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# DETERMINATION OF AMOXICILLIN IN BODY FLUIDS BY REVERSED-PHASE LIQUID CHROMATOGRAPHY COUPLED WITH A POST-COLUMN DERIVATIZATION PROCEDURE

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## SUMMARY

Quantitative methods for determination of amoxicillin in body fluids are described. They comprise separation by reversed-phase chromatography (LiChrosorb RP-8, 5 μm) of the aqueous supernatants obtained from plasma or urine after purification steps involving protein precipitation followed by extraction in the case of plasma, or a double extraction procedure in the case of urine, post-column derivatization with air segmentation, and finally measurement of the UV absorbance at 310 nm. The derivatization involves formation of the mercuric mercaptide of penicillenic acid and is specific for compounds with an intact penicillanic acid ring system.

Detection limits achieved on injecting 200 μl of plasma and 20 μl of urine are about 25 ng/ml and 200 ng/ml, respectively, but it is possible to improve the sensitivity further by injecting larger volumes. Precisions ($s_{rel}$) obtained for determination of 0.10 and 0.45 μg/ml in plasma were 3.72 and 1.40%, respectively.

Some problems regarding column stability originating from the injection of biological samples are discussed.

## INTRODUCTION

The analysis of ampicillin and mecillinam in biological material using high-performance liquid chromatography combined with post-column derivatization has previously been described [1]. The derivatives are penicillenic acid mercuric mercaptides formed by reaction of the penicillins with imidazole and mercuric chloride according to the method of Bundgaard and Ilver [2]. The same method is also, in principle, applicable to amoxicillin, but since this is a very polar compound which elutes early in reversed-phase liquid chromatographic systems, there are some problems regarding the appearance of the

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blank chromatograms. The disturbances were especially serious for urine samples. It was therefore found necessary to include cleaning-up extraction steps before the chromatographic step.

An analytical method for the determination of amoxicillin in plasma, urine and saliva based on reversed-phase liquid chromatography and direct UV absorption measurements has been published [3]. The limit of determination is about 0.5 μg/ml and the method can consequently not be applied for rigorous pharmacokinetic studies. Some methods based on fluorimetric assays of amoxicillin in plasma have also been published [4, 5].

## EXPERIMENTAL

### *Apparatus*

In the liquid chromatography experiments, the following components were used: Constametric II pump for the mobile phase, Haskel AO 15 pump for column packing, and the peristaltic pump Gilson Minipuls II for the reagent solution; LDC Spectromonitor II photometric detector; W. & W. 1100 recorder; Rheodyne Model 7120 injecting valve; columns of precision-bore stainless steel from Handy & Harman (length 100 mm, I.D. 4.0 mm, O.D. 1/4 in.) with modified zero dead volume, Swagelok® connections as column end-fittings.

Coils, mixing tees, debubblers, feed lines (polyethylene) and heating bath for the post-column derivatization were of Technicon quality. The eluent is mixed with the reagent in a mixing tee (PT2 116-B 000) connected to the column with a Teflon tube (3 cm long, I.D. 0.5 mm), and the outlet of the mixing tee, at an angle of 90° to the reagent and eluent streams, is connected to the injection fitting (116-0492-01C), where the air bubbles are introduced, by another Teflon tube (6—8 cm long, I.D. 0.9 mm, O.D. 1.3 mm) provided with a short (1 cm) and wide (I.D. 1.2 mm, O.D. 1.9 mm) Teflon tube to make a better fit into the injection fitting. The sequented stream then passes a small mixing coil (1 × 7 turns, I.D. 1.6 mm) before entering the reaction coil (6 cm long, I.D. 1.6 mm; 105-1128-01) that is kept at 40° in a heating bath. Into the debubbler (116-0203-01) a Teflon tube (10 cm long, I.D. 0.5 mm, O.D. 1.0 mm) is inserted in order to minimize the dispersion. At the end of this thin tube a wider Teflon tube is provided (I.D. 1 mm, O.D. 1.6 mm) for connection to the detector inlet stainless-steel capillary (I.D. 0.5 mm). At the outlet the detector is connected by a Teflon tube (I.D. 0.5 mm) to the peristaltic pump which sucks the liquid stream through the detector.

