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AUTOMATED DETERMINATION OF AMOXYCILLIN IN BIOLOGICAL FLUIDS BY COLUMN SWITCHING IN ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHIC SYSTEMS WITH POST-COLUMN DERIVATIZATION

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SUMMARY

Amoxycillin, a polar aminopenicillin, is rather unstable in biological fluids. Degradation can be prevented by fast sample pretreatment and storage at −70°C or below. After pH adjustment, it is stable in biological fluids for over 16 h. The samples were handled by automated chromatography overnight. The chromatographic system consisted of a small guard column, two analytical columns separated by a switching valve, a post-column reactor and a fluorescence detector. The chromatographic events and the calculation of results were handled by a computing integrator.

The chromatography was based on ion-pairing principles. An efficient clean-up of the biological fluids was obtained by a heart cut from the first column, where the neutral mobile phase contained hexyl sulphate. In the second column the organic anion was exchanged for a large quaternary ammonium compound; amoxycillin was then retained as an ion pair. The composition of the mobile phases had to be designed carefully in order to avoid a disturbance of the chromatographic performance on the last column. An adequate selectivity and sensitivity was obtained by a post-column derivatization with fluorescamine.

Detection limits were 10 and 25 ng/ml for plasma and urine, respectively, and the inter-assay precisions at low levels (350 and 2000 ng/ml for plasma and urine, respectively) were ca. 5% (R.S.D.).

INTRODUCTION

The quantification of antibiotics in biological material has previously been

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performed by microbiological techniques. Such assays have the disadvantages of being non-selective (i.e. active metabolites are co-determined) and of giving non-precise results: the R.S.D. is generally ca. 15%. During recent years, however, many chemical assays based on modern column liquid chromatography (LC) have been introduced offering a selective and precise method of determination for this kind of compounds (see ref. 1 for review).

Amoxycillin, a polar aminopenicillin with a broad antibacterial spectrum, has been determined in body fluids by column LC with direct UV detection [2—4] and after post-column derivatization with imidazole and mercuric chloride followed by UV detection [5] and in urine by fluorimetric detection after reaction with fluorescamine [6]. The use of direct UV detection permits a limit of detection of ca. 0.5 μg/ml in plasma, and the post-column derivatization procedure [5] improves this to 25 ng/ml and in urine to 200 ng/ml.

However, in careful pharmacokinetic studies the requirements on detection limits may be even harder to meet, which made it necessary to develop new methods for both plasma and urine determinations. Earlier methodology [5] was also quite elaborate, involving purifying extractions before the samples could be injected into the chromatographic system. Pharmacokinetic studies generate a large number of samples, which necessitates the development of methods with a high sample throughput requiring a minimum of supervision for routine determinations. The aims of this study were to make the sample work-up as simple as possible, to develop methodology for automated injection of the samples and to achieve a high selectivity and the necessary low detection limits.

EXPERIMENTAL

Chemicals and reagents

Methanol, perchloric acid, citric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, all of pro analysi quality, and sodium hydroxide 5 M (Combi-Titrisol®) were all from Merck (Darmstadt, F.R.G.). Acetonitrile for high-performance liquid chromatography and Fluram (fluorescamine) puriss, p.a. were from Fluka (Buchs, Switzerland). Sodium hexyl sulphate (Research Plus, Bayonne & Denville, NJ, U.S.A.) and tetrahexylammonium hydrogen sulphate (Niels Clauson-Kaas, Denmark) were used as ion-pairing additives in the mobile phase. Sodium amoxycillin was kindly supplied by Beecham (U.K.).

Citrate/phosphate buffer (pH 5.4) was made from 19.9 g of disodium hydrogen phosphate and 40.0 ml of 1 M citric acid dissolved in deionized water and made up to 250 ml.

The solution for protein precipitation consisted of perchloric acid and citrate/phosphate buffer (pH 5.4) (10:90, v/v).

