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Determination of amoxicillin in plasma by high-performance liquid chromatography with fluorescence detection after on-line oxidation

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SUMMARY

A simplified high-performance liquid chromatographic method for the determination of amoxicillin in plasma is described. Specific and sensitive fluorescence detection was achieved by on-line post-column electrochemical oxidation, using an electrochemical detector. Owing to the high specificity of the detection system, deproteinized plasma samples could be injected directly without prior treatment. This method permits a very fast and reproducible determination of amoxicillin in plasma on a routine basis at levels, down to 50 ng/ml. The absolute detection limit is about 10 pg injected.

INTRODUCTION

Amoxicillin (α-amino-*p*-hydroxybenzylpenicillin; Fig. 1) is an orally absorbed, acid-stable, broad-spectrum antimicrobial agent. Using high-performance liquid chromatographic (HPLC) methods with UV detection at low wavelength, *e.g.*, 230 nm[1–3], the determination of amoxicillin in body fluids was difficult and the detection limits were *ca.* 500 ng/ml. In order to enhance the selectivity, different HPLC methods with pre-column[4–6] or post-column[7–9] reactions have been developed.

Derivatization of amoxicillin to a fluorescent compound in an on-line electrochemical reactor proved to be a useful approach, resulting in higher reproducibility and increased sensitivity. This paper describes a reliable HPLC method for amoxicillin especially applicable to routine work. It includes the optimization of the on-line electrochemical reaction and the development of a new HPLC separation system. In order to reduce sample manipulation, protein precipitation with perchloric acid was used.

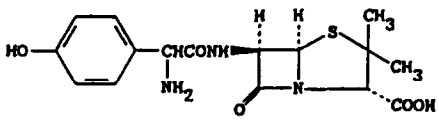

Fig. 1. Structure of amoxicillin.

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EXPERIMENTAL

Materials

All reagents were of analytical-reagent grade or better and were purchased from Merck (Darmstadt, F.R.G.) or Fluka (Buchs, Switzerland). Solvents used for chromatography were obtained as HPLC far-UV grade (acetonitrile) or were purified in the laboratory (water).

An isocratic HPLC system consisting of the following components was used: a Kontron (Zürich, Switzerland) Model 420 solvent delivery pump, a Rheodyne (Cotati, CA, U.S.A.) Model 125 injection valve with a 20-μl loop, a Merck Hitachi Model F1000 fluorimetric detector (Merck) and an LDC CI-10B chromatographic integrator (Milton-Roy, Riviera Beach, FL, U.S.A.). The post-column electrochemical reaction was monitored with a Model 5010 electrochemical detector (ESA, Bedford, MA, U.S.A.).

Methods

Amoxicillin was separated on an 80 × 4 mm I.D. stainless-steel column packed with Nucleosil 120 $3C_{18}$ (SRD, Vienna, Austria) and 0.02 *M* methanesulphonic acid–acetonitrile (92.5:7.5, v/v) as the mobile phase. The effluent was monitored after

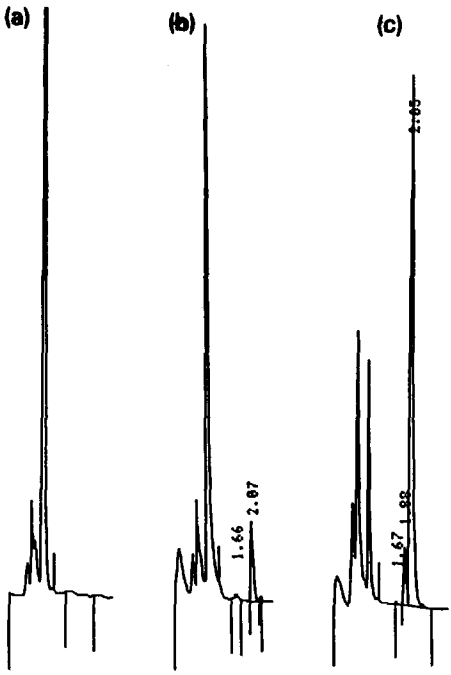

Fig. 2. Chromatograms obtained after injection of 20 μl of deproteinized plasma: (a) pre-dose plasma; (b) plasma spiked with 0.42 μg/ml of amoxicillin (t_R = 2.1 min); (c) plasma from a volunteer given a single oral dose of amoxicillin, 15 min after application, with an amoxicillin plasma level of 1.63 μg/ml. Column, Nucleosil $3C_{18}$, 80 × 4 mm I.D.; eluent, 0.02 *M* methansulphonic acid–acetonitrile (92.5:7.5, v/v); fluorimetric detection, $\lambda_{ex.}$ 255 nm, $\lambda_{em.}$ 400 nm, after post-column oxidation at 0.78 V. Numbers at peaks indicate retention times in min.

on-line post-column electrochemical reaction at 0.78 V at an excitation wavelength of 255 nm and an emission wavelength of 400 nm.

Plasma samples were stored at −30°C until analysis. After thawing, 1.0 ml of a plasma sample (standard or volunteer plasma) was mixed with 0.15 ml of 20% perchloric acid and centrifuged at *ca.* 2000 *g* for 4 min. Supernatants were used for further analysis. As amoxicillin is unstable in this supernatant, HPLC analysis was carried out within 15 min after protein precipitation.

Blank plasma samples were spiked with amoxicillin. These samples were analysed as described above. Finally, peak areas were plotted against concentration.

