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ANALYSIS OF ISOXAZOLYL PENICILLINS AND THEIR METABOLITES IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H.H.W. THIJSSEN

Department of Pharmacology, University of Maastricht, Biomedical Centre, P.O. Box 616, 6200 MD Maastricht (The Netherlands)

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SUMMARY

A high-performance liquid chromatographic assay method to quantitate the isoxazolyl penicillins, their active metabolites, and their penicilloic acids in serum or urine is described. Separation and analysis is performed using reversed-phase chromatography. Urine samples, after the appropriate dilution, can be assayed directly. Serum samples (0.1 ml) are either extracted with methylene chloride or treated with perchloric acid—methanol. Serum levels as low as 0.4 μg/ml (extraction procedure) can be assayed accurately.

INTRODUCTION

In studying the disposition of antibiotics, an accurate and rapid assay procedure that differentiates between the different compounds or metabolites is preferred over the well-known bioassay, especially when it is recognized that active metabolites are formed. High-performance liquid chromatography (HPLC), particularly the reversed-phase mode, has found wide application as an analytical tool to quantitate, among others, antibiotic drugs in biological fluids [1—5].

The formation of active metabolites of the isoxazolyl penicillins (the active metabolites are the 5-hydroxymethyl derivatives of the parent compounds [6]) has been described in some detail and it was shown that in anephric patients the active metabolite may represent 40—50% of the total penicillin activity [7]. The parent compound and the metabolite were assayed by bioassay after separation by reversed-phase thin-layer chromatography. A further investigation of the clinical relevance of the formation of active metabolites of the iso-

xazolyl penicillins in anephric patients [8] prompted the development of a rapid and reliable assay technique. This paper describes a reversed-phase HPLC method to determine the isoxazolyl penicillins, their 5-hydroxymethyl metabolites and their penicilloic acids in plasma, serum or urine.

EXPERIMENTAL

HPLC instrumentation and conditions

The HPLC system consisted of a Waters M-6000A pump equipped with a U6K injector. UV absorbance at 220 nm was monitored with a Perkin-Elmer LC-55 spectrophotometer. A Lichrosorb RP-8 column (particle size 5 μm; 250 mm × 4.6 mm I.D.; Chrompack, Middelburg, The Netherlands) was used in combination with a guard column (ODS pellicular material; 50 mm × 2.1 mm I.D.) to extent life time. The mobile phase was either a buffer—methanol or a buffer—acetonitrile mixture.

Materials

Sodium oxacillin and sodium dicloxacillin were gifts of Bristol Labs. (The Netherlands); sodium cloxacillin and sodium flucloxacillin were gifts of Beecham Research Labs. (The Netherlands). The active metabolites were isolated from rat urine as described previously [6]. The corresponding penicilloic acids were prepared by treating solutions of the isoxazolyl penicillins with alkaline (50 μl 1 *N* sodium hydroxide to 1 mg of the penicillin dissolved in 1 ml of water) for 30 min at room temperature whereafter the reaction mixture was neutralized with hydrochloric acid.

Sample preparation

Samples were analyzed with the aid of an internal standard. One of the isoxazolyl penicillins was added to the sample before the work-up procedure. Plasma (serum) samples were either treated with methanol—perchloric acid or extracted with methylene chloride. In the former case, to 100 μl sample spiked with the internal standard, 300 μl of ice cold 0.05 *N* perchloric acid in methanol was added. The mixture was allowed to stand for 10 min in ice. Aliquots of the mixture, clarified by centrifugation at 0°C, were injected. In the extraction procedure, to 100 μl sample spiked with the internal standard, 100 μl citric acid buffer (0.5 *M*, pH 2.2) and 20 μl 0.5 *N* hydrochloric acid were added. Extraction was performed with 2.5 ml methylene chloride. The organic phase was evaporated to dryness at 35°C by a stream of nitrogen. The residue was redissolved in an appropriate volume of mobile phase. Urine samples were diluted accordingly with water.

