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Determination of amoxycillin in plasma by ion pair column extraction and reversed-phase ion pair high-performance liquid chromatography

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Abstract: A quantitative method is described for the determination of amoxycillin in plasma. The method utilizes ion pair extraction of amoxycillin on disposable columns packed with Baker-10 SPE™ octadecyl, with tetrabutylammonium ion as the counter ion and methanol as the eluent. Separation and quantitation is performed by reversed-phase ion pair high-performance liquid chromatography (Nucleosil® C-18) using the same counter ion and a mobile phase of methanol–phosphate buffer (pH 6.0) (31:69 v/v) with detection at 229 nm.

Keywords: *Amoxycillin; reversed-phase high-performance liquid chromatography; ion pair column extraction.*

Introduction

For the assay of amoxycillin (D-(−)-α-amino-*p*-hydroxybenzyl penicillin, Fig. 1), a semisynthetic broad-spectrum antimicrobial agent, in a large number of plasma samples from pharmacokinetic studies, a selective, sensitive and rapid method is required.

In general, the microbiological assays presently available (e.g. [1–3]) are tedious and time-consuming and have limited accuracy in the lower concentration range; those assays are therefore unsuitable for use in pharmacokinetic studies.

More sensitive methods for the measurement of amoxycillin in plasma [4–7] and urine [7–8] are based on spectrofluorimetry but such methods are still laborious. This is partly caused by the difficulty in isolating the drug from plasma by normal liquid–liquid extraction due to the highly polar character of the compound, which results in relatively high water solubility.

At present a few methods based on high-performance liquid chromatography (HPLC) have been published. Vree *et al.* [9] circumvented the problem of the difficult extractability of the drug by directly injecting into the chromatograph the supernatant that is obtained after deproteinization of plasma with perchloric acid. This method is

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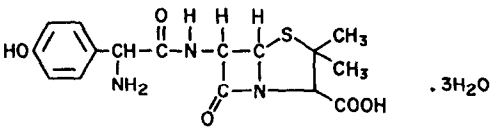
Figure 1
The structural formula of amoxycillin.



very rapid and very suitable for immediate assay of samples in the hospital for drug monitoring purposes. However, the suitability of the method for the assay of large numbers of samples is limited since the column performance tends to deteriorate after the injection of thirty to forty samples. Carlqvist and Westerlund [10] described a method based on protein precipitation followed by separation by reversed-phase HPLC, post-column derivatization with air segmentation and finally measurement of UV absorbance at 310 nm. The method is very sensitive but still time-consuming. The recently published method of Foulstone and Reading [11] is based on that of Vree *et al.* [9] but involves ultrafiltration of the serum sample. Since amoxycillin is an amphoteric compound, it can be considered to be potentially a good candidate for ion pairing processes [12–15]. Therefore, it was decided to develop a rapid, straightforward assay based on ion pair column extraction and ion pair reversed-phase HPLC.

Experimental

Chemicals and solvents

Amoxycillin trihydrate was provided by Beecham Pharmaceuticals (Betchworth, UK). Disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate and tetrabutylammonium hydrogen sulphate (TBA) were of analytical reagent grade and were obtained from E. Merck (Darmstadt, FRG). Benzoic acid was also of analytical reagent grade (Analar®, The British Drug Houses, Ltd., Poole, UK). Methanol was of HPLC grade and was supplied by Fisons (Loughborough, UK).

The internal standard solution was 0.025 mM benzoic acid with 0.01 M TBA in 0.1 M phosphate buffer (pH 7.0). The extraction was performed on 1-ml extraction columns packed with Baker-10 SPE™ octadecyl (≈ 100 mg C_{18}). The wash solvent was 0.01 M TBA in 0.1 M phosphate buffer (pH 7.0). Methanol was used as the eluent.

The mobile phase for HPLC was methanol–0.005 M TBA with 0.05 M sodium dihydrogen phosphate monohydrate (pH 6.0, adjusted with NaOH) (31:69, v/v). Before use, the mobile phase was filtered through a Millipore filter type D.A. 0.65 μm and degassed by ultrasonification for 10 min.

Instruments and instrumental conditions

The assay was performed on a chromatograph comprising a Waters M 45 solvent delivery system and a Waters Model 441 fixed wavelength ultraviolet detector equipped with a filter (229 nm) (Waters Assoc., Milford, Ma, USA).

The 150 × 4.6 mm i.d. column was packed with reversed-phase 5-μm Nucleosil C-18 (Machery-Nagel, Düren, FRG).

The flow-rate was 1.0 ml. min^{-1} and the column was immersed in a water-bath at room temperature.

Injections were made by means of a Waters Intelligent Sample Processor, Model 710 B (Waters Assoc., Milford, Ma, USA).

Data analysis was performed with a Spectra-Physics SP 4100 computing integrator (Spectra-Physics, Santa Clara, Ca, USA), using measurements of peak height ratio.

Analytical method

The extraction columns were conditioned with two portions of 1.0 ml of methanol followed by 1.0 ml of the wash solvent under vacuum.

