and line of the blank sample which result from endogenous urine components were subtracted by computer. The extraction ratio, obtained using the calibration curve and benzalkonium chloride as phase-transfer catalyst, was 82.5%.

The results of the statistical evaluation are summarized in Table I.

TABLE I

STATISTICAL PARAMETERS OF AUC VALUES USED FOR DENSITOMETRIC DETERMINATION OF AMPICILLIN

	Plate 1	Plate 2	Plate 3	Plate 4
Arithmetic mean	378.4	384.7	1050.0	1136.4
Variance	219.5	299.4	902.8	1255.1
Standard deviation	14.8	17.3	30.0	35.4
Relative standard deviation (%) (coefficient of variation)	3.9	4.5	2.9	3.1
Standard error	4.7	5.4	9.5	11.2
Relative standard error (%)	1.2	1.4	0.9	1.0
95% Confidence interval of the mean	±10.6	±12.3	±21.5	±25.3

Quantitative analysis

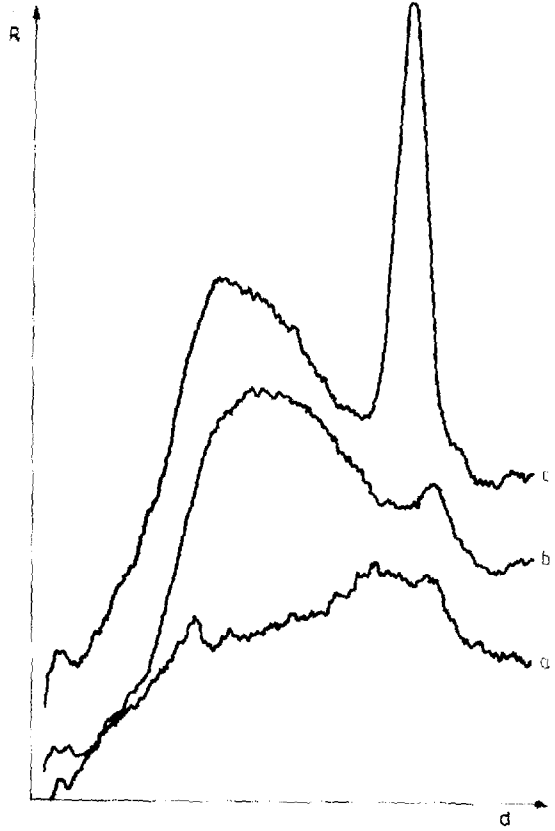
Figs. 2 and 3 illustrate use of the computer to subtract the base line and the line of the blank sample to give the corrected ampicillin curve.

Figs. 4 and 5 show curves for ampicillin in urine after extraction of known and unknown quantities of ampicillin.

Chromatographic parameters for the samples and their quantitative evaluation are given in Table II. Ampicillin quantities in spots are calculated considering the extraction ratio. Table III shows the calculation of concentrations of unmetabolized ampicillin in urine after oral administration of a 500-mg capsule.

DISCUSSION

The results obtained show that the HPTLC method for the determination of unchanged ampicillin can be used in in vitro as well as in in vivo ampicillin



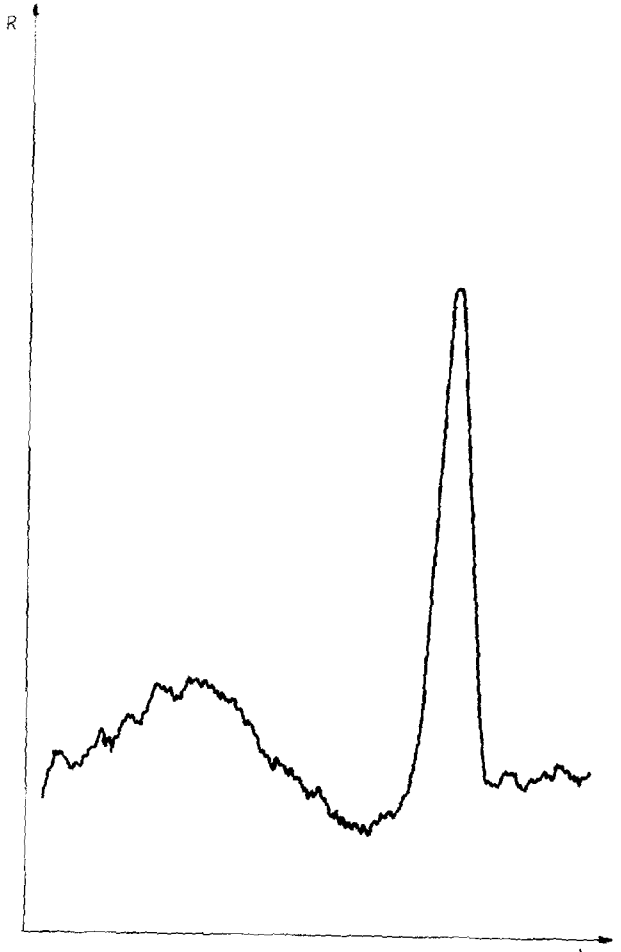


Fig. 2. Base line curve (a), black sample curve (b) and ampicillin curve (c) when ampicillin solution is spotted together with urine extract. R = percentage remission, d = distance in cm.

Fig. 3. Corrected ampicillin curve after subtracting base line and line of blank sample.