The solutions for pH adjustments were: for plasma, citrate/phosphate buffer (pH 5.4) and 1 M sodium hydroxide (110:40, v/v); for urine, 100 ml of 0.5 M disodium hydrogen phosphate and 350 ml of deionized water adjusted with 1 M citric acid to pH 4.85 before addition of water to 500 ml.

Apparatus

The LC system consisted of: a computing integrator CCM 2 (Laboratory Data Control, Riviera Beach, FL, U.S.A.) for microprocessor control of the equipment and for the computation of results; an autosampler WISP 710 A (Waters Assoc., Milford, MA, U.S.A.); two pumps, Constametric III and I (Laboratory Data Control) for the two analytical columns; a switching valve, the pneumatic Rheodyne 7001 (Rheodyne, Cotati, CA, U.S.A.); a UV detector, Spectromonitor III (Laboratory Data Control). The post-column reaction system had an LDC minipump (Laboratory Data Control) or an Eldex E-60-S (Eldex Labs., San Carlos, CA, U.S.A.) to pump the reagent; the reactor was a knitted [14] PTFE tube (5 m × 0.4 mm I.D.; Habia, Sollentuna, Sweden); a Valco zero-volume tee (Valco, Houston, TX, U.S.A.) was used for mixing the eluate and the reagent; the fluorescence detector was either Schoeffel SP 970 (Kratos, Ramsey, NJ, U.S.A.) or Shimadzu SF 30 [Shimadzu (Europa), Düsseldorf, F.R.G.].

The microanalytical balance was a Mettler M 5 SA (Mettler, Zürich, Switzerland), the centrifuge was Labsystems OY CF 510-A (Labsystems, Helsinki, Finland); a vortex-evaporator (Buchler, Fort Lee, NJ, U.S.A.) was used for mixing; and a Microlab® M (Hamilton, Bonaduz, Switzerland) was used for diluting and dispensing.

Sample handling

The collected blood samples were quickly centrifuged and filtered with Sera-Clear® (Technicon, Tarrytown, NJ, U.S.A.) and the plasma was frozen in a carbon dioxide—ethanol (or acetone) bath within 20 min after the collection of the whole blood. For urine samples, after measurement of the total volume, an aliquot of the fluid was frozen in the freezing bath within 20 min after collection of the samples. Both plasma and urine samples were stored at −70°C or below until analysis.

Sample work-up

Plasma sample preparation. To 0.5 ml of plasma in a 4-ml plastic tube, 250 μl of the solution for protein precipitation were added. After mixing by vortex for ca. 1 min and centrifugation at ca. 2400 g for 10 min, 450 μl of the supernatant were transferred to a new tube, which contained 500 μl of the solution for adjustment of plasma pH. After a short vortex treatment, the mixture was transferred to a sample vial and placed in the sample carousel of the autoinjector. Depending on the expected concentration level, 25–100 μl of the sample were injected.

Urine sample preparation. A 500-μl volume of urine (diluted for expected levels higher than 200 μg/ml) was transferred to a sample vial (4 ml) which contained 2 ml of the solution for pH adjustment of urine. After vortex treatment the vial was put into the autoinjector and, depending on the expected concentration, 10–25 μl of the mixture were injected.

Chromatography

The chromatographic system (Fig. 1) contained one guard column, 5-μm Spherisorb S5 ODS (5 × 4 mm I.D.) (Phase Separations, Queensferry, U.K.)

