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Reversed-phase high-performance liquid chromatographic assay methods for the analysis of a range of penicillins in in vitro permeation studies

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Abstract

Simple, rapid and selective HPLC methods have been developed for the analysis of amoxycillin, ampicillin, epicillin, mecillinam and propicillin. A Hypersil ODS 5 μm (150×4.6 mm I.D.) column was used with mobile phases containing aqueous phosphate buffers, pH 3–4.6 and either methanol or acetonitrile as the organic modifier. Samples were detected by their optimal UV absorption (210–230 nm). The lower limits of quantitation of the compounds (100 μl injection volume) were 0.1 μg/ml. The assays were linear in the range of 0.1–100 μg/ml with r^2 values greater than 0.99. The methods have been applied successfully for the measurement of the flux of the compounds across Caco-2 cells monolayers. © 1998 Elsevier Science B.V.

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1. Introduction

The study of β-lactam antibiotics has been pursued vigorously for over 50 years [1]. Since the discovery of penicillins, several analytical techniques have been developed for their analysis including microbiological, colorimetric and chromatographic techniques. Microbiological assays are time consuming and lack specificity. Colorimetric assays are also nonspecific thereby limiting their frequent application. High-performance liquid chromatography (HPLC) is currently the most widely used technique because it combines specificity with sensitivity, reproducibility and cost-effectiveness.

Presently, there is interest in the study of penicillins in in vitro models [2] in order to understand the mechanisms of transport of these drugs across the gastrointestinal tract and to assist in the development of new therapies. The models frequently used include animal tissue isolates and tissue cultures such as Caco-2 cells. In addition, the usefulness of amoxycillin in the treatment of *Helicobacter pylori* and the increasing resistance of *H. pylori* to metronidazole and clarithromycin (which are frequently used in the eradication of *H. pylori* infections) has increased the need for the evaluation of other penicillins as suitable alternatives since amoxycillin effectively eradicates *H. pylori* when combined with other drugs such as omeprazole and metronidazole [3].

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The chromatographic methods for the analysis of many penicillins have been reported previously [4–7]. These include those of amoxycillin, ampicillin, cloxacillin, metampicillin, penicillin G and penicillin V. The method reported for the determination of carbenicillin in plasma and urine [8] is for the analysis of the epimers of the compound and is unlikely to prove useful for routine analysis of the compound in large number of samples. A reversed-phase HPLC assay method for the analysis of mecillinam has also been mentioned in the literature but only as a note [9]. These existing methods for the analysis of the penicillins have diverse HPLC conditions, and are not specifically designed for in vitro studies where simplicity and speed are important.

The purpose of this study is to develop HPLC assay methods for the simple, rapid and specific analysis of amoxycillin, ampicillin, epicillin, mecillinam and propicillin in in vitro experiments involving the penetration of these compounds across biological membranes.

2. Experimental

2.1. Chemicals

Potassium dihydrogen phosphate, orthophosphoric acid, triethylamine, sodium dihydrogen phosphate, disodium hydrogen phosphate and anhydrous sodium hydroxide were purchased from Fluka (Poole, UK). Acetonitrile, methanol and perchloric acid were purchased from Fisons (Loughborough, UK). Amoxycillin, ampicillin, epicillin, dimethylsulfoxide (DMSO), fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM) nonessential amino acid (100×), glucose (cell culture grade), Hank's balanced salt solution (HBSS), HEPES buffer (1 *M*, pH 7.4) and sodium bicarbonate were purchased from Sigma (Poole, UK). Mecillinam was obtained from Leo Laboratories (Ballerup, Denmark) while propicillin was obtained from Bayer (Newbury, UK). Phosphate buffer saline, pH 7.3 (PBS) was purchased from Unipath (Basingstoke, Hampshire, UK). All chemicals were of HPLC grade or better.