RESULTS AND DISCUSSION

Different liquid chromatographic systems were used for the determination of amoxicillin in plasma. With a C_{18} column and methanesulphonic acid–acetonitrile (92.5:7.5, v/v) as the eluent, amoxicillin is rapidly and efficiently separated from plasma matrix components (Fig. 2a and b).

Selective separation and specific fluorescence detection allow the direct injection of small volumes of plasma into the HPLC system without further purification. Fig. 2c shows a chromatogram obtained after injection of 20 µl of deproteinized plasma from a volunteer after a single oral dose of amoxicillin.

Although the assay was performed without the use of an internal standard, the day-to-day variation for the range 0.09–21.0 µg/ml was less than 10% standard deviation (median, 3.7%) (Table I). The absolute recovery for amoxicillin from plasma was >94%.

Electrochemical oxidation of amoxicillin results in the formation of two major reaction products, one with intense fluorescence ($\lambda_{em.}$ 400 nm, $\lambda_{ex.}$ 255 or 280 nm) and the other with a UV absorption maximum at 255 nm. In off-line experiments, the amoxicillin peak was eluted from the column and, after passage through the electrochemical detector cell, was collected and injected into a more selective HPLC

TABLE I

DAY-TO-DAY REPRODUCIBILITY (FOUR DIFFERENT DAYS) AND ACCURACY OF THE DETERMINATION OF AMOXICILLIN IN SPIKED HUMAN PLASMA

Fluorescence detection: excitation 255 nm, emission 400 nm.

Amoxicillin added (µg/ml)	*Amoxicillin found (µg/ml)*[a]	*Relative standard deviation (%)*	*Recovery (%)*
0.087	0.079 ± 0.007	8.9	90.8
0.210	0.189 ± 0.007	3.7	90.0
0.420	0.411 ± 0.034	6.3	97.9
0.842	0.882 ± 0.057	6.5	104.8
1.684	1.741 ± 0.078	4.5	103.4
3.368	3.522 ± 0.062	1.8	104.6
6.736	6.599 ± 0.109	1.7	98.0
10.521	10.521 ± 0.247	2.3	103.2
21.042	20.794 ± 0.200	1.0	98.9

[a] Mean ± S.D. (n = 4).

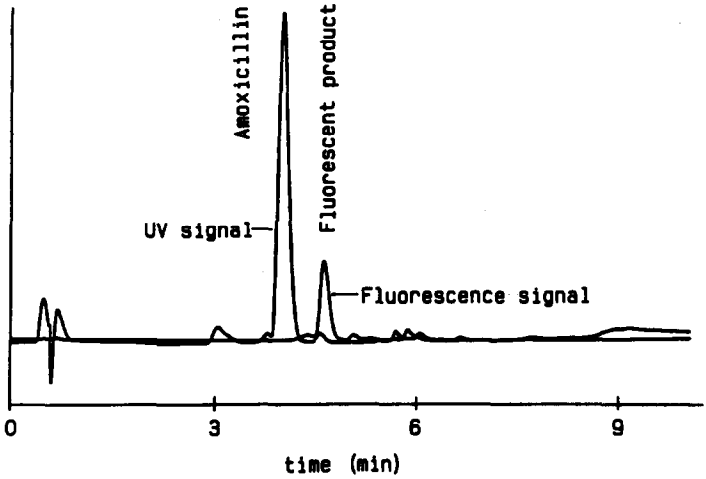

Fig. 3. Chromatogram obtained after injection of oxidized amoxicillin. Column, Nucleosil $3C_{18}$, 80 × 4 mm I.D.; eluent, 0.02 *M* methansulphonic acid–acetonitrile (98:2, v/v). (A) Fluorimetric detection, $\lambda_{em.}$ 255 nm, $\lambda_{em.}$ 480 nm; (B) UV detection at 250 nm.

system [methansulphonic acid–acetonitrile (98:2, v/v)]. This system separates native amoxicillin and the major reaction products (Fig. 3).

A decrease in peak area at higher potential applied to the electrochemical detector cell could be monitored only when the excitation wavelength was set at 255 nm. Setting the excitation wavelength to 280 nm resulted in an increase in the peak area at higher potential. The increase in the peak area was limited only by the detector's ground current, caused by the mobile phase (see Fig. 4).

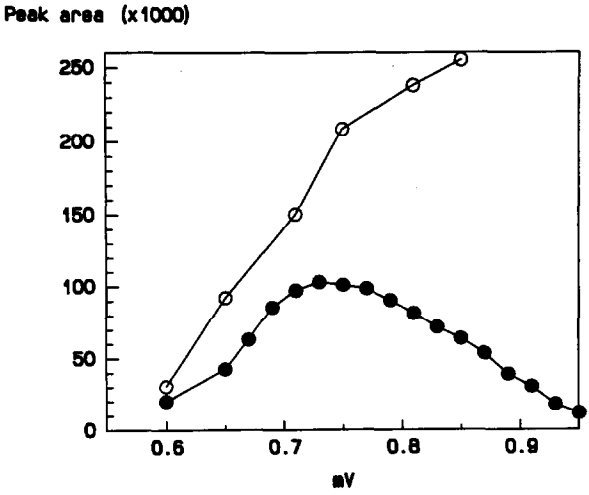

Fig. 4. Potential course of fluorescence change for one-line electrochemically oxidized amoxicillin at two excitation wavelengths: ● = 255 nm; ○ = 280 nm.

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