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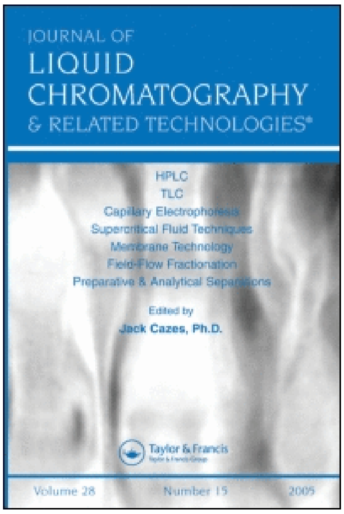

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The Analysis of Penicillins in Biological Fluids and Pharmaceutical Preparations by High-Performance Liquid Chromatography: A Review

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THE ANALYSIS OF PENICILLINS IN BIOLOGICAL FLUIDS AND PHARMACEUTICAL PREPARATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A REVIEW

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ABSTRACT

High performance liquid chromatographic methods for the analysis of penicillins in biological fluids and pharmaceutical preparations are reviewed. In particular, sample preparation and handling procedures, chromatographic conditions and detection methods are discussed. A summary of published high performance liquid chromatographic assays for individual penicillins is included.

INTRODUCTION

The penicillins are a group of bactericidal antibiotics derived from 6-aminopenicillanic acid (figure 1). The penicillin nucleus itself is the principal structural requirement for biological activity but the beta-lactam ring side-chain (R of figure 1; position 6) determines many of the

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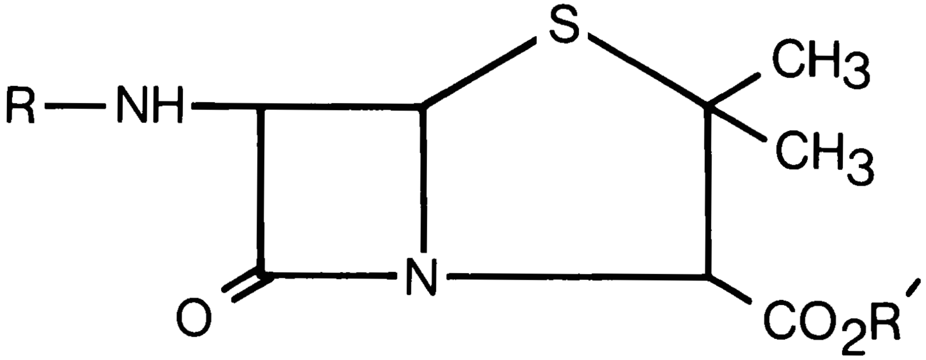

FIGURE 1

Chemical structure of penicillins

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antibacterial and pharmacological characteristics of a particular type of penicillin. Thus, the past three decades has seen the introduction of a range of 6-substituted penicillins in an effort to provide penicillins resistant to staphylococcal penicillinase as well as congeners with a broad spectrum of activity.

With the recognition of the valuable clinical advantages of many of these congeners it has become necessary to characterise their individual pharmacological and pharmacokinetic behaviour. For example, the penicillins differ markedly in their acid stability and oral absorption (1). It is also clearly important that the stability of pharmaceutical preparations of these drugs is defined. Consequently numerous techniques have been developed for the analysis of penicillins in biological fluids and pharmaceutical preparations. At this stage it is worth noting that while it is obviously necessary to characterise the chemical and biological properties of penicillins, they are among the least toxic of all drugs; conventional dosage regimens of these drugs allow plasma concentrations far in excess of minimum inhibitory concentrations of most susceptible pathogens to be safely achieved. Determination of plasma concentrations to ensure effective dosage of penicillins is therefore rarely necessary (2).

The analysis of penicillins has traditionally been performed using microbiological methods. However, this technique is slow and suffers from poor precision and specificity, since active metabolites and co-administered antimicrobials may interfere (3). Although numerous chemical and physical techniques have been reported for the assay of penicillins, they suffer from a variety of disadvantages. For example, spectrophotometric assays generally lack sensitivity (4,5) and a colourimetric method involving the reaction of hydroxylamine with penicillins lacks both sensitivity and specificity (6). An iodometric titration technique is not applicable to penicillins with an unsaturated side-chain (7,8). Spectrofluorometric assays are applicable only to fluorophoric penicillins (9) or those which form a fluorescent product on hydrolysis (10,11).