Injector

Pump A

C1

C2

Pump B

Loop

C3

UV-
Detector

Waste

Fluorescence
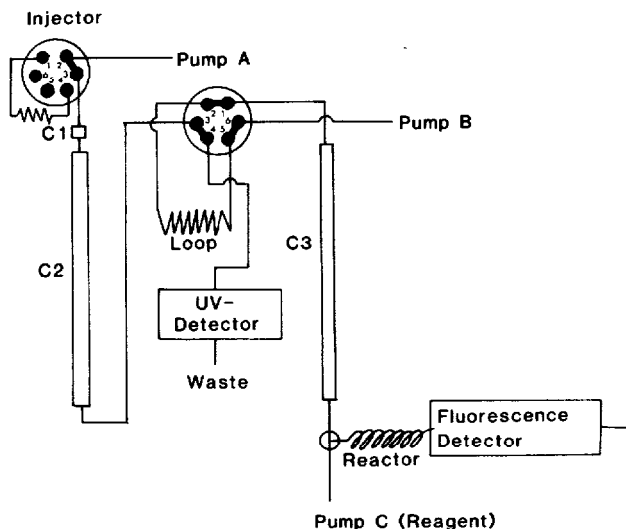Detector

Reactor

Pump C (Reagent)

Fig. 1. Chromatographic system. C1 = Guard column; C2, C3 = analytical columns; for details, see text.

and two analytical columns, 3-μm CP Microspher C_{18} (100 $\times$ 4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands), which were separated by the switching valve. Mobile phases were: for the first column, 10--11% methanol in phosphate buffer (pH 7.4) (ionic strength 0.1), containing 10^{-3} M sodium hexyl sulphate; for the second column, 30—35% methanol in phosphate buffer (pH 7.4) (ionic strength 0.1) containing 10^{-3} M tetrahexylammonium hydrogen sulphate. The methanol concentration was adjusted daily, within the range indicated, according to the performance of the columns. Flow-rates were 0.75 and 1.0 ml/min, respectively.

The eluate fraction containing amoxycillin was collected in a loop (0.5 mm I.D., 1 ml) in the separating switching valve; it eluted for ca. 40 sec with a retention time of ca. 5 min from the first column (C2). Before and after this capture, the eluate from this column was directed to waste. Meanwhile the second analytical column (C3) was equilibrated with its relevant mobile phase, in which the retention time of amoxycillin was similar (ca. 5 min).

A UV detector was connected to the "waste" channel after column C2 to check the performance of this column at regular intervals following the injection of standard solutions of amoxycillin. After column C3, the eluate was mixed with a solution of fluorescamine in acetonitrile (0.16 mg/ml), pumped at a flow-rate of 0.2 ml/min. After a reaction time of 0.5 min, the fluorescence at 470 nm was monitored; the excitation wavelength was 372.5 cm (Shimadzu SF 30). During quantification of routine samples, injections could generally be made at 14-min intervals, and the injections of standard and control samples were spread evenly among the unknowns.

For the preparation of standard and control solutions, three separate solutions of amoxycillin were prepared. Depending on the actual unknown samples, the concentrations of amoxycillin in the standards were in the ranges 1—100 μg/ml and 0.5—6 μg/ml for urine and plasma, respectively. Four to six standards containing the same concentration of amoxycillin were used to

construct a standard curve through the origin for quantification of the unknowns. In addition, to secure the accuracy, control samples were run at three different concentration levels, chosen according to the expected levels in each case.

RESULTS AND DISCUSSIONS

Sample handling

In bioanalytical methods intended for quantitative determinations it is essential to have control over the whole procedure from sample collection to calculation of the results.

Suitable routines for the different steps in the analytical procedure for amoxycillin were worked out. Studies of the stability of the drug in whole blood, plasma and urine at physiological and ambient temperatures indicated the importance of rapid handling of specimens. The recommendations, which were based on the experimental results on the stability, indicate that blood samples must be quickly centrifuged and filtered (use of Sera-Clear gave quantitative recoveries). The obtained plasma samples were then frozen in a carbon dioxide—ethanol bath within 20 min after the collection of the whole blood. The plasma samples were kept frozen at $-70°C$ before analysis; studies have shown that the drug is stable for at least one year under such conditions. No studies of the stability at $-20°C$ were performed for amoxycillin, the reason being that earlier studies on ampicillin [7] had revealed that this chemically closely related compound decomposed at this temperature if stored for longer than a couple of weeks. Urine samples were treated accordingly, after measurements of the total volumes excreted, an aliquot of the fluid was rapidly frozen and kept at $-70°C$ until analysis. Interestingly, higher concentrations of the compound in urine at the physiological temperature seemed to be more unstable than the lower (Table I). The very high concentration of 20 mg/ml degraded faster than concentrations 10 and 100 times lower.