Pump tubes: reagent and air — orange—green (116-0549 PO 4), 0.32 ml/min; from detector — orange—white (116-0549 PO 6), 0.68 ml/min. Pump speed: 22 rpm.

Photometric measurements were performed on a Zeiss DMR 21 spectrophotometer with 10-mm quartz cells. The samples were mixed on a Fisons Whirlimixer and centrifuged in a Wifug X-1. The pH measurements were made with an Orion Digital Ionalyzer Model 801 A.

An ultrasonic bath (Bransonic 220) was used for homogenization and degassing of solvents.

*Reagents and chemicals*

LiChrosorb RP-8 (5 μm) (Merck, Darmstadt, G.F.R.) was used as chromatographic support.

Amoxicillin and ampicillin were obtained from the Department of Antibacterial Chemotherapy at Astra Läkemedel. Imidazole puriss p.a. (Fluka, Buchs, Switzerland) was recrystallized from toluene (375 g in 1.5 l), the precipitate being filtered and washed with diethyl ether (800 ml) and dried under vacuum in a desiccator overnight. The absorbance of an 8% aqueous solution at 310 nm is then about 0.06 (1-cm cell).

Mercuric chloride "pronalys" (May & Baker, Dagenham, Great Britain), methanol "zur Analyse", phosphoric acid p.a., sodium dihydrogen phosphate p.a. and disodium hydrogen phosphate p.a. from Merck were all used as received.

For protein precipitation and cleaning-up extractions the following reagents were used: tetrapropylammonium hydrogen sulphate (Labkemi, Gothenburg, Sweden), perchloric acid "zur Analyse", 70% (Merck), 1,2-dichloroethane, certified (Fisher Scientific, Pittsburgh, Pa., U.S.A.) and *n*-amyl alcohol, certified (Fisher).

The water was deionized. The phosphate buffer had an ionic strength of 0.1.

*Analytical methods*

*Sample preparation*

*Plasma.* To 1.00 ml of plasma, 1.0 ml of dichloroethane is added followed by 20 μl of perchloric acid (70%), and mixing is carried out on a whirlimixer for about 30 sec. After centrifugation at 1600 *g* for 3 min, the aqueous (upper) phase is filtered through a piece of cotton wool applied at the tip of a Pasteur pipette, and 200 μl of the clear solution are injected without delay on to the chromatographic column.

*Urine.* The special reagents used were: reagent A, 0.05 *M* tetrapropylammonium hydrogen sulphate in a mixture of *n*-amyl alcohol—dichloroethane (2:1, v/v), saturated with water; and reagent B, a mixture of *n*-amyl alcohol—dichloroethane (3:4, v/v), saturated with water. A 1.00-ml volume of urine is extracted with 4.0 ml of reagent A by mixing for about 20 sec on a whirlimixer. After centrifuging at 350—800 *g* for 2 min, the organic (upper) phase is discarded by aspiration and 4.0 ml of reagent B are added. After extracting by mixing on the whirlimixer for another 20 sec, the mixture is centrifuged at 350—800 *g* for about 2 min and 10—20 μl of the aqueous (upper) layer are injected without delay on to the chromatographic column.

*Chromatographic system*

The column dimensions were 100 × 4 mm I.D. The mobile phase was phosphate buffer (pH 8, ionic strength 0.1)—methanol (92:8, v/v) at a flow-rate of 1.0 ml/min.

*Post-column derivatization*

The derivatization reagent was an aqueous solution of imidazole (33%) and

mercuric chloride (0.11%) adjusted to pH 7.2 by hydrochloric acid and containing Brij 35 (0.12%). The flow-rate was 0.3 ml/min, the air bubble rate 1 $sec^{-1}$. The absorbance was measured at 310 nm.