The convenience and versatility of high performance liquid chromatography (h.p.l.c.) has led to its acceptance as one of the most useful techniques for the analysis of drugs in biological fluids (12). Compared to chemical methods and other chromatographic techniques h.p.l.c. offers advantages in terms of precision, rapidity, reproducibility, sensitivity and specificity and it is therefore not surprising that the applicability of h.p.l.c. to the analysis of penicillins has become recognised. This is apparent when the number of h.p.l.c. assays for penicillins published in recent years (and reviewed here) is compared to previous reviews (3,12). The present article aims to review general approaches to the analysis of penicillins in biological fluids and pharmaceutical preparations by h.p.l.c. using examples from the literature. A list of methods for individual drugs is also included.

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SAMPLE PREPARATION AND HANDLING

Penicillins are relatively unstable in aqueous solutions, the degradation being catalysed by both acids and bases (13). In particular, the penicillins are extremely susceptible to nucleophilic attack and the inactivation of ampicillin in base is about 1,400 times faster than in acid. Optimum stability for monobasic penicillins generally occurs at a pH between 6 and 7, while for the zwitterionic penicillins it coincides with the isoelectric point. Buffer components may also affect the rate of penicillin degradation.

Not only does the aqueous instability of penicillins have important consequences for extraction and chromatography conditions (see below), but it is also an important consideration for the stability of stock solutions and storage of biological samples. Thus, amdinocillin degrades rapidly in plasma stored at -25°C, with only 80% and 50% remaining unchanged after 1 and 3 months respectively (14). Storage at -70°C slows down the degradation considerably. Amdinocillin has also been reported to be unstable in frozen (-20°C) urine samples (15).

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In general, the penicillins are strong organic acids (figure 1, R' = H) with pKa values less than 3. Certain penicillins, such as the ureidopenicillins, contain a basic group in the side-chain (R figure 1) and are therefore ampholytes. The polar nature of penicillins makes them difficult to partition from biological fluids into organic solvents. The majority of h.p.l.c. assays for the analysis of penicillins in plasma have therefore taken the approach of precipitating plasma proteins with perchloric acid, trichloroacetic acid or acetonitrile and directly injecting the separated supernatant onto the chromatography column. Although this approach is generally the most suitable and convenient, it should be recognised that it may give rise to a number of problems. Ampicillin has been shown to decompose at the rate of 15% per hour in the supernatant of a trichloroacetic acid treated plasma sample (14). Similarly, perchloric acid treated plasma samples containing amoxicillin must be chromatographed without delay to avoid drug loss (16). The instability of penicillins in acid media requires that methods involving an acid protein precipitation step should be performed in a highly reproducible fashion so that each standard and sample is subjected to the different steps of the procedure for the same (minimal) length of time (14). For extremely polar, zwitterionic penicillins such as amdinopenicillin, interference by endogenous compounds is a major problem during the reversed-phase chromatography of acetonitrile-plasma supernatants. The introduction of a diethyl ether wash of the protein-free supernatant has been shown to remove a large proportion of the interfering endogenous compounds and also to effectively concentrate the supernatant by extracting the acetonitrile (17). This procedure does result in some loss of the analyte, the reported recovery being 74%, but the use of an appropriate internal standard overcomes this problem. Other problems associated with the protein-precipitation method are lack of sensitivity compared to extraction procedures (see below) and comparatively short column life. The direct injection of perchloric acid and trichloroacetic acid supernatants is known to accelerate column deterioration, although in an analytical procedure for amoxicillin it has been reported that frequent (every fifteen injections) replacement of a few millimetres of the support at the top of the column with new material maintains column performance for up to six months (16).