2.2. Chromatographic system

The HPLC system consisted of an automatic sample injector 231, diluter 401, 302 solvent pump, pressure measurement unit 802C and computer software 715 (Gilson Medical Electronics, Villiers le Bel, France) and an Applied Biosystems (Foster City, CA, USA) 759A UV absorbance detector. The analytical column used was a Hypersil ODS 5 μm (150×4.6 mm I.D.) purchased from Hypersil (Runcorn, UK) maintained at ambient temperature. A guard column (20×2 mm I.D.) packed with Hypersil ODS 5 μm material was placed between the injector and the analytical column. The chromatographic method development was carried out isocratically at ambient temperature. Different mobile phases made up of phosphate buffers and different proportions of methanol or acetonitrile were prepared with or without triethylamine (0.1% v/v). Each mobile phase was filtered through a 0.45 μm membrane filter and degassed for 30 min with helium before use. The flow-rate of the mobile phase was 1 ml/min and the sample injection volume was 100 μl. The chromatographic system was flushed with at least 20 column volumes of the mobile phase before each penicillin was analysed.

The proportion of the organic phase in each mobile phase was varied from 7 to 35% as required. The pH of the phosphate buffers (with or without triethylamine) was varied from pH 3 to 7 by the addition of appropriate volumes of orthophosphoric acid or sodium hydroxide solution. The decision to change the pH, buffer, proportion of organic phase or to add triethylamine to the mobile phase used to analyse each compound was based on the knowledge of the pK_a and lipophilicity (as represented by log D) of each compound. These pK_a and log D values were determined using a Sirius PCA1O1 automated potentiometric titrator (Sirius Analytical Instruments Ltd., Forest Row, UK) as previously described [10]. The peak symmetry and retention time of each penicillin were optimised.

In order to obtain the wavelengths of optimal UV absorption of the penicillins used for the method development and analysis (Table 1), UV scan of each compound (20 μg/ml, prepared in deionized water) was carried out using a Biochrom 4060 UV–visible

Table 1
Mobile phases and ultraviolet detection wavelengths for the analysis of a range of penicillins and the pK_a and log D values of the compounds

Compound	Mobile phase	Detection wavelength (nm)	pK_a value(s)	Log $D_{7.4}$
Amoxycillin	1	230	2.68, 7.49, 9.63	−1.89
Ampicillin	2	229	2.52, 7.13	−2.25
Epicillin	3	220	2.42, 7.47	−1.60
Mecillinam	4	218	2.45, 8.86	−1.67
Propicillin	5	210	2.56	−1.85

1=Aqueous 50 m*M* phosphate buffer containing 0.1% v/v triethylamine and adjusted to pH 3 with orthophosphoric acid–methanol (90:10, v/v).
2=Aqueous 50 m*M* phosphate buffer containing 0.1% v/v triethylamine and adjusted to pH 3 with orthophosphoric acid–methanol (70:30, v/v).
3=Aqueous 50 m*M* phosphate buffer containing 0.1% v/v triethylamine and adjusted to pH 3 with orthophosphoric acid–methanol (65:35, v/v).
4=Aqueous 50 m*M* phosphate buffer, pH 4.6–acetonitrile (86:14, v/v).
5=Aqueous 50 m*M* phosphate buffer, pH 7–acetonitrile (70:30, v/v).

spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, UK).

2.3. Calibration standards

Calibration samples of each compound were prepared in the mobile phase and HBSS supplemented with 20 m*M* glucose, 9 m*M* sodium bicarbonate and 25 m*M* HEPES buffer, pH 7.4 used in the in vitro studies. Calibration lines were performed over concentration ranges of 0.05 to 100 μg/ml for each individual penicillin.

2.4. Validation of methods

Calibration lines were constructed from peak areas against the drug concentrations. Linear regression analysis was used to calculate the slope, intercept and the correlation coefficient (r^2) of each calibration line. The assay precision (C.V.%) was assessed by expressing the standard deviation of repeated measurements as a percentage of the mean value. Intra-day precision was estimated from six replicates of measured peaks. Inter-day precision was estimated from the analysis of freshly prepared control samples on three separate days.