*Quantitation*

Quantitations were performed from standard curves constructed by adding small volumes ($\leqslant$10 $\mu$l) of solutions of amoxicillin in citrate buffer (pH 5) to pooled plasma or urine, and plotting peak heights against added concentrations.

## RESULTS AND DISCUSSION

*Sample preparation*

Considerable caution must be exercised in the analysis of penicillins owing to their comparative instability in aqueous environments (*cf.* ref. 1). Plasma and urine samples are therefore stored at —70° before analysis, and the analytical procedures are performed in a highly reproducible fashion for each step.

The analysis of ampicillin and mecillinam [1] is performed by the direct injection of plasma/whole blood (after protein precipitation) or urine. Owing to the polar character of amoxicillin use of the optimal chromatographic mobile phase for this compound results in interference in the chromatograms due to polar endogenous compounds, which necessitates the introduction of purification steps. Precipitation of proteins by the addition of trichloroacetic acid did not prove to be possible with amoxicillin because unexpected deterioration of the chromatographic performance occurred on injecting such samples. Instead, perchloric acid was used, but the addition of an organic solvent, dichloroethane, was also necessary to achieve complete precipitation of the proteins. Obviously, some endogenous compounds will also be extracted into the organic phase in this procedure providing further purification of the plasma sample. In choosing the organic solvent its solubility in the aqueous phase must be considered, due to the risk of the appearance of serious interference in the chromatogram, with dichloromethane, for example.

Interference by endogenous compounds in blank chromatograms from urine samples was far more serious than in those from plasma samples, and a double extraction procedure was found necessary. Low solubility in the aqueous phase of the organic solvents used is essential, see discussion above, and mixtures of a higher alcohol, *n*-amyl alcohol, together with dichloroethane were chosen. The proportions of the solvents in the mixtures used were chosen so that their densities were such that the aqueous phase was the heavier one in the first step, where the organic phase is discarded, but the lighter one in the last step, after which the sample is injected on to the chromatograph. This obviously facilitates sample handling and increases the analytical capacity.

The presence of the salt of the quaternary ammonium compound in the first step results in an acidic solution (pH about 1), and since the salt is distributed in the aqueous phase and will be present in the injected sample, an increase in the retention volume occurs (about 15%) probably by an ion-pair effect. The achievement of the longer retention time is essential in order to eliminate the influence of interfering peaks.

*Chromatography and post-column derivatization*

The chromatographic performance of several amphoteric penicillins has been studied [1], and it was found that they are retained more in their charged forms. The selectivity for different penicillins using phosphate buffer (pH 8)—methanol (7:3, v/v) showed that amoxicillin was the least retained of the compounds investigated. As an example the selectivity factors for ampicillin/amoxicillin and carbenicillin/amoxicillin are 8.10 and 1.30, respectively under these conditions. A further illustration is given in Fig. 1, which shows the

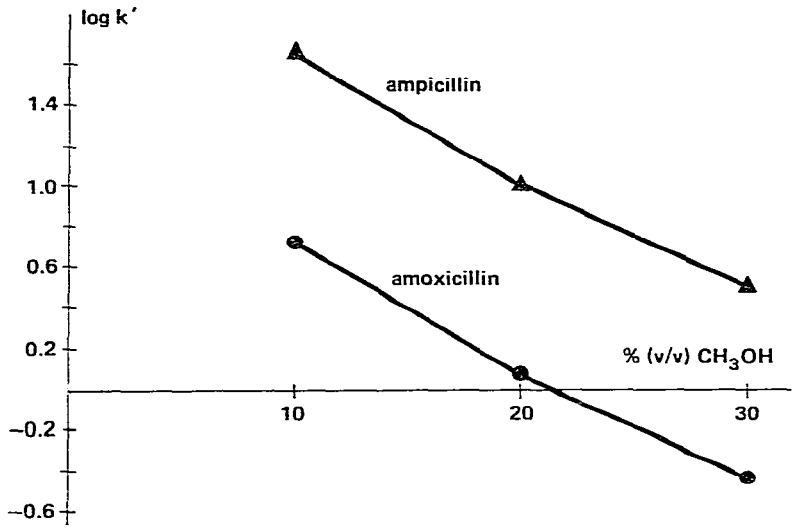


Fig. 1. Dependence of capacity ratios ($k'$) on amount of methanol in the mobile phase. Support: LiChrosorb RP-8 (5 μm). Mobile phase: phosphate buffer (pH 8, ionic strength = 0.1)—methanol. ●, Amoxicillin; ▲, ampicillin.