TABLE I

STABILITY OF AMOXYCILLIN IN URINE AT 37° C

Aliquots of the sample were taken from a thermostatted water bath and immediately frozen by a carbon dioxide—ethanol mixture and stored at $-70°C$ before analysis by HPLC.

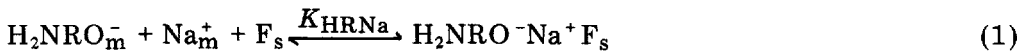| Time (h)	Recovery (%)		
	20.0 mg/ml	2.06 mg/ml	0.19 mg/ml
0	98.0	101	94.6
0.5	100	101	97.0
1	97.0	101	95.5
2	92.8	98.8	96.2
4	85.0	97.6	93.5
6	82.1	94.7	91.0
8	74.3	91.3	90.9
24	46.4	80.7	79.5
28	36.6	74.8	78.4
32	40.2	71.3	75.7

The reason is probably a tendency for aminopenicillins to polymerize at high concentrations, which is a well known reaction [8].

Amphoteric penicillins often have their optimal stability at the isoelectric point, i.e. for amoxycillin at pH 4.8. The addition of buffers around this value gives conditions in which the compound is reasonably stable at ambient temperature in biological fluids. The addition of 2 ml of phosphate/citrate buffer (pH 4.85) to 0.5 ml of urine, or ca. 1 ml of citrate buffer (pH 5.4) to 1 ml of protein precipitated plasma, provided conditions that kept amoxycillin intact for at least 16 h at ambient temperature. This is an adequate time to perform the determinations in an automated fashion, i.e. the samples can be put in the sample carousel of an automated injector for analysis overnight.

Studies on gentamicin [9], furosemide [10] and tocainide [11], which all are primary amines, have indicated a complication in the form of reaction with endogenous aldehydes to form a Schiff base [cf. ref. 12]. This may be a reversible reaction, and results from plasma determinations of the three compounds gave low extraction yields (tocainide) or a strange dependence on the time course for handling of the samples (gentamicin and furosemide). However, incubations of amoxycillin in plasma for different times before analysis according to the method described here showed no such pecularities for this compound.

Chromatography

Ion-pairing principles were used in earlier applications for the assays of penicillins [5, 13], and this approach was also utilized in the actual application. Amoxycillin is an amphoteric compound with $pK_1 = 2.4$ and $pK_2 = 7.2$. On alkyl-bonded silica gel, it will be an anion or cation only at extreme pH values; it will be present as a zwitterion at the most commonly used pH values. Multi-dimensional chromatography, i.e. the use of several coupled columns, may lead to very high selectivities. The technique is utilized to its full extent only when the chromatographic principles used in the different columns are completely unrelated to each other [14]. However, in practice it may not be possible to combine freely different retention principles, since practical problems may arise because of incompatibilities between different systems. The basic idea for the development of a method was to combine different ion-pairing principles and to use as many columns as necessary in order to achieve an adequate selectivity. During the work to develop suitable conditions it was found that chromatographic instabilities may result when two different ion-pairing principles are combined. The discussion of the effects obtained in the chromatographic system is based on the retention model, ion-pair adsorption, as given by Tilly-Melin and co-workers [16, 17]. Ion pairs are adsorbed on the solid phase, which has a limited capacity. Amoxycillin is a zwitterion $(H_3N^+\!-\!RO^-)$ at intermediate pH (3—6) and is mainly cationic at pH 2, and anionic at pH 7.2. The mobile phase was kept at pH 7.4 in this study, which means that amoxycillin will have an anionic character during the chromatography. The main equilibria in the system will depend on whether an anionic or cationic ion-pair reagent is present. (Subscripts m and s mean mobile and stationary phase, respectively.)
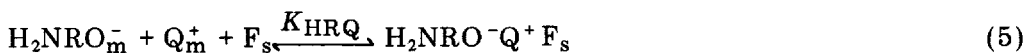
Anionic organic ion-pair reagent (S⁻)