Specificity was assessed by the absence of interference at the retention time of the peak of each compound by any other peaks. The concentrations of the measured peaks were calculated from the calibration lines by linear regression analysis and the sample accuracy was estimated as the percentage of each measured concentration from the nominal (added) concentration.

The limits of detection were assessed as the minimum detectable quantity of each compound (signal-to-noise ratio of 3:1) that could be detected without interference from the baseline noise. The limits of quantitation were assessed as the minimum quantity of each compound (which is not less than three times the detection limit) that could be quantified.

In order to assess the stability of the compounds, samples of the compounds in buffer solution (HBSS supplemented with 20 m*M* glucose, 9 m*M* sodium bicarbonate and 25 m*M* HEPES buffer, pH 7.4) were analysed before and after an overnight storage in the autosampler at ambient temperature.

2.5. Application in in vitro permeability studies

2.5.1. Permeability of penicillins across Caco-2 cells monolayers

Caco-2 cells of passage number 20 purchased from the European Collection of Animal Cell Culture (ECACC) (Salisbury, UK) were used in the study. Cells of passage numbers 42–47 were used in the experiments as described previously [11]. Briefly,

Caco-2 cells maintained in cell culture flask were passed (1:10) every week and harvested at approximately 95% confluence. The cells were seeded at a density of 500 000 cells per Millicel®-PCF membrane insert (30 mm diameter, 3 μm pore size, Millipore Corporation, Bedford, MA, USA) and grown in DMEM growth medium supplemented with 10% fetal bovine serum and 1% MEM nonessential amino acid which was replaced every other day.

The apical-to-basolateral permeability of the penicillins was carried out on cell monolayers having transepithelial electrical resistance (TEER) of 700–800 ohms cm^2, 18–24 days postseeding and 24 h postfeeding. Antibiotic permeability across the monolayers was measured in a six-well cluster tissue culture plate in which the insert containing the monolayer was placed on top of a perforated cylindrical teflon disc in the well of the plate. The transport medium (5.5 ml of HBSS supplemented with 20 m*M* glucose, 9 m*M* sodium bicarbonate and 25 m*M* HEPES buffer, pH 7.4), containing the penicillin (2 m*M*) was placed in the insert while blank transport medium (2.5 ml) was placed in the well and stirred with a magnetic stirrer placed at the centre of the cylindrical disc. Aliquots (0.5 ml each) were sampled from the well at 0, 10, 20, 30, 45, 60, 90 and 120 min. Transport medium (0.5 ml) was used to replace each aliquot removed. The experiment was conducted at 37°C in an atmosphere of 5% $C0_2$ and 90% relative humidity. All experiments were carried out in 5 replicates. The aliquots (vortex-mixed and centrifuged at 11 600 *g* for 5 min) were analysed for the penicillins by HPLC.

2.5.2. Analysis of data

The measured concentrations of each compound were calculated from the slope and intercept of the calibration line of the compound in modified HBSS, pH 7.4 by linear regression analysis. The permeability of the penicillins was calculated as the amount transferred per unit area ($nmol/cm^2$). The flux of the penicillins ($nmol/cm^2\,h$) was calculated from the linear portion of the plot of the amount transferred ($nmol/cm^2$) against time using linear regression analysis. Statistical analysis was carried out using the unpaired Student *t*-test.

3. Results and discussion

3.1. Chromatographic conditions

The mobile phase that produced the best peaks in terms of symmetry and peak efficiency of each of the penicillins is given in Table 1. The respective chromatograms of the penicillins are given in Fig. 1. Different buffer pH values and solvent systems were used in the methods developed for the range of penicillins. The pH of the mobile phase affects the retention of compounds by determining the degree of ionization [12]. The ionization constant (pK_a) is the