dependence of the capacity factors on the methanol content in the mobile phase for ampicillin and amoxicillin. The selectivity relative to the most important metabolite, the penicilloic acid which is even more polar than the parent compound, can also be expected to be very high in analogy with ampicillin and ampicilloate where the selectivity factor with 10% of methanol in the mobile phase and pH 8 is about 13.

The penicilloates will furthermore not react, or at least only to a very limited extent, in the derivatization procedure [2], which does not yield a quantitative reaction for amoxicillin under these conditions (*cf.* ref 1). A compromise between reaction time and completeness of the reaction is essential because of the band broadening that occurs in the reactor part [1].

*Quantitative determinations*

Representative blank and sample chromatograms obtained from plasma and urine are shown in Figs. 2 and 3, respectively. Endogenous peaks elute before the penicillin with only one exception in the case of plasma. Detection limits, defined as that concentration which gives a signal corresponding to twice the

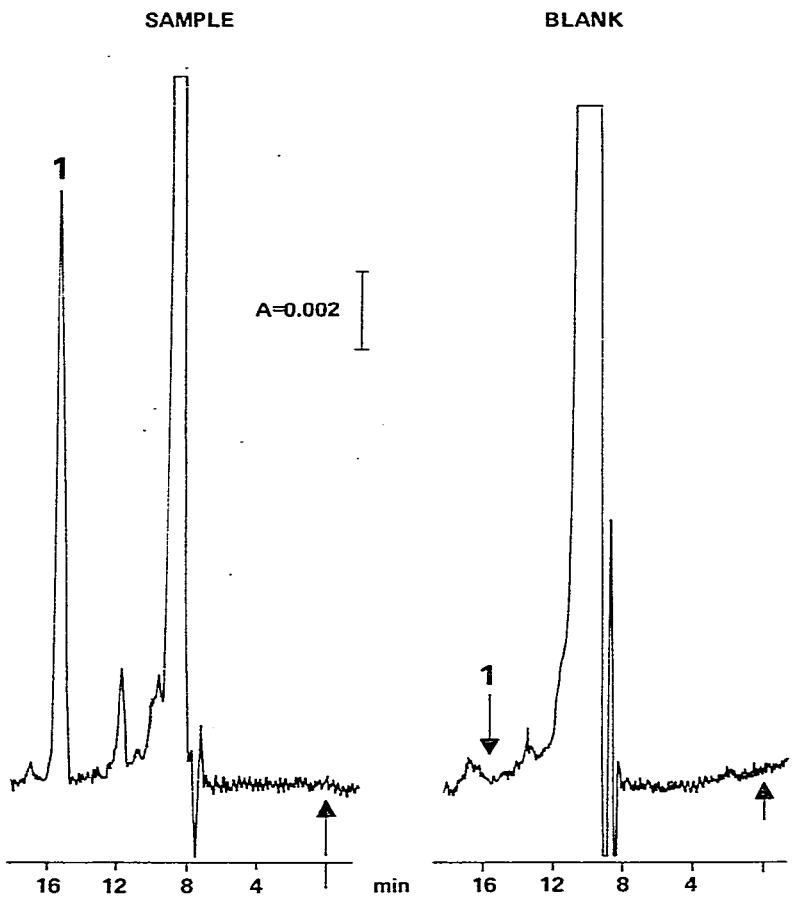


Fig. 2. Blank and sample chromatograms from plasma. Column: LiChrosorb RP-8 (5 μm) in 100 × 4 mm I.D. column. Mobile phase: phosphate buffer (pH 8)—methanol (92:8, v/v). 1 = amoxicillin.

