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Note

High-performance liquid chromatographic determination of amoxicillin in human plasma using a bonded-phase extraction

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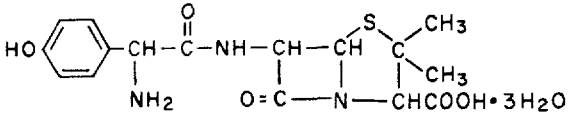
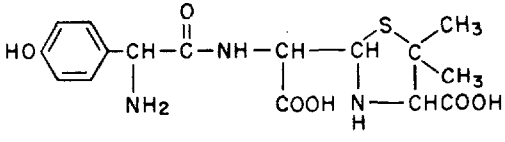
The analysis of amoxicillin (α-amino-*p*-hydroxybenzyl penicillin, I) (Fig. 1) in biological fluids by high-performance liquid chromatographic (HPLC) assay with UV detection at 225 nm [1, 2], post-column derivatization to the mercuric mercaptide derivative of penicillenic acid and UV detection at 310 nm [3], and post-column derivatization to the fluorescamine derivative with fluorometric detection (excitation/emission 385 nm/490 nm) [4], have been reported. The assay of amoxicillin in plasma [1—3] requires the formation of a protein-free filtrate with perchloric acid and subsequent direct injection of this supernatant which typically results in rapid column deterioration [3]. In addi-



[I]



[II]

Fig. 1. Chemical structures of amoxicillin and its penicilloic acid metabolite.

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tion, the drug is unstable in this supernatant requiring its immediate analysis after protein precipitation [3]. Recent studies have demonstrated that a variety of drugs can be isolated from biological matrices using bonded-phase extraction techniques [5—9]. These methods are extremely rapid and can often avoid the use of solvent extraction and/or protein precipitation steps with strong acids as was reported for amoxicillin [1—3].

The present study describes a sensitive and selective HPLC assay for the determination of amoxicillin in human plasma using a rapid bonded-phase extraction. The methodology avoids the use of the highly acidic protein-free filtrate with resultant drug stability to allow for the assay of amoxicillin using auto-injection.

The bonded-phase extraction method involves the activation of the bonded-phase extraction material, application of the diluted plasma sample onto the bonded-phase resin, clean up and selective elution by the appropriate choice of buffers and water—methanol mixtures. The final eluent is assayed by reversed-phase HPLC using a C_8 column with UV detection at 225 nm. The recovery of amoxicillin in the concentration range of 0.5—7.5 μg/ml of plasma is 90.4 ± 6.6% (S.D.) with a limit of detection equivalent to 0.5 μg/ml plasma using a 1-ml specimen per assay.

The assay was applied to the determination of plasma concentration of amoxicillin following a single 500-mg oral dose of Larotid (amoxicillin) to one human subject.

EXPERIMENTAL

Column

The column used was a 25 cm × 4.6 mm I.D. stainless-steel prepacked column containing approximately 6-μm reversed-phase Zorbax C_8 (DuPont, Wilmington, DE, U.S.A.).

Instrumentation

The HPLC system consisted of a Model 6000A pumping system, a Model 710B automatic sample injector (WISP™) (Waters Assoc., Milford, MA, U.S.A.). An LDC Model spectromonitor III variable-wavelength UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.), was used for quantitation at 225 nm. A back-pressure coil (approximately 3 m × 1.5 mm O.D., 0.3 mm I.D. Altex PTFE tubing) was connected to the sample outlet of the flow cell. The isocratic mobile phase used for reversed-phase chromatography was a mixture of water—methanol—1 *M* phosphate buffer, pH 7 (94 : 6 : 0.5). The chromatographic system was operated at ambient temperature, with a flow-rate of 2.0 ml/min at a head pressure of $1.5 \cdot 10^3$ p.s.i. (10.35 MPa). Under the above conditions the retention time of amoxicillin was approximately 7 min (capacity factor, $k' = 3.3$). The injection of 0.16 μg of amoxicillin per 80 μl yielded a peak of nearly 40% full scale response at a detector sensitivity of 0.01 a.u.f.s. The chart speed on the 10-mV Hewlett-Packard, Model 7132A strip chart recorder was 6.35 mm/min.