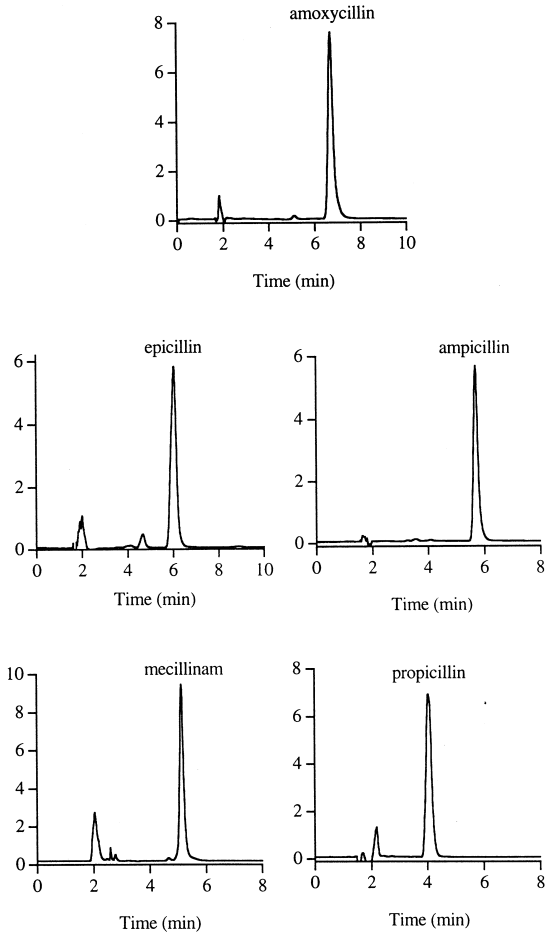

Fig. 1. Chromatograms of a range of penicillins in buffer soluion (Hank's balanced salt solution supplemented with glucose, HEPES buffer and sodium bicarbonate, pH 7.4).

pH at which 50% of the compound exists in solution as the ionized form. Knowledge of the pK_a values for the penicillins used in this study (Table 1) is useful in guiding the selection of the aqueous buffer pH. A correctly chosen aqueous buffer pH ensures that the functional groups in an analyte occur in a single form, whether ionized or unionized. The existence of a partially ionized analyte during a chromatographic run can often result in peak tailing, particularly with basic compounds. In addition, the lipophilicity profiles of amoxycillin, ampicillin, epicillin and propicillin (Fig. 2) were equally useful in the selection of appropriate buffer pH. The lipophilicity profiles are plots of the log D (logarithm of distribution coefficient) values against pH. Log D is a measure of the lipophilicity of a compound and a higher value indicates a more lipophilic compound [13]. The lipophilicity of a compound gives an indication of its interaction with the stationary phase and hence can predict its retention behaviour.

At a methanol concentration of 10% v/v in the mobile phase containing phosphate buffer and triethylamine 0.1% v/v (pH 3), the peaks of the amino-penicillins, ampicillin and epicillin were very broad and had retention times of more than 30 min, whereas amoxycillin produced a symmetrical peak.

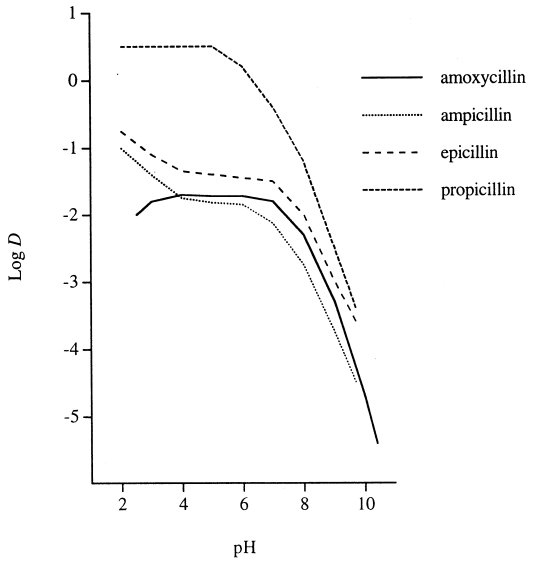

Fig. 2. Lipophilicity profiles of amoxycillin, ampicillin, epicillin and propicillin.