baseline noise, are for plasma about 25 ng/ml and for urine about 200 ng/ml. These figures are valid for the injection of 200 μl plasma and 20 μl urine, but because of the trace enrichment effect [1, 6] that operates in chromatographic systems of this kind it is probably possible to improve the detection limits considerably by injecting larger volumes, as already demonstrated in the case of ampicillin and mecillinam [1]. To illustrate the performance of the analytical methods some data are presented on within-run recoveries and precision for the determination of low concentrations in plasma (Table I) and a standard curve obtained in a urine analysis, covering a rather broad concentration range and with the 99.9% confidence limits marked out (Fig. 4). Some examples of confidence limits (95%) by inverted predictions at different concentration levels (4.98—6.31, 35.7—36.9, 59.2—60.5, and 99.1—100.6 μg/ml) demonstrate the performance of this special standard curve.

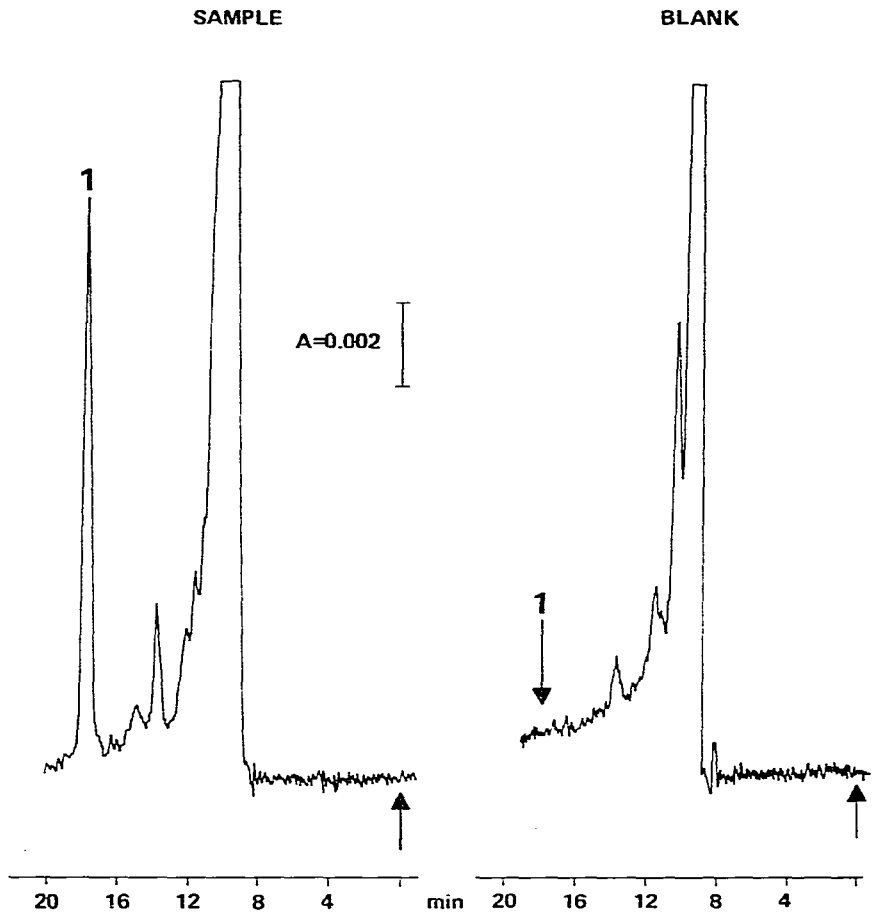


Fig. 3. Blank and sample chromatograms from urine. The same chromatographic conditions as in Fig. 2. 1 = amoxicillin.