Standard solution

Stock solution of 1 mg/ml (Stock A) of amoxicillin, 6-[(*R*)-2-amino 2-(*p*-hydroxphenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate ($C_{16}H_{19}O_5N_3S \cdot 3H_2O$, mol. wt. 419.45 corrected for 86% potency) was prepared by dissolving 11.63 mg in 10 ml of water. Stock solution A was diluted 1 : 10 with water to prepare solution B containing 100 μg/ml of amoxicillin. Aliquots of 5, 10, 25, 50 and 75 μl of B (equivalent to 0.5, 1.0, 2.5, 5.0 or 7.5 μg of amoxicillin) are added to 1.0 ml of control plasma to establish a calibration curve of the recovered standards for the quantitation of the concentration of amoxicillin in the unknowns and for the determination of percent recovery.

A series of external standards containing 0.5, 1, 2 and 4 μg of amoxicillin per ml of water respectively, are prepared by appropriate dilution of stock solution B. Aliquots (80 μl) of these solutions (equivalent to 0.04, 0.08, 0.16 or 0.32 μg of amoxicillin) are assayed as external standards to verify the performance of the HPLC system and for the calculation of the recovery of the assay.

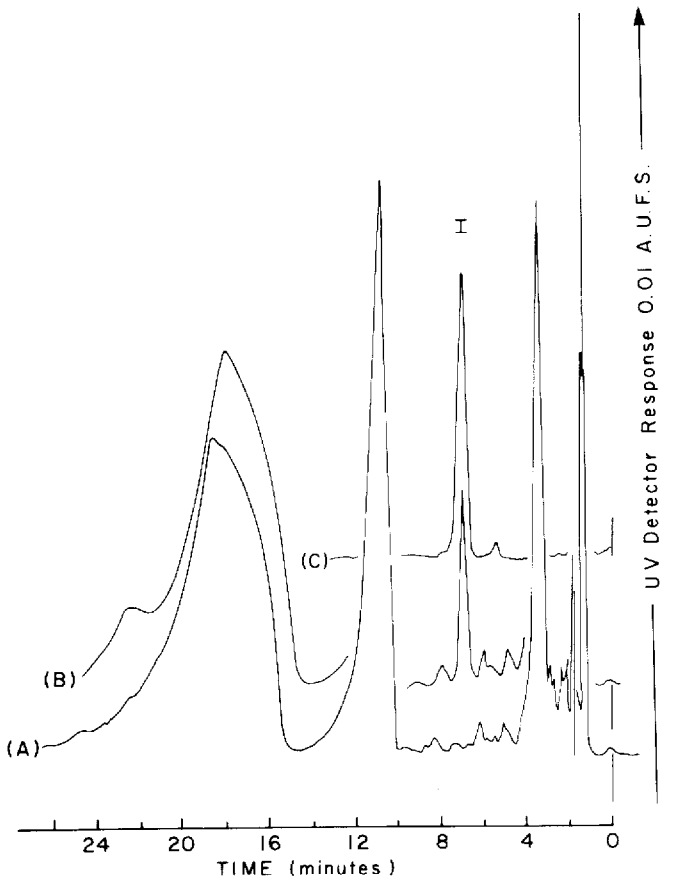

Fig. 2. HPLC analysis of extracts of (A) control plasma (from hospital supply); (B) control plasma containing 2.5 μg amoxicillin per ml added authentic standard (injection aliquot 80/1600 μl, i.e., assuming final volume of collection); (C) authentic standard 0.16 μg (80 μl injection).