When the concentration of the methanol used to analyse ampicillin and epicillin were increased to 30% v/v and 35% v/v, respectively, symmetrical peaks of these compounds were produced. Amoxycillin has three pK_a values while ampicillin and epicillin have two each (Table 1). Thus the compounds would exist as ionized species for the entire pH range from pH 1–14. The existence of these compounds in two different species leads to peak tailing for the entire pH range when using silica based columns such as the ODS column used in this study. The addition of triethylamine to the mobile phase at the pH of the aqueous buffer used prevents the peak tailing by suppression of interactions of the ionized amine groups with the residual silanol groups of the HPLC packing material. The lipophilicity profiles of the three penicillins showed lower water solubility of ampicillin ($\log D \sim -1.4$) and epicillin ($\log D \sim -1.2$) at the mobile phase pH when compared with amoxycillin ($\log D \sim -1.8$). The differences in the proportion of organic modifier (methanol) used in the mobile phases for the analysis of the compounds can therefore be explained in terms of their relative solubilities.

Using aqueous phosphate buffer, pH 4.6 with methanol concentrations of 25–35% v/v, propicillin produced split peaks. A symmetrical peak was produced when the methanol was changed to acetonitrile (30% v/v). The choice of the aqueous buffer pH used for the analysis of propicillin can also be explained in terms of its pK_a values of 2.56. The splitting of the peaks produced by propicillin may be due to the diastereoisomers since the compound has chiral centres. However, at the mobile phase pH chosen for its analysis, the compound is fully ionized as a single species thus preventing any peak tailing. The experience gained from the methods developed for the analysis of the other penicillins described above was used to select phosphate buffer at pH 4.6 and acetonitrile at 14% for the preparation of the mobile phases used for the analysis of mecillinam.

Organic modifiers used in mobile phases are known to be adsorbed at the surface of the column packing material and are in competing equilibrium with lipophilic ions for absorption sites on the stationary phase [12]. Unlike acetonitrile, methanol forms hydrogen bonds resulting in decrease in the

ability to elute solutes from the adsorption sites in the column when compared with acetonitrile. Thus if methanol is to be replaced by an equal volume of acetonitrile as the organic modifier, a decrease in retention of the analyte is expected. Methanol is a cheaper solvent than acetonitrile but the higher viscosity leads to higher pressure drop along the analytical column [12]. Therefore the choice between methanol and acetonitrile in this study was based on cost, availability of the solvent, retention time and peak shape of the analyte.

3.2. Validation of methods

The assays were validated using 13-point calibration lines of each compound in the mobile phase and HBSS, pH 7.4. The calibration lines were all linear in the range of 0.1–100 μg/ml with r^2 values greater than 0.99. The validation data for the penicillins are shown in Table 2. The lower limits of detection of the compounds were about 0.02 μg/ml while the lower limits of quantitation were 0.1 μg/ml. The peaks of the penicillins had no interference from any other peaks. The compounds were stable in the aqueous samples after an overnight storage at ambient temperature.

3.3. Application in in vitro transport studies

It was possible to measure the apical-to-basolateral flux across Caco-2 cells monolayers for all the penicillins using the assay methods described. Examples of the measured flux plotted against time are given in Fig. 3. The calculated flux (nmol/cm^2.h) of ampicillin, epicillin, mecillinam and propicillin were similar but significantly lower than that of amoxycillin ($P<0.01$).

4. Conclusions

HPLC assay methods have been described for the rapid analysis of amoxycillin, ampicillin, epicillin, mecillinam and propicillin. The methods are simple, selective, reproducible and are useful for the analysis of these compounds in in vitro experiments involving the use of aqueous solutions. The ultraviolet detection of the compounds provides adequate sen-

Table 2
Accuracy and precision for the analysis of a range of penicillins ($n=6$)