A 250 µl sample of plasma and a 250 µl sample of internal standard solution were mixed for 30 s with a vortex mixer; 250 µl of this mixture was transferred to the extraction column and left for 10 min. The column was washed three times with 500 µl of the wash solvent under vacuum. The column was eluted three times with 50 µl of methanol under vacuum and then once with 350 µl of 0.005 M TBA with 0.05 M sodium dihydrogen phosphate monohydrate (pH 6.0). The eluent was shaken for 5 s with a vortex mixer and stored at 5°C until just before injection. An aliquot of 10 µl was injected into the chromatograph.

Results

A chromatogram derived from a blank plasma sample is depicted in Fig. 2. A chromatogram derived from a plasma sample obtained from a subject after administration of amoxycillin capsules is illustrated in Fig. 3.

The applicability of the method is demonstrated (Fig. 4) by a curve of plasma concentration against time for a subject who had taken 750 mg of amoxycillin orally.

The results of the recovery studies are given in Table 1. The analytical recovery was satisfactory (more than 80%) at all amoxycillin concentrations. Table 2 shows that intra-day variability was satisfactory (relative standard deviation (R.S.D.) 4.1–5.7%). Table 3 shows satisfactory results for inter-day variability (R.S.D. up to 5.0%). The limit of sensitivity was 0.1 mg l^{-1}.

Discussion

Extraction procedure

Amoxycillin (Fig. 1) is an amphoteric compound. The pK_a values of the COOH, NH_2 and OH groups are 2.4, 7.4 and 9.6, respectively [16]; thus the drug has a slightly acid character. A 0.2% (m/v) solution of the drug in CO_2-free water has a pH of 3.5–5.5. Owing to its strongly polar character amoxycillin is relatively highly soluble in water. The solubility at pH 4–8 varies from 4.2 g l^{-1} to 9.0 g l^{-1}. The drug is also very soluble in polar solvents such as methanol (7.5 g l^{-1}), ethanol (3.4 g l^{-1}) and acetone (1.3 g l^{-1}); it is insoluble in ethyl acetate, chloroform, ethylether, *n*-hexane or benzene [16].

Moreover, amoxycillin is unstable in the strongly acid solutions required for the drug to be present in the unionized form (pH values about 2 below 2.4). As a consequence of the physico-chemical properties it is almost impossible to isolate amoxycillin from plasma by liquid–liquid batch extraction.

In contrast, the drug is a potential candidate for ion pair extraction. Ion pair batch extraction was not considered to be appropriate because an evaporation procedure was required and because the drug was very unstable at elevated temperatures. It was decided therefore to develop an ion pair extraction method with disposable octadecyl columns. A pH value of 7 was selected since it is within the pH range of the maximum stability of amoxycillin. Tetrabutyl-ammonium has been shown to be a very good counter-ion for the formation of ion pairs with acid groups in bulky molecules [12–15]. It was found that the TBA–amoxycillin ion pair could be easily retained by the C-18 extraction columns. Hydrophilic compounds from the plasma could be washed from the

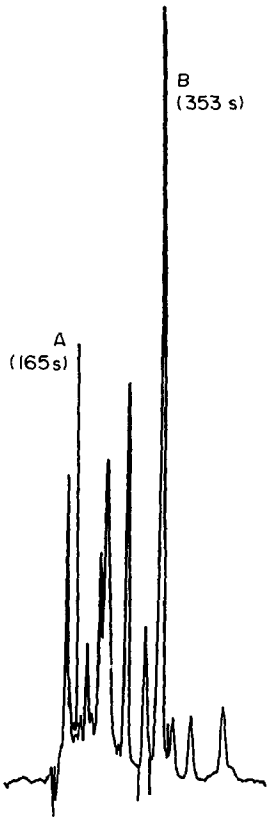


Figure 2
Chromatogram derived from a "blank" plasma sample that was drawn from a subject just before amoxycillin administration. A = corresponding retention time for amoxycillin. B = internal standard (benzoic acid).

Table 1
Analytical recovery of amoxycillin at three initial plasma concentrations

Initial concentration of amoxycillin in plasma (mg l^{-1})	Absolute recovery (%) (mean ± S.D.)	No. of determinations
1.25	80.0 ± 6.5	7
2.50	82.9 ± 6.0	7
5.00	88.8 ± 4.8	7

Table 2
Intra-day variability

Initial plasma concentration (mg l^{-1})	Measured plasma concentration (mg l^{-1})		
	Mean*	S.D.	R.S.D. (%)
1.25	1.17	0.053	4.5
2.50	2.44	0.128	5.2
5.00	4.94	0.284	5.7
10.00	10.28	0.420	4.1

*$n = 7$.

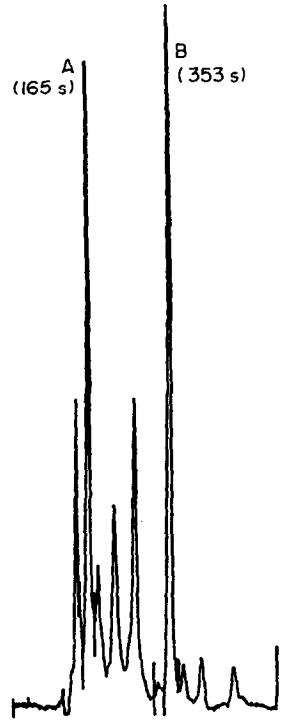


Figure 3
Chromatogram derived from a plasma sample drawn from the same subject as in Fig. 2 after administration of amoxycillin. A = amoxycillin (7.6 mg l^{-1}). B = internal standard (benzoic acid).