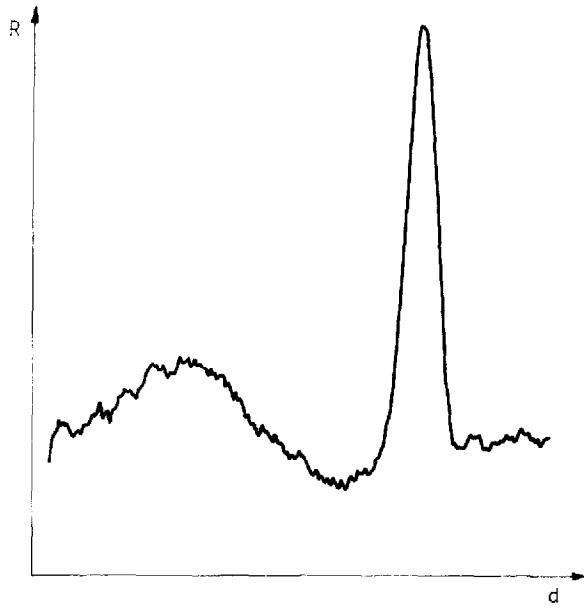


Fig. 4. Ampicillin curve after extraction from urine (original concentration = 0.86 μg/μl, concentration after extraction = 0.71 μg/μl).

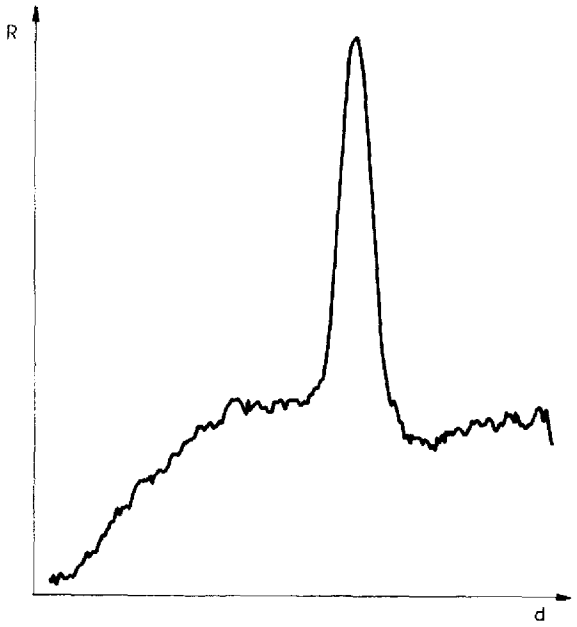


Fig. 5. Ampicillin curve after oral administration and sample extraction for VIII, third experiment.

studies. In drug quality control it could be the method of choice for several reasons: it is rapid, it enables the simultaneous measurement of a large number of samples under the same conditions, and it shows great reproducibility and

TABLE II

CHROMATOGRAPHIC PARAMETERS FOR AMPICILLIN DETERMINATION IN URINE SAMPLES

Sample	Peak height (cm)	Peak width at half height (mm)	AUC (mm^2)	Ampicillin quantity in spot (μg)
VI 1	5.2	7.0	364.0	0.606
VII 1	4.5	6.5	292.5	0.436
VIII 1	3.6	6.2	223.2	0.327
VIII 2	5.1	6.8	346.8	0.569
VIII 3	6.3	6.5	409.5	0.690
VIII 4	6.3	6.5	409.5	0.690

TABLE III

URINARY AMPICILLIN CONCENTRATIONS AND QUANTITIES, APPROPRIATE URINE VOLUMES, AND PERCENTAGE OF UNMETABOLIZED AMPICILLIN IN URINE AFTER ORAL ADMINISTRATION OF 500 mg OF AMPICILLIN

Sample	Urinary ampicillin concentration (mg/ml)	Urine volume (ml)	Quantity of urinary ampicillin (mg)	Percentage of unchanged ampicillin excreted
VI 1	0.606	300	181.8	42.27
VII 1	0.436	410	178.7	41.55
VIII 1	0.327	450	147.6	34.33
VIII 2	0.569	250	142.2	33.08
VIII 3	0.690	220	151.8	35.30
VIII 4	0.690	210	144.9	33.69

sensitivity compared to other methods, especially the official ones. The method is particularly suitable for pharmacokinetic studies. The criteria for an analytical method to be used in pharmacokinetics are fulfilled and the results obtained for urinary ampicillin concentrations coincide well with those in the literature [20].

Since the urinary ampicillin concentrations in the kinetic study (urine sample collection during appropriate time intervals) were higher than 0.1 mg/ml, the sensitivity of the method was not tested separately. However, the limit of quantitation per ml of urine can be given, i.e. 0.05 mg/ml. The proposed method for ampicillin, whose metabolites are therapeutically inactive and are present in very low concentrations in urine [12], proved to be very useful for ampicillin antimicrobial activity evaluation under in vivo conditions.

The proposed method demonstrates some advantages of HPTLC over other chromatographic methods: the simultaneous measurement of a large number of samples, a very flexible system for absorption and fluorescence measurements, scanning of spectra directly from densitograms, separate chromatographic and detection systems, impure samples do not interfere (clean-up is not needed), and very dilute solutions can be spotted repeatedly. Significant improvement was attained using the Apple microcomputer, which had control

of scanning on the densitometer, preserves the data on discs and defines the integrating conditions for AUC calculations. It computes, comparing with standard values, appropriate statistical parameters and prints out the results in full. Using the specific programs for subtracting the base line and the line of blank sample, better reproducibility and accuracy were obtained.

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