$$H_2NRO_m^- + Na_m^+ + F_s \xrightleftharpoons{K_{HRNa}} H_2NRO^-Na^+F_s \tag{1}$$

$$S_m^- + Na_m^+ + F_s \xrightleftharpoons{K_{NaS}} NaSF_s \tag{2}$$

The total adsorption capacity of the support is given by:

$$K^0 = [F_s] + [H_2NRO^-Na^+F_s] + [NaSF_s] \tag{3}$$

An expression for the capacity ratio according to earlier outlined principles [16] will be:

$$k' = \frac{qK^0K_{HRNa}[Na]}{1 + K_{NaS}[Na] \, [S]} \tag{4}$$

Cationic ion-pair reagent (Q⁺)

$$H_2NRO_m^- + Q_m^+ + F_s \xrightleftharpoons{K_{HRQ}} H_2NRO^-Q^+F_s \tag{5}$$

$$2Q_m^+ + P_m^{2-} + F_s \xrightleftharpoons{K_{Q_2P}} Q_2PF_s \tag{6}$$

A combination of eqns. 1, 3 (modified), 5 and 6 gives:

$$k' = \frac{qK^0(K_{HRQ}[Q^+] + K_{HRNa}[Na^+])}{1 + K_{Q_2P}[Q^+]^2 [P^{2-}]} \tag{7}$$

It is highly probable that the term $K_{HRQ}[Q^+]$ will dominate so that the term $K_{HRNa}[Na^+]$ can be neglected in this equation. The adsorption of the zwitterionic form of the analyte has, as an approximation, been neglected in deriving the equations.

The retention time for amoxycillin decreased continuously by ca. 3% per injection, when the compound, dissolved in a slightly alkaline buffer (with 5% of methanol) containing a high level (10^{-2} M) of hexyl sulphate, was injected into a system with the same buffer (but 20% of methanol) as the mobile phase but containing a comparatively low concentration of tetrahexylammonium (10^{-3} M). This effect may be interpreted as a consequence of the retention of an ion pair between the hexyl sulphate anion (S⁻) with the hydrophobic quaternary ammonium compound (Q⁺). The effective concentration of Q⁺ in the mobile phase will decrease owing to an adsorption of the Q⁺S⁻ ion pair on the solid phase. [Q⁺] in eqn. 7 will thus decrease, which may explain the decreasing retention times of the analyte in this case. Injecting amoxycillin dissolved in pure water did not affect the retention volumes.

A difference in pH values between the injected (or transferred) solution may also affect the chromatographic performance. The injection of 200 μl of amoxycillin dissolved in 10% methanol in phosphate buffer (pH 7.4) with 10^{-3} M hexyl sulphate into a chromatographic system with a mobile phase containing the same organic anion concentration, but at a lower pH (2) and a higher methanol content (25%), decreased the efficiency by ca. 60%. The injection of a ten-fold higher concentration of hexyl sulphate even resulted in the appearance of split peaks. In the injected sample, amoxycillin will be dis-

tributed to the solid phase as an ion pair with sodium according to eqn. 1, and this ion pair will compete for adsorption sites with hexyl sulphate ion pairs (eqn. 4). When the acidic mobile phase starts to mix with the alkaline sample there will be a continuous change in the retention mechanism, so that the analyte will be adsorbed as an ion pair with hexyl sulphate. The decrease in efficiency may depend on an effective increase of the injected sample volume, owing to the competition for adsorption sites in the alkaline solution; the peak splitting may be an effect of the different roles played by the organic anionic ion-pair reagent in such a system.