A number of analyses for penicillins in biological fluids use solvent extraction procedures. Solvent extraction is a convenient means for the simultaneous selective enrichment and concentration of samples. Thijssen reported (18) that assay

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sensitivity for isoxazolyl penicillins is two to three fold higher with an extraction procedure compared to direct injection of the supernatant from a perchloric acid treated plasma sample. The extraction procedure also gave rise to considerably cleaner chromatograms. However, extraction of penicillins normally requires acidification of the plasma and this may result in degradation. For example, 6.4% of a sample of nafcillin is degraded during five minutes exposure to sulphuric acid treated plasma (19). Generally extraction efficiencies are less than quantitative, even when polar solvents such as chloroform-pentanol (20) and ethyl acetate (21) are used, and inclusion of an internal standard is essential. The use of an ion-pairing agent (tetrabutylammonium phosphate) for the extraction of the ureidopenicillins azlocillin and mezlocillin has been reported (22), but extraction efficiency using this approach was 50% or less.

Special problems arise with the analysis of ester (figure 1, R' = alkyl) pro-drugs of penicillins, such as bacmecillinam and bacampicillin. Bacmecillinam is extremely unstable in blood (approx. 30% of the compound is lost within 1 minute at 37^{0}C) and a fast procedure for the collection of blood samples is essential; typically blood samples containing ester pro-drugs are collected into fluorinated tubes and immediately cooled to -70^{0}C (23,24). Bacmecillinam also has a tendency to adsorb at certain interfaces. Silanisation of glassware or use of plastic extraction tubes does not appear to diminish adsorption and only by the addition of a large excess of a related pro-drug, such as bacampicillin, can extractions be made reproducible.

As indicated earlier, interference from endogenous compounds may cause major problems during the reversed-phase chromatography of plasma supernatant samples containing polar, zwitterionic penicillins. An h.p.l.c. assay for the determination of amoxicillin is plasma using a rapid

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bonded-phase extraction technique has recently been reported (25). This procedure results in significantly cleaner chromatograms than those normally obtained from injection of protein-free plasma supernatants. By avoiding the use of acidic plasma supernatants, drug stability is improved and an auto-injector may be used if large numbers of samples are involved.

CHROMATOGRAPHY

Since penicillins contain a carboxylic acid group, an anion exchange column was utilised in early papers (26,27) for the assay of ampicillin and benzylpenicillin. However, the pH of the mobile phases used in these assays was less than optimum with respect to the stabilities of the compounds being analysed. Thus, most recent methods for penicillin analysis have employed reversed-phase or ion-pair reversed-phase h.p.l.c. on microparticulate ($< 10\,\mu m$) bonded packing materials. High chromatographic efficiencies and short analysis times for penicillins are generally achievable with reversed-phase h.p.l.c. Furthermore, since penicillins are generally difficult to extract from biological samples reversed-phase chromatography provides the additional advantage of enabling the analysis to be performed in an aqueous environment.

Westerlund et al. (14) have investigated the influence of the pH of the buffer component of the mobile phase on the capacity ratios of four amphoteric (ampicillin, epicillin, cyclacillin and amdinocillin) and one acidic penicillin (benzylcillin) for reversed-phase chromatography on a C-8 column. For the amphoteric penicillins the retention increases both at lower (< 3.5) and higher (> 6.0) pH while, as expected, it continuously decreases with increasing pH for benzylpenicillin.

Thus, the charged forms of the amphoteric compounds appear to be retained to a larger extent than the neutral form, opposite to the trend observed for benzylpenicillin. The dramatic effect of mobile phase pH on the retention of the acidic penicillin, ticarcillin (also on a C-8 column) has similarly been shown by Kwan et al. (21); using a constant proportion of organic modifier (acetonitrile, 12.5%) the retention time was observed to increase from 1.5 to 11.0 minutes as the pH was decreased from 5.0 to 2.0. Although sharp, symmetrical peaks may be obtained by ion-suppression reversed-phase h.p.l.c. of acidic penicillins (21) the large majority of published h.p.l.c. assays for penicillins use a mobile phase pH in the range 4.5 - 7.5, presumably due to the instability of these compounds under more acidic conditions (14).

The influence of mobile phase organic modifier on the reversed-phase chromatography of ampicillin and amdinocillin has also been investigated by Westerlund et al. (14). The capacity ratios decreased as expected with increasing concentration of methanol or acetonitrile, although the relationship was curvilinear with acetonitrile which is the stronger solvating agent for penicillins. The increase in selectivity with increasing organic modifier was more marked with methanol than with acetonitrile, an effect which probably also reflects larger differences in solvation of the two penicillins in the organic solvents.