RESULTS AND DISCUSSION

Good resolution of the isoxazolyl penicillins and their 5-hydroxymethyl metabolites could be achieved on LiChrosorb RP-8 with either buffer—methanol mixtures [for instance: 0.02 *M* sodium acetate (pH 5.5)—methanol (10:8, v/v)] or buffer—acetonitrile mixtures [for instance: 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v)]. The former mobile phase system only was

TABLE I

CAPACITY FACTORS, k', OF THE ISOXAZOLYL PENICILLINS, THE 5-HYDROXYMETHYL METABOLITES (M) AND THE PENICILLOIC ACIDS (PA)

Compound	Mobile phase*	
	I	II
Oxacillin	2.70	1.92
M	1.43	0.96
PA	0.86	–
Cloxacillin	3.55	2.95
M	1.80	1.46
PA	1.20	–
Flucloxacillin	4.00	4.19
M	2.10	2.19
PA	1.53	–
Dicloxacillin	6.00	6.23
M	2.70	2.96
PA	2.30	–

* I, 0.02 *M* sodium acetate (pH 5.5)—methanol (10:8, v/v); II, 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v).

suitable for analysing the penicilloic acids. In the buffer—acetonitrile mobile phase the penicilloic acids eluted with the unretained solute (Table I). At first these results made it obvious to select the buffer—methanol system. Once in use, however, some disadvantages were discovered. Besides a higher working pressure, the main problem appeared to be the instability of the penicillins. Fig. 1 shows the degradation of cloxacillin with time. The reaction product, which probably was the monomethyl ester of the α-penicilloate [9], was less

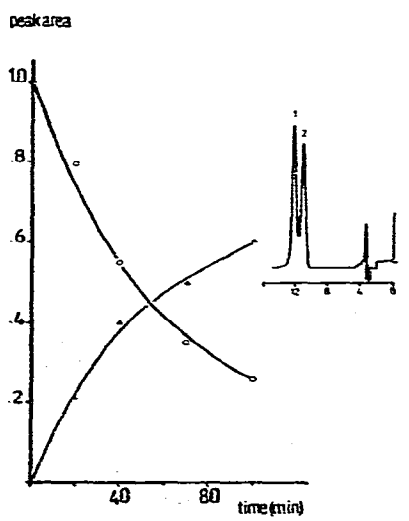

Fig. 1. Degradation of cloxacillin in methanol. Cloxacillin solution (100 µl) (1 mg cloxacillin in 1 ml 0.9% sodium chloride in water) was diluted with methanol to 1 ml. At different time intervals aliquots were injected for analysis. Cloxacillin, O; product, ▲, Insertion: chromatogram of the reaction mixture after 40 min at room temperature. Cloxacillin, 1; product, 2. Chromatographic conditions: mobile phase, 0.02 *M* sodium acetate (pH 5.5)—methanol (100:75, v/v); flow-rate, 1.2 ml/min; timescale in minutes.

retained on the column. The observed methanolic degradation was slowed down considerably in the cold.

The extent of the detector response per absolute amount (expressed in peak area) at 220 nm appeared to be the same for the isoxazolyl penicillins (Table II). UV absorption per mass equalled the parent compounds for the 5-hydroxymethyl metabolites and the penicilloic acids. It was therefore obvious to use one of the isoxazolyl penicillins as the internal standard for the sample analysis.

TABLE II

DETECTOR RESPONSE (PEAK AREA, mm^2) AT 220 nm

Chromatographic conditions: mobile phase, 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v); flow-rate, 1 ml/min; detector sensitivity, 0.01 a.u.f.s.; chart speed, 5 mm/min.

Amount injected (ng)	Penicillin*			
	O	Cl	FCl	Cl_2
40	144	140	144	146
100	358	361	362	361
500	1785	1838	1822	1800

* Oxacillin, O; cloxacillin, Cl; flucloxacillin, FCl; dicloxacillin, Cl_2.

Plasma or serum samples in which the amount of penicilloic acid was also to be analyzed were handled by the methanol—perchloric acid treatment (see Experimental). The extraction procedure, which resulted in much cleaner samples, could not be applied because the recovery of penicilloic acids was shown to be poor and most often degradation was observed due to the acid treatment [9]. When the serum penicilloic acid levels were not important, the methylene chloride extraction procedure was used. By preference, the buffer—acetonitrile system was chosen as the mobile phase.

Examples of chromatograms obtained under different chromatographic conditions from biological specimens containing isoxazolyl penicillin and its metabolic products are shown in Fig. 2.