	Nominal conc. (μg/ml)	Accuracy (%)		Precision (C.V.%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Amoxycillin	50.0	95.8	99.0	2.3	1.2
	6.25	99.4	97.8	1.6	1.9
	0.1	98.9	95.8	0.8	4.2
Ampicillin	50.0	97.7	98.3	2.3	2.1
	6.25	98.8	98.0	3.2	2.3
	0.1	94.7	94.6	1.7	1.5
Epicillin	50.0	100.8	101.1	2.7	2.0
	6.25	99.0	101.8	1.8	3.3
	0.1	94.3	100.9	3.2	9.1
Mecillinam	50.0	100.4	100.2	2.0	5.3
	6.25	92.1	96.0	2.1	5.6
	0.1	84.9	90.2	11.3	10.0
Propicillin	50.0	96.9	100.4	7.0	7.1
	6.25	92.0	97.7	4.1	8.2
	0.1	99.6	105.4	5.7	8.8

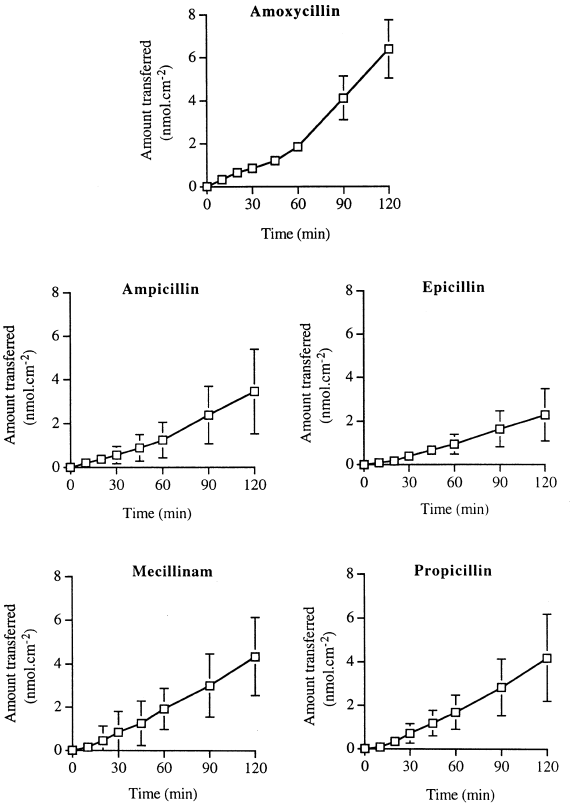

Fig. 3. Apical-to-basolateral permeability of a range of penicillins across Caco-2 cells monolayers. The error bars indicate the standard deviation.

sitivity and specificity for application in many in vitro experiments.

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References

[1] J.P. Hou, J.W. Poole, J. Pharm. Sci. 60 (1971) 503–521.
[2] L. Mizen, V. Berry, G. Woodnutt, J. Pharm. Pharmacol. 47 (1995) 725–730.
[3] G. Treiber, Am. J. Gastroenterol. 91 (1996) 246–257.
[4] W.A. Moats, J. Chromatogr. 593 (1992) 15–20.
[5] H.S. Huang, J.R. Wu, M.L. Chen, J. Chromatogr. 564 (1991) 195–203.
[6] H.Y. Lee, J.S. Lee, H.S. Lee, J. Chromatogr. B 664 (1995) 335–340.
[7] T.B. Vree, Y.A. Hekster, A.M. Baars, E. van der Kleijn, J. Chromatogr. 145 (1978) 496–501.
[8] M. Ishida, Y. Tsuda, Y. Onuki, T. Itoh, H. Shimada, H. Yamada, J. Chromatogr. B 652 (1994) 43–49.
[9] H.Y. Aboulenein, S.A. Bakr, Pharmazie 43 (1988) 212.
[10] A. Avdeef, Quant. Struct. Act. Relat. 11 (1992) 510–517.
[11] M. Hu, J. Chen, Y. Zhu, A.H. Dantzig, R.E. Stratford, M.T. Kuhfeld, Pharm. Res. 11 (1994) 1405–1413.
[12] J. Weiss, Ion Chromatography, 2nd ed., VCH, New York, 1995, pp. 239–255.
[13] C.N. Manners, D.W. Payling, D.A. Smith, Xenobiotica 18 (1988) 331–350.