However, a trace enrichment effect can be exploited by injecting amoxycillin in water containing tetrabutylammonium (see eqn. 5) into a mobile phase containing hexyl sulphate (pH 7.4) and 10% of methanol. The efficiency of the amoxycillin peak more than doubled compared with the injection of a pure water solution. It was also found that a mobile phase at pH 7.4 with 10^{-3} M tetrabutylammonium could handle the injection (200 μl) of an equimolar concentration of hexyl sulphate at the same pH but with half the amount of alcohol.

These introductory experiments formed the basis for the construction of the first system of coupled columns. Preliminary results on urine samples with this system indicated severe selectivity problems when using the same detection principles as in earlier papers [5, 13] on the determination of penicillins, which was a post-column derivatization to form the penicillenic acid mercuric mercaptide, detected by UV at 310 nm. As an alternative a post-column reaction with fluorescamine was developed (see below), and three coupled columns were tried initially. The first column was a short (5 cm) one with a C_{18} support placed in a loop. The mobile phase was acidic and contained a small percentage (2%) of methanol and the anion (hexyl sulphate). Amoxycillin will, under such conditions, have a cationic character and was retained as an ion pair. The sample was, after entrapment on the loop column, backflushed into a longer C_{18} column (10 cm) with a mobile phase that still contained the hexyl sulphate but at a higher pH (7.4) and methanol content (10%). The relevant amoxycillin fraction was captured in a loop and finally introduced into the third C_{18} column with a third mobile phase, which also had the neutral pH but instead of the anion a hydrophobic cation, tetrahexylammonium, and double the amount of methanol (20%).

However, when the system was applied in routine determinations there were problems with the first column, the retention times changed continuously and it became difficult to control the column-switching event so that the correct fraction was delivered to the second column. Furthermore, similar problems were encountered with the second column: after some days use, the retention times slowly but continuously changed. This instability seems to be related to the fact that the pH is drastically changed between the two columns, in analogy with the experiences from the introductory experiments (above). These experiences led to the development of an alternative system, in principle based on only two columns (Fig. 1). The first column was, however, preceded by a short (5 mm) guard column, which eliminated the stability problems encountered during the injection of biological fluids. It is readily handpacked and easily replaced, generally after three to five days of use. The two new analytical

columns were commercially available ready-packed C_{18} supports with 3-μm particles. They proved to be very stable under the conditions used.

The final system then involved the injection of buffered diluted urine or protein-precipitated plasma onto the guard column and the first analytical column. Amoxycillin was then chromatographed with a neutral mobile phase containing 10—11% of methanol and 10^{-3} M hexyl sulphate. In this environment amoxycillin is negatively charged, and the retention principle involved competition for the adsorption sites on the support between the anions (eqn. 4): the analyte and hexyl sulphate. The fraction containing amoxycillin (Fig. 2A) was captured in a large loop (1 ml) before the final chromatography. In this step the mobile phase had the same buffer system as in the first column, but a higher methanol content (30—35%), and the organic anion was exchanged for a hydrophobic quaternary ammonium compound, tetrahexylammonium. The negatively charged amoxycillin will in this column be retained as an ion pair with the large cation (eqn. 7), which is present in a comparatively high concentration. The injection of the large volume required the existence of an enrichment effect in order to avoid excessive band broadening, and this was provided by the difference in methanol content and by the ion-pair retention with tetrahexylammonium.

A very clean chromatogram resulted from this system as demonstrated for a urine blank in Fig. 2B. Amoxycillin eluted without any interference.

Detection

Amoxycillin has UV absorbance only in the low wavelength region (below 230 nm), and a derivatization of the compound was necessary for selectivity and sensitivity reasons for the determinations in biological material. Another possibility might be electrochemical detection of the phenol group; this, however, has not been tested here.