Using the internal standard procedure, the accuracies for the different sample preparation methods were 100 ± 16 (S.D., n = 12, concentrations $\geqslant$ 1 μg/ml) and 100 ± 6 (S.D., n = 11, concentrations $\geqslant$ 0.4 μg/ml) for the methanol—perchloric acid method and the extraction method, respectively. Serum concentration—time profiles and the cumulative excretions of cloxacillin and flucloxacillin and their respective metabolites after their oral administration to a healthy fasting individual (1.5 g in capsules) are shown in Fig. 3. The same volunteer participated in these experiments. The time between the first and second experiment was two weeks. As can be seen, the formation of the 5-hydroxymethyl metabolites was low. Serum levels were low (peak ratios for metabolite and parent compound 1/16 and 1/25 for cloxacillin and flucloxacillin, respectively) and small amounts were excreted in urine (10% or less of the total penicillin amount).

The amount of the penicilloic acids excreted in urine over the time period of collection was even less. The serum levels, however, appeared to be higher and, most striking, their half-lives were at least twice as long as the half-lives of the

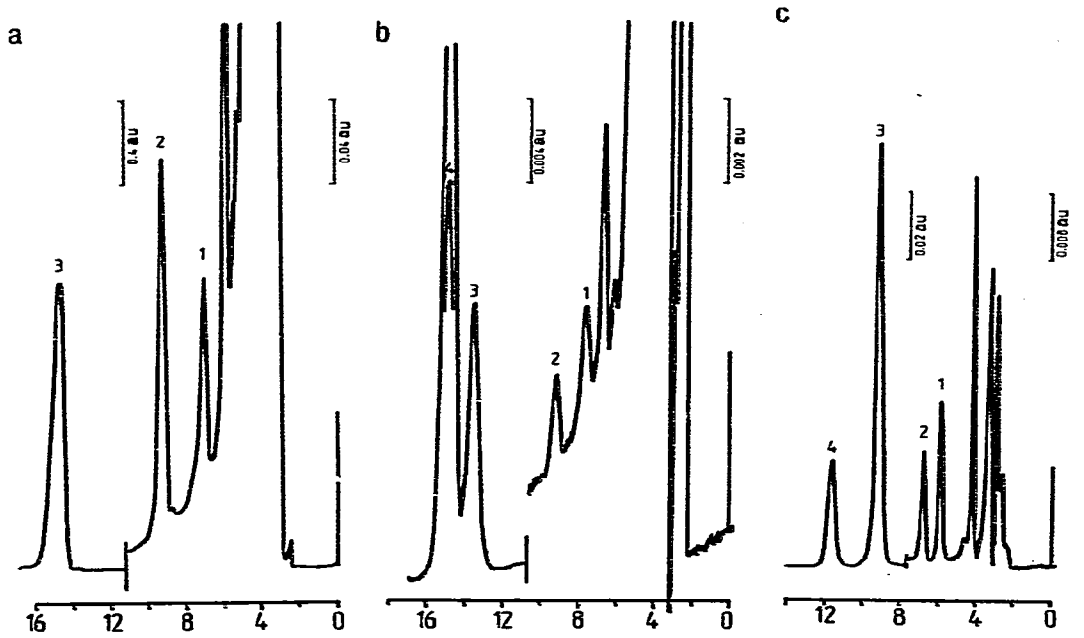

Fig. 2. Chromatograms of (a) urine sample after the oral administration of flucloxacillin. The urine sample was diluted twenty fold with water, 20 μl were injected; peaks: 3, flucloxacillin, 1320 μg/ml; 2, 5-hydroxymethyl metabolite, 122 μg/ml; 1, penicilloic acid, 54 μg/ml. Chromatographic conditions: mobile phase, 0.02 *M* sodium acetate (pH 5.5)—methanol (10:8, v/v), flow-rate 0.8 ml/min; timescale in minutes. (b) Serum sample after the oral administration of flucloxacillin. 100 μl serum, spiked with cloxacillin, 10 μg/ml, was treated with 300 μl 0.05 *N* perchloric acid in methanol (see Experimental); peaks: 4, flucloxacillin, 35.2 μg/ml; 3, cloxacillin, 10 μg/ml; 2, 5-hydroxymethyl metabolite, 1.3 μg/ml; 1, penicilloic acid, 1.5 μg/ml. Chromatographic conditions as in (a). (c) Serum sample after the oral administration of cloxacillin. 100 μl serum, spiked with flucloxacillin, and oxacillin, (5 μg/ml and 1 μg/ml respectively), were extracted with methylene chloride (see Experimental); peaks: 4, flucloxacillin, 5 μg/ml; 3, cloxacillin, 14.6 μg/ml; 2, oxacillin, 1 μg/ml; 1, 5-hydroxymethyl metabolite, 1.3 μg/ml. Chromatographic conditions: mobile phase, 0.02 *M* ammonium acetate (pH 6.6)—acetonitrile (100:34, v/v); flow-rate, 0.8 ml/min; timescale in minutes.

parent compounds (for instance, 170 and 69 min for the penicilloic acid and cloxacillin, respectively). A clear picture of the fate of the penicilloic acids in plasma (serum) after the administration of a penicillin is not yet known. Cole et al. [10] assumed on the basis of the prolonged urinary excretion of the penicilloic acids that their plasma half-lives should be longer than the half-lives of the corresponding penicillins.