Reagents and materials

All reagents are of analytical-grade purity and are prepared in deionized distilled water. Phosphate buffer (1.0 *M*, pH 7.0) is prepared by mixing 390 ml 1 *M* KH_2PO_4 (136.1 g/l) and 610 ml of $K_2HPO_4 \cdot 3H_2O$ (228.2 g/l). (Mix well and adjust to pH 7 with 1 *M* phosphoric acid as needed). Methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

The bonded-phase extraction cartridges containing C_{18} reversed-phase packing (Sep-Pak C_{18} cartridges, Catalogue No. 51910) and the Sep-Pak Cartridge Rack (Catalogue No. 22030) which is capable of holding eight cartridges for simultaneous processing were both obtained from Waters Assoc.

The cartridges were activated with 4 ml of methanol followed by 1 ml of 0.02 *M* KH_2PO_4 at a flow-rate of 1—2 ml/min using a water aspirator as a vacuum source connected to the cartridge rack.

Assay procedure

An aliquot (1 ml) of control plasma, spiked control plasma (0.5—7.5 μg/ml of amoxicillin) or unknown samples was diluted with 1 ml of 0.02 *M* KH_2PO_4 and passed through the respective cartridges. The cartridges were washed with

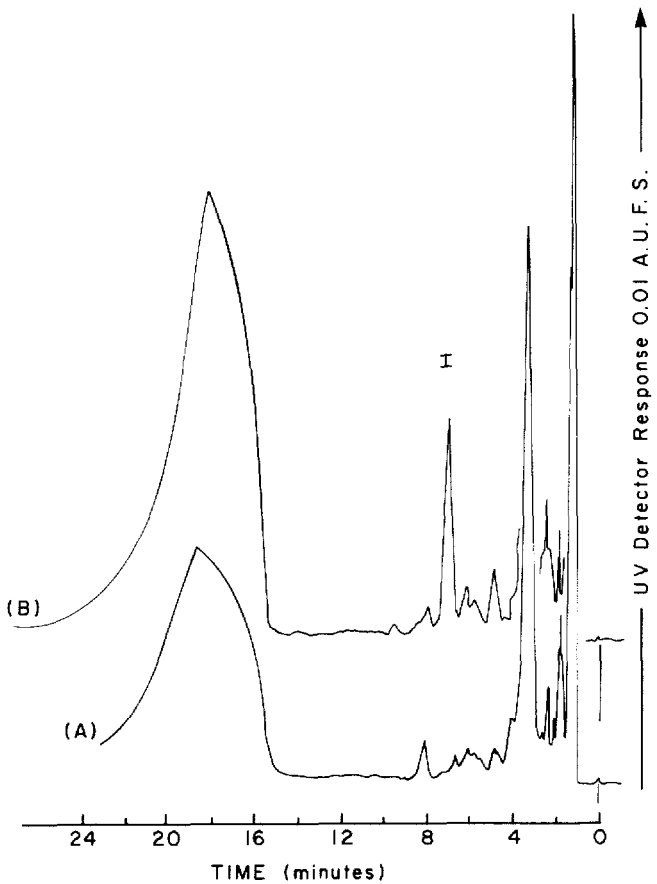

Fig. 3. HPLC analysis of extracts of (A) patient control plasma (0 h) and (B) patient plasma 1 h post 500-mg oral dose of Larotid® (amoxicillin).

1 ml of 0.02 *M* KH_2PO_4 and 0.5 ml of water. The amoxicillin was then eluted from the cartridge with a 2-ml aliquot of water—methanol (85 : 15). The eluent was collected and transferred to a WISP vial, which was placed in the WISP carousel for automatic injection. The WISP was programmed for a 80-μl injection volume and for a 25-min run time per sample. Typical chromatograms are shown in Figs. 2 and 3. The recovered standards were used to establish a calibration curve (linear regression analysis) for the direct quantitation of the concentration of compound I in the unknowns.

Selectivity

Biotransformation studies in urine have indicated that following oral administration of amoxicillin, 50—68% and 22—30% of the dosed drug is excreted in 8 h as the intact drug and penicilloic acid [6-D-(−)-α-amino-*p*-hydroxyphenyl penicilloic acid, II] metabolite, respectively [4], (Fig. 1). The HPLC assay is selective for amoxicillin in the presence of the penicilloic acid metabolite which is far more polar than the parent and is eluted near the void volume using the parameters described. Simultaneous assay for the metabolite is not feasible due to endogenous interferences in the control plasma (Figs. 2 and 3).