As indicated earlier, most published h.p.l.c. assays for penicillins use a mobile phase pH in the range 4.5 - 7.5, but some degradation of certain penicillins may still occur in this range (14). There appears to be no clear preference for either methanol or acetonitrile as organic modifier and mobile phases containing either solvent generally enable good separation of penicillins from metabolites, other drugs and endogenous plasma/urine constituents on either C-18, C-8 or phenyl columns.

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Ion-pair reversed-phase chromatography has been shown to be particularly useful for the separation of penicillins and their degradation products in pharmaceutical preparations (28-30). Typically the tetrabutylammonium cation has been used as the counterion. A mobile phase containing N,N-dimethyl-N-octylamine has been shown to improve the selectivity and analysis time for the measurement of ampicillin in urine (24). Similarly, heptylsulphonic acid has been used for the separation of a range of penicilins with an amine function in their side-chain in urine (31).

Special problems may occur with the chromatography of ester pro-drugs, such as bacmacillinam, which have a tendency to adsorb at certain interfaces. Reversed-phase chromatography of bacmecillinam was shown (23) to give unacceptable asymmetric peaks when the support was packed in conventional stainless steel columns. With glass-lined stainless steel columns the peaks were symmetrical but non-reproducible peak heights were obtained. This phenomenon was interpreted as being due to adsorption of the compound to the glass wall and was overcome by the addition of an adsorption inhibitor, N-hexyl-N-methylamine, to the mobile phase.

Some penicillins exist as diastereoisomers and there is evidence to suggest that the biological properties of the individual diastereoisomers are markedly different. For this reason there has been some interest in the separation of individual penicillin diastereoisomers. The diastereoisomers of ampicillin and phenoxyethyl-penicillin (32) and of carbenicillin (28) are separable by reversed-phase or ion-pair reversed-phase h.p.l.c. In all cases, mobile phase pH has been the critical factor in achieving satisfactory resolution of diastereoisomers.

DETECTION

The majority of h.p.l.c. assays for penicillins in biological fluids have employed ultraviolet detection (see Table

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TABLE 1

H.p.l.c. assays for penicillins

Drug	Biological fluid[a]	Column[b]	Chrom. time (min.)	Detection[c]	Sensitivity (mg/L)	Reference
Adicillin	Ph	RP	15	UV(220)	-	30
Amdinopenicillin	P,U,L	RP-8	20	D,UV(310)	0.1	14
	P,U	RP-Ph	8	UV(220)	0.5	17
	U	RP	7	UV(220)	50	15
Amoxicillin	P,S,U	RP-8	12	UV(225)	0.5	36
	P	RP-8	30	UV(225)	1.0	25
	U	RP-8	9	D,F(395,485)	2.5	35
	Ph	RP-8	20	UV(254)	-	37
Ampicillin	P,S,U	RP-8	10	UV(225)	0.5	36
	P,U,L	RP-8	15	D,UV(310)	0.1	14
	Ph	AX	12	UV(254)	-	27
Apalcillin	P,U	RP	15	UV(254)	0.5	33
	P	RP	-	UV(254)	0.5	38
Azlocillin	P	RP	5	UV(220)	1.5	39
	P	RP	15	UV(220,254)	0.4	34
	U	RP	12	UV(220)	1.0	40
Bacampicillin	Bl	RP	-	D,F(-)	0.008	24

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Bacmecillinam	Bl,Bi	RP	10	UV(220)	0.6	23
Benzathine-penicillins	Ph	RP	-	UV(258)	-	41
Benzylpenicillin	Ph	AX	22	UV(254)	-	26
Carbenicillin	Ph	RP	12	UV(254)	0.25	28
Cloxacillin	P,U	RP-8	8	UV(210)	0.05	19
	P,U	RP-8	10	UV(220)	0.4	18
Flucoxacillin	P,U	RP-8	18	UV(220)	0.4	18
Mezlocillin	P	RP	5	UV(220)	4.0	22
	P	RP	5	UV(220)	1.5	39
Nafcillin	P	RP	10	UV(218)	0.08	42
Oxacillin	P,U	RP-8	8	UV(220)	0.4	18
	U	RP	30	UV(254)	80	43
Penicillin	Ph	RP	8	UV(254)	-	29

(continued)

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TABLE I (continued)