TABLE I

QUANTITATIVE DETERMINATION OF AMOXICILLIN IN PLASMA

Standard curve with six standards in the range 85—1015 ng/ml.

| Added (ng/ml) | Found (%) | $s_{rel}$ (%) | $n$ |
|---|---|---|---|
| 100 | 101.7 | 3.72 | 6 |
| 454 | 99.6 | 1.40 | 5 |

*Routine use*

The method presented as well as the methods for ampicillin and mecillinam [1] have been used routinely in this laboratory for about 1.5 years. Performance has been excellent except for one point regarding column stability; namely, in order to maintain the column performance it has been found necessary to frequently (after about every fifteen injections of biological

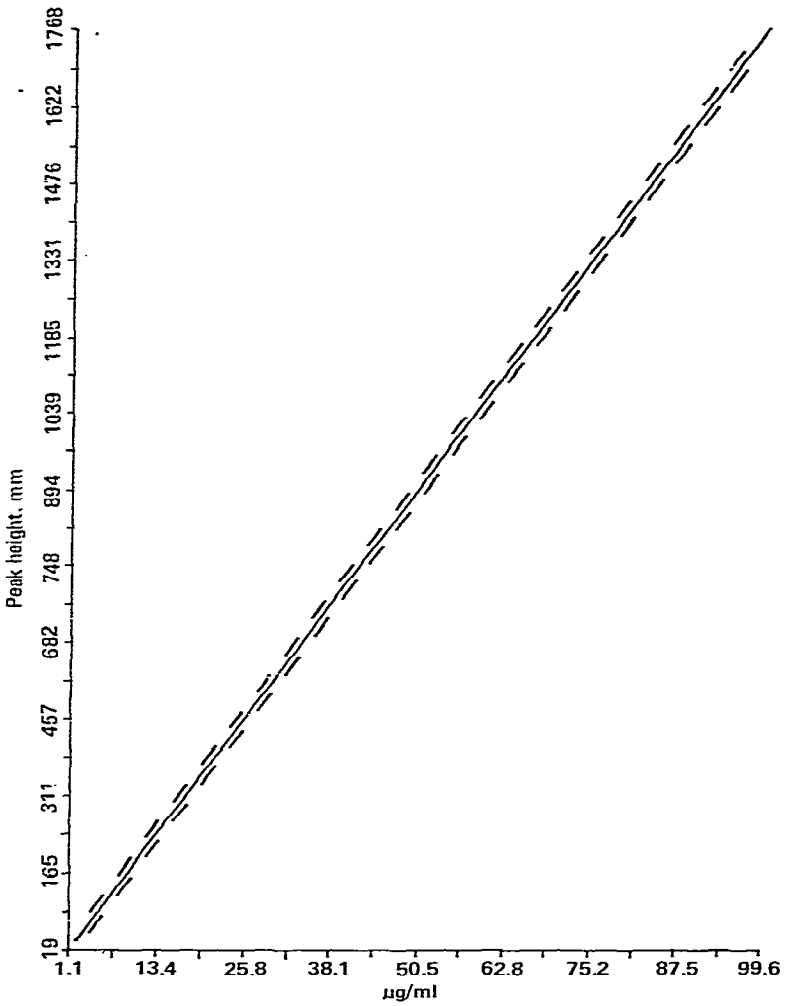


Fig. 4. Determination of amoxicillin in urine — standard curve. Analysis performed as described in "Analytical methods". Regression equation: $y = 2.13 + 17.69\,x$. Coefficient of correlation: 0.99989. 99.9% confidence limits are marked out. At 95% confidence limits: $17.57 < \text{slope} < 17.81$, $-0.68 < \text{intercept} < 4.94$.

samples) replace some millimeters of the support at the top of the column with new material. This procedure restores the chromatographic performance almost to its original condition and for runs carried out on the same day excellent standard curves are usually obtained. Columns treated in this way have been in use for more than six months when both routine and development work were carried out alternately. The cause of the column degradation is probably partly dependent on the absorption of non-polar endogenous compounds, such as fatty acids and lipids, that remain at the top of the column. However, this does not seem to be the only reason for the deterioration since it also occurs on injecting aqueous buffer samples, although in such cases it is then only discernible after a larger number of injections.

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