The assay is selective for amoxicillin in the presence of ampicillin (retention time, $t_R \geqslant 26$ min). Based upon another study using a C_8 column with a mixture of phosphate buffer and methanol as the mobile phase [10], interferences in the assay of amoxicillin would not be expected from a large number of penicillin antibiotics which all were reported to have retention times much greater than both amoxicillin and ampicillin.

Statistical evaluation of the method

The assay was validated in the concentration range of 0.5—7.5 μg of amoxicillin per ml of plasma. One sample at each concentration (Table I) was taken through the assay procedure using different lot numbers of the C_{18} cartridges on each run in a total of ten analytical runs. The mean inter-assay coefficient of variation for the ten experiments was 5.7% (Table I). The percent recovery of amoxicillin from plasma, calculated from the ratio of peak heights of amoxicillin external and recovered standards (assuming the final volume of collection of the effluent to be equal to 1.6 ml) was 90.4 ± 6.6% (S.D.). The sensitivity limit of the assay was 0.5 μg/ml of plasma.

TABLE I

LINEARITY AND INTER-ASSAY PRECISION OF THE HPLC ASSAY FOR AMOXICILLIN IN PLASMA (n = 10)

Concentration added (μg/ml)	Mean concentration found ± S.D. (μg/ml)	Coefficient of variation (%)
0.50	0.53 ± 0.04	7.6
1.00	0.97 ± 0.07	7.1
2.50	2.47 ± 0.18	7.3
5.00	5.06 ± 0.14	2.8
7.50	7.47 ± 0.27	3.6
	Mean	5.7

Application of the method to biological specimens

Plasma concentrations of amoxillin were determined using the bonded-phase extraction HPLC technique in one human subject following a single oral dose of 500 mg of Larotid. The HPLC data are in fair agreement with the analysis of the same samples by microbiological assay (Table II) and also with previously reported serum concentrations of amoxicillin in normal human subjects by microbiological assay [11]. Additional data will be needed to further statistically validate the correlation between the two assays.

TABLE II

PLASMA CONCENTRATION OF AMOXICILLIN (μg/ml) IN ONE HUMAN SUBJECT FOLLOWING SINGLE ORAL DOSE OF 500 mg OF LAROTID MEASURED BY HPLC AND MICROBIOLOGICAL ASSAY

Sampling time (h)	Concentration of plasma (μg/ml)	
	HPLC	Microbiological assay
0.5	N.M.*	N.M.
1	2.4	1.4
2	8.8	8.1
4.6	2.4	1.6
6.5	0.5	0.4**

*N.M. = Non-measurable, < 0.5 μg/ml of plasma.
**Sensitivity limit for microbiological assay = 0.4 μg/ml plasma.

DISCUSSION

A suitable internal standard with UV and chromatographic properties similar to amoxicillin could not be found. The problem was due to large interfering peaks with retention times of greater than 8 min in hospital supplied control plasma (Fig. 2) and greater than 14 min in patient control plasma (Fig. 3). These peaks also limit the total number of samples per analytical run, since approximately 30 min is required per sample.

Preliminary experiments utilizing an acetonitrile protein precipitation with a diethyl ether wash with or without an evaporation step of the final aqueous phase (similar to that used for amdinocillin [12]) did not yield sufficiently clean chromatograms to allow quantitation below 1.0—2.0 μg/ml.

In addition, experiments with a Bondapak C_{18} column (Waters Assoc.) with an eluting solvent of water—methanol—acetic acid (90 : 10 : 0.5) at a flow-rate of 1.0 ml/min yielded a retention time of 9.0 min for amoxicillin. However, interfering peaks from the control plasma again limited the assay sensitivity to 1—2 μg/ml with an unacceptably long time of analysis of 1.0 h per sample.

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