As already mentioned, the post-column reaction used earlier [13] was not adequate. Fluorescamine, which reacts with primary amino groups to form highly fluorescent products, has been used earlier for determinations of
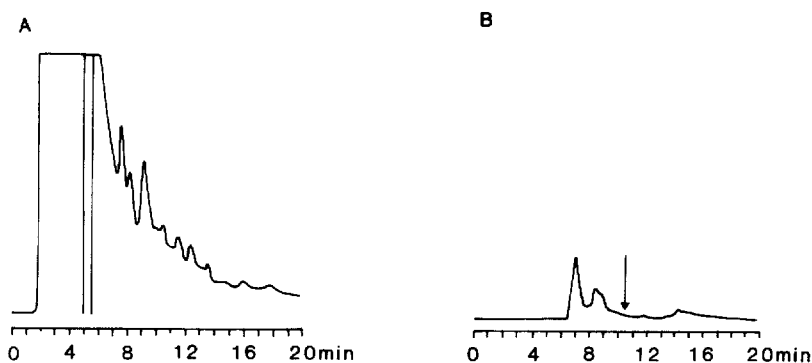

Fig. 2. Clean-up efficiency by column switching; analysis of a blank urine sample according to the described method. (A) Post-column reactor and fluorescence detector attached directly to the first analytical column (C1 + C2). The marked area indicates the retention time for amoxycillin. (B) Post-column reactor and fluorescence detector attached after the second analytical column (C1 + C2 + C3). Arrow indicates the retention time for amoxycillin.

amoxycillin in urine [6], and was tested in this study. The eluate was mixed directly with the reagent dissolved in acetonitrile. Initially the reaction was performed in a packed-bed (150 × 4 mm) reactor of 10-μm glass beads. An alternative that proved to give slightly better peak efficiency involved the use of knitted teflon capillaries [15]. In this application the band broadening of such devices was independent of their length (at least up to 10 m). Optimal conditions of reagent concentration, flow-rate and capillary length were determined by experiments where the parameters were systematically varied. Two different fluorescence detectors were compared, Schoeffel SP 970 and Shimadzu SF 30. The signal-to-noise ratio was a factor of 2 better for the latter, for which the optimal wavelengths (excitation and emission) were 372/470 nm. The excitation lamp of the Schoeffel detector has a higher intensity in the low wavelength range, demonstrated by giving on optimal excitation at 273 nm. This may also be a drawback regarding selectivity towards endogenous fluorescent compounds.

Routine determinations

The method has been used frequently for more than a year for quantifications of samples obtained from clinical phase I trials. It was possible to perform the determinations in an automated fashion — the samples were worked up manually (protein precipitation, buffering) and then placed in a sample carousel of an autoinjector for automated chromatography, which normally were run overnight. The microprocessor governed the times for injection of samples, the column-switching times, and the start and finish of the chromatograms. The amoxycillin peaks were automatically integrated and evaluated against a standard curve constructed from the standards in the same run as the unkowns.

Initially two different kinds of standard curve were run. The first was of a traditional type, involving standards with different concentrations covering the actual concentration range; the second type was constructed from only four standards containing the same nominal concentration and the origin. Excellent correlations were obtained from both plasma and urine for the two

TABLE II

STANDARD CURVES AND RECOVERIES

The quantitations were performed in two ways (urine samples): (1) twelve standards in the range 0.05—75.2 μg/ml (r = 1.0000); (2) Four standards with the same concentration of 7.84 μg/ml (R.S.D. = 1.6%). Injected volume, 20 μl.

Concentration added (μg/ml)	Found (%)		R.S.D.[*] (%)
	Method 1	Method 2	
0.08	94	92	6.9
0.80	102	100	2.1
7.84	102	—	—
78.2	101	99	1.3

[*]Quantitation from standard curve 2; n = 4.