Helström et al. [11] observed a plasma radioactivity half-life of about 150 min after the administration of [^{35}S]cloxacillin which is compatible with the serum half-life of the cloxacillin penicilloic acid found in the present study.

Some relevant pharmacokinetic data, describing the experiments from Fig. 3, are summarized in Table III. Although the data are from one subject only, the kinetic parameters show that one of the factors determining the higher serum levels after an oral dosage of flucloxacillin as compared with cloxacillin is the slower elimination of the former (compare renal clearances of the penicillins and the area under the curve values of the penicilloic acids). These findings are in agreement with earlier data [12, 13].

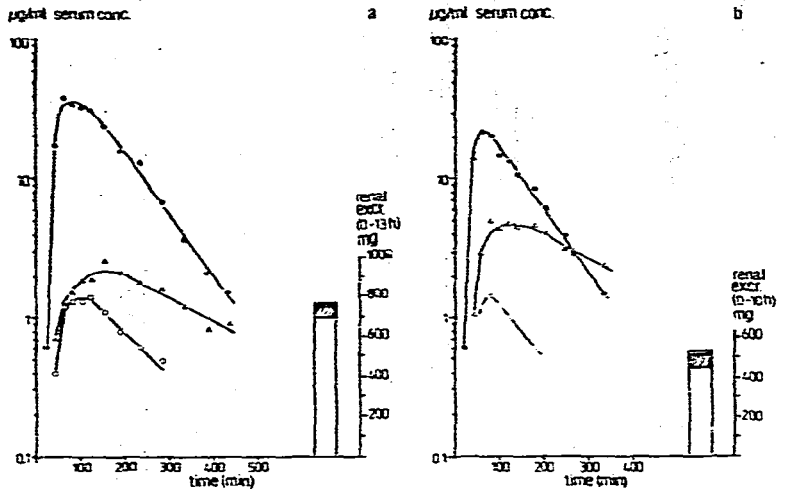

Fig. 3. Serum concentration—time profile and cumulative renal excretion of (a) flucloxacillin and its metabolites after an oral dose of flucloxacillin (1.5 g, capsules); serum: flucloxacillin, ●; 5-hydroxymethyl metabolite, O; penicilloic acid, △ and urine: flucloxacillin, □; 5-hydroxymethyl metabolite, ■; penicilloic acid, ▣. (b) Cloxacillin and its metabolites after an oral dose of cloxacillin (1.5 g, capsules); serum: cloxacillin, ●; 5-hydroxymethyl metabolite, ○; penicilloic acid, △ and urine: cloxacillin, □; 5-hydroxymethyl metabolite, ▨; penicilloic acid, ▤.

TABLE III

PHARMACOKINETIC PARAMETERS OF CLOXACILLIN AND FLUCLOXACILLIN AFTER ORAL ADMINISTRATION (FIG. 3)

M = 5-hydroxymethyl metabolite, PA = penicilloic acid. The serum half-life ($t_{½}$) was estimated from the elimination phase by the least-squar.. method. The area under the curve (AUC) was estimated by the trapezoidal rule. The area from the last serum data to infinity was calculated by $C_t \times t_{½} \times (\ln 2)^{-1}$. Renal clearance was estimated by dividing the amount excreted in urine by AUC.

Compound	$t_{½}$ (min)	AUC (µg/ml·min)	Renal clearance (ml/min)	fr*
Cloxacillin	69	2913	160	31.0
M	78	217	220	3.2
PA	170	1675	17	2.3
Flucloxacillin	68	7300	96	46.5
M	76	285	166	3.2
PA	170	819	26	1.4

* The percentage of the dose cumulatively excreted in the urine.

CONCLUSIONS

The HPLC analysis for the determination of the isoxazolyl penicillins, their 5-hydroxymethyl metabolites and their penicilloic acids is a simple, sensitive and reliable method. Its advantages over microbiological assays is evident because of its ability to differentiate between the various antibacterial active species in a sample.

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