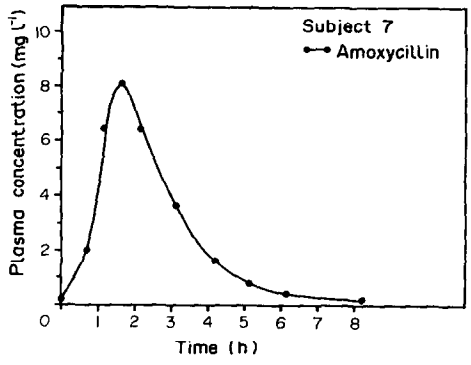


Figure 4
Curve of plasma concentration of amoxycillin against time after administration of 750 mg of the drug (two capsules of Clamoxyl® 375 mg).

Table 3
Inter-day variability

Initial concentration of amoxycillin plasma (mg l^{-1})	Measured plasma concentration (mg l^{-1}) Mean*	S.D.	R.S.D. (%)
1.25	1.26	0.063	5.0
2.50	2.53	0.099	3.9
5.00	4.94	0.167	3.4
10.00	10.02	0.054	0.5

*$n = 7$.

column with a phosphate buffer that contained TBA to keep the amoxycillin in the state of the ion pair. The TBA-amoxycillin ion pair could be eluted from the column with methanol.

Preferably the internal standard should have some structural similarity with the ion pair forming group (−COOH) in the molecule. Benzoic acid was shown to have an extraction behaviour during this procedure similar to that of amoxycillin itself.

HPLC procedure

As the expected plasma concentrations after therapeutic use of amoxycillin are in the range 0–15 mg l^{-1} and because the drug has good UV absorbing properties (molar absorptivity in 0.1 N HCl at 229 nm = 9500), UV absorption was selected as the method of detection. Since maximum absorbance of the drug occurs at 229 nm, however, straight phase systems with mobile phases containing solvents such as chloroform or dichloromethane could not be used because those solvents absorb radiation at this wavelength.

Therefore, a reversed-phase system was selected. Because of its extremely polar character, amoxycillin is poorly retained on reversed-phase columns; however, the hydrophilicity of the drug is increased when chromatographed as the TBA ion pair. The best separation from other unidentified compounds from the plasma extract was achieved with a mobile phase with an apparent pH of 6 and containing 30% (v/v) of methanol. Attempts were made to eliminate some peaks that were retained close to the TBA–amoxycillin ion pair although these peaks did not interfere. It appeared to be possible to remove those peaks by washing the column with a solution of about 5% (v/v) methanol, but this process resulted also in a decrease to about 60% in the recovery of amoxycillin.

Attempts to use other antibiotics of the penicillin group (e.g. ampicillin, penicillin G, penicillin V, oxacillin, cloxacillin or flucloxacillin) were unsuccessful because these substances are considerably less polar than amoxycillin and their TBA–ion pairs have retention times that are at least ten times greater than that of the TBA–amoxycillin ion pair.

The retention time of TBA–amoxycillin is still short probably because the TBA^+-ion neutralizes only the $-COO^-$ group in the amoxycillin. The polar groups $-NH^+$, $-OH$, $-\overset{\overset{O}{\|}}{C}-$ and $-C-$ also contribute substantially to its strongly polar character. The TBA–ion pair of benzoic acid was found to have suitable chromatographic properties and therefore benzoic acid was selected as the internal standard. In extracts of some plasma samples a small peak (of reproducible height) was found with about the same retention time as the TBA ion pair of benzoic acid.

Quantitation, reproducibility and accuracy

The results of the recovery experiments show that amoxycillin can be extracted efficiently and reproducibly at plasma concentrations attained after administration of therapeutic doses of the drug. The analytical recovery of amoxycillin at the three concentrations tested was 84.9 ± 6.7% (mean ± SD; $n = 21$). Both the intra-day (within-day) variability (R.S.D. of 4.1 to 5.7%) and the inter-day (day-to-day) variability (R.S.D. up to 5.0%) were satisfactory. The low variability was probably also due to the preparation of a new calibration curve each day with a new series of samples.

Calibration graphs were rectilinear and passed through or near the origin. For a typical

calibration graph, the regression equation for peak height ratio (y) against concentration (x) was: $y = 0.1101\ x - 0.0022$ ($r > 0.998$; $n = 7$).

Amoxycillin proved to have a limited stability in plasma at room temperature; therefore frozen plasma samples were thawed and extracted shortly before injection into the chromatograph.

The limit of sensitivity (defined as three times the baseline noise) under the conditions described above was 0.1 mg l^{-1}, which was sensitive enough for therapeutic monitoring or for use in pharmacokinetic studies. An example of a typical curve of plasma concentration against time (Fig. 4) shows the applicability of the method.

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