Sample   Blank

T
Range
0.4
⊥
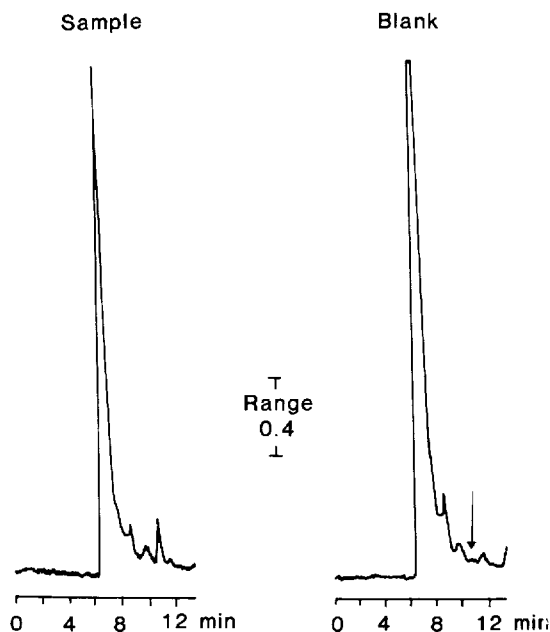
0   4   8   12 min   0   4   8   12 min

Fig. 3. Chromatograms from urine; analysis according to the described method. The sample contained 100 ng/ml amoxycillin. The arrow in the blank chromatogram indicates the retention time for the analyte.

Sample   Blank

T
Range
0.4
⊥
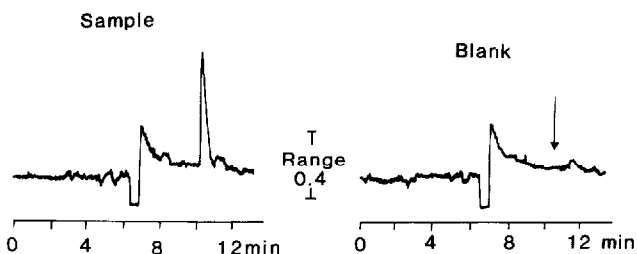
0   4   8   12min   0   4   8   12 min

Fig. 4. Chromatograms from plasma; analysis according to the described method. The sample contained 50 ng/ml amoxycillin. The arrow in the blank chromatogram indicates the retention time for the analyte.

TABLE III

INTER-ASSAY PRECISION

Level (μg/ml)	Found (%)	R.S.D. (%)	n	Interval (days)
Urine (six standards at 41.6 μg/ml)				
1.9	100	5.1	22	11
804	96.6	1.4	22	11
3110	97.9	1.5	16	8
Plasma (five standards at 17.0 μg/ml)				
0.35	100.3	5.0	8	4
8.77	98.6	2.0	8	4
38.8	97.9	1.3	8	4

different types of standard curves (Table II). For the routine determinations the simpler type 2 was therefore used regularly. Typical chromatograms are shown in Figs. 3 and 4.

Detection limits, defined as the concentration that gives a peak height corresponding to three times the noise of the blank baseline at the actual retention time, were: for urine, 26 ng/ml; for plasma, 10 ng/ml. Some examples of the inter-assay precisions obtained are given in Table III. Low (in the therapeutic practice) concentrations, 350 ng/ml in plasma and 2 μg/ml in urine, can be determined with an R.S.D. of ca. 5%.

In general, 50—60 samples were run each night; after the last sample an automated shut-down procedure started. It involved first, for control of chromatographic performance, the injection of amoxycillin dissolved in a buffer and detection by UV after column C2; the same column was then washed with methanol—water (8:2, v/v) for 30 min and was then shut off together with the reagent flow. For column C3 the flow-rate was reduced to half its normal value and put on recycling overnight. Before the start of the next series of samples the chromatographic performance was tested by an injection of amoxycillin: if it was unacceptable, the guard column was exchanged for a new one. This was generally necessary after each third to fifth day.

ACKNOWLEDGEMENT

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