H.p.l.c. assays for penicillins

Drug	Biological fluid[a]	Column[b]	Chrom. time (min.)	Detection[c]	Sensitivity (mg/L)	Reference
Piperacillin	P	RP	7	UV(254)	0.25	20
Pivmecillinam	Ph	RP	15	UV(220)	-	44
Ticarcillin	P,U	RP	8	UV(210)	1.0	21

[a] Biological fluid: P, plasma/serum; U, urine; L, lymph; Bl, blood; Bi, bile; Ph, pharmaceutical preparation

[b] Chromatographic column; AX, anion exchange; RP, reversed phase - the absence of a numeral following this implies octadecylsilane (C-18), while the presence of a numeral/ symbol indicates any other alkyl bonded phase used, i.e. RP-8 denotes octylsilica, RP-Ph phenylsilica.

[c] Detection: D, determined as a derivative; UV, ultraviolet detection with wavelength monitored (nm) in parentheses; F, fluorescence detection with excitation, emission wavelengths in parentheses.

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1). Ultraviolet detection generally provides sensitivity and specificity which is adequate for most bioanalytical purposes. The usual detection range for penicillins is 210-230nm, which is the region of the available detection range where these compounds exhibit maximum absorbance. However, it should be recognised that many endogenous compounds in plasma and urine absorb light in this area of the spectrum and additional measures are occasionally necessary to improve specificity. As indicated in the section on sample handling and stability, some methods remove potential interfering substandes from the analytical sample by solvent extraction or bonded-phase extraction. Alternative approaches to improving specificity rely on utilising other optical properties of the molecule or derivatisation with specific reagents.

Some penicillins contain a beta-lactam ring substituent (R of figure 1) which have sufficiently high molar absorptivity to allow their detection at longer wavelengths. For example, apalcillin, which contains the 4-hydroxy-1,5-naphthyridine group, also exhibits maxima at 254 and 310nm (33). Using a wavelength of 254nm the detection limit for the quantitation of apalcillin in plasma was 0.5mg/L and no interference from endogenous compounds was observed. Dual wavelength monitoring has also been employed to confirm assay specificity. In a method for azlocillin, column effluent was monitored at both 220nm and 254nm (34). The ratio of the peak height at 220nm to that at 254nm remains constant under normal conditions and a value different to this constant ratio therefore indicates the presence of an interfering compound.

Westerlund et al. (14, 16, 24) have developed a post-column derivatisation procedure for penicillins which gives a product with high molar absorbance around 310nm. Both the selectivity of the derivatisation reaction and the higher detection wavelength minimise the risk of interference from endogenous compounds. The derivatisation reaction proceeds by self-catalysed attack of imidazole on the beta-lactam bond of the penicillin followed by the formation of the mercuric mercaptide of the penicillenic acid formed. The reaction is specific for penicillins with an intact beta-lactam ring. The product has a molar absorbance of approximately 20,000 in the wavelength range 308-345nm, depending on the type of penicillin. Post-column derivatising procedures of the amino side-chain penicillins amoxicillin and bacampicillin with fluorescamine have been reported (24, 35). This procedure has the advantage that penicilloic acid metabolites may also be determined. In addition, formation of a fluorescent derivative enhances both specificity and sensitivity, the reported detection limit for the determination of bacampicillin in whole blood being 0.8 µg/L.

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METHOD COMPARISON

Where determined, excellent correlations (r 0.96 - 0.99) have been demonstrated for the measurement of penicillins by h.p.l.c. and microbiological assays (17,21,25,33-35). However, while the correlation coefficient for the measurement of azlocillin by h.p.l.c. and bioassay was 0.96, the microbiological technique consistently overestimated plasma azlocillin concentrations (34). A similar phenomenon has been observed for ticarcillin (21), where plasma concentrations measured by a microbiological assay were consistently 13% higher than those measured by h.p.l.c.

SUMMARY OF ASSAYS

Published h.p.l.c. analyses of individual penicillins are summarised in Table 1. Information has been provided on the type of biological fluid (or pharmaceutical preparation) to which the analysis has been applied, the type of chromatographic column and detection system used, total chromatography time, assay sensitivity and the reference. Where many methods for a particular drug have been published, an assessment has been made by the author as to which are the best available and only these have been listed.

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