J. Pharm. Pharmacol. 1988, 40: 506–509
Communicated February 3, 1988

Facile plasma-catalysed degradation of penicillin alkyl esters but with no liberation of the parent penicillin

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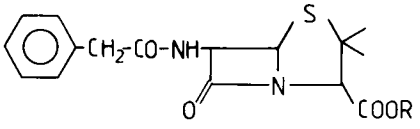
Abstract—The methyl ester and some glycolamide esters of benzylpenicillin and ampicillin were shown to be rapidly degraded by human plasma at 37 °C with no parent penicillin being produced. The plasma-catalysed degradation which was also observed in rat plasma proceeds most likely through hydrolytic cleavage of the β-lactam bonds of the penicillin esters and is suggested to be due to the presence of an ester-specific β-lactamase in plasma. The results show that the failure of simple alkyl esters of penicillins to function as prodrugs is not due to a high enzymatic stability of the esters, as widely believed, but rather to a pronounced susceptibility to undergo hydrolytic cleavage of their β-lactam ring in-vivo. Since double ester prodrugs of penicillins, such as the pivaloyloxymethyl ester of ampicillin, are readily hydrolysed in plasma to yield the parent penicillin although at a rate lower than e.g. that of inactivation of a simple methyl ester, the plasma enzyme apparently attacking the β-lactam bond of penicillin esters appears to have a high degree of specificity for the ester structure.

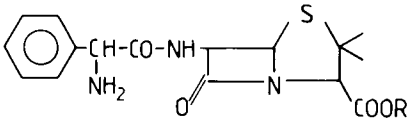
Esterification is a commonly used approach to obtain bioreversible derivatives (prodrugs) of carboxylic acids or alcohols with the aim of, for example, improving the absorption characteristics (Bundgaard 1985). The usefulness of this approach stems mainly from the fact that the organism is rich in enzymes capable of hydrolysing esters. The distribution of esterases is ubiquitous and several types can be found in the blood, liver and other organs or tissues (LaDu & Snady 1971; Banerjee & Amidon 1985). In addition, by appropriate esterification of molecules containing a carboxyl or hydroxyl group, it is feasible to obtain derivatives with almost any desirable hydrophilicity or lipophilicity as well as in-vivo lability.

Sometimes, however, many aliphatic or aromatic esters are not sufficiently labile in-vivo to ensure a sufficiently high rate and extent of prodrug ester conversion. This is the case for penicillin esters. It has long been recognized that, although the methyl ester and other simple alkyl or aralkyl esters of benzylpenicillin are readily hydrolysed to the free penicillin acid in some animals such as mice, they possess little antibacterial activity in-vivo in the larger mammals including man (Richardson et al 1945; Cavallito et al 1945; Ungar 1947). The reason for this lack of activity of simple esters of benzylpenicillin when given to e.g. the dog, monkey and man has consistently been claimed to be a lack of enzymes in these species capable of hydrolysing the esters (Hamilton-Miller 1967; Ferres 1980, 1983). This shortcoming of simple esters of penicillins for use as prodrugs has more recently been overcome by the development of double ester types such as (acyloxy) alkyl esters, which are readily hydrolysed to release the free penicillin in the blood and tissues of several species, including man (for reviews see Ferres 1980, 1983). The double ester prodrug principle has been used successfully to improve the oral bioavailability of ampicillin, and three ampicillin prodrug forms are now on the market, namely the pivaloyloxymethyl ester (pivampicillin), the phthalidyl ester (talampicillin) and the ethoxycarbonyloxyethyl ester (bacampicillin).

In a study of the stability of various penicillin esters, we found that the simple methyl ester (II) and some glycolamide esters (III, IV and VII) of benzylpenicillin (I) and ampicillin (V) were destroyed unexpectedly rapidly in human plasma solutions but that no free penicillin was formed. In contrast, pivampicillin (VI) and bacampicillin readily regenerated the parent ampicillin under the same conditions although at a *slower* rate than that of the loss of the simple alkyl esters. These findings, to be reported here, make it necessary to revise the explanation for the lack of in-vivo activity of simple alkyl esters of penicillins. The *N,N*-disubstituted glycolamide esters of the penicillins were included in the study since such esters of benzoic acid and several other acids have been shown to be cleaved exceptionally rapidly by pseudocholinesterase in human plasma (Bundgaard & Nielsen 1987; Nielsen & Bundgaard 1988).



I R=H
II R=-CH_3
III R=-$CH_2CON(CH_3)_2$
IV R=-$CH_2CON(C_2H_5)_2$



V R=H
VI R=-CH_2-OOC$C(CH_3)_3$
VII R=-$CH_2CON(C_2H_5)_2$

Materials and methods

HPLC was carried out with a system consisting of a Kontron T-414 LC pump, a Kontron Uvikon 740 LC detector operated at 215 nm and a Rheodyne 7125 injection valve with a 20 μL loop. A column, 100 × 3 mm, packed with Chromspher C 8 (5 μm particles) and equipped with a ChromSep guard column packed with a 30–40 μm pellicular reversed phase material was used. ^{1}H-NMR spectra were run on a Varian 360L instrument. Elemental analysis was performed at the Microanalytical Laboratory, University of Copenhagen. Benzylpenicillin sodium, ampicillin sodium and pivampicillin sodium were obtained from Leo Pharmaceutics, Ballerup, Denmark.

Synthesis of penicillin esters

Benzylpenicillin methyl ester (II) was prepared as described by

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Bell et al (1972), mp 95–96 °C; rep. mp 95–96·5 °C. The *N,N*-dimethylglycolamide ester (III) was prepared by stirring a mixture of benzylpenicillin sodium (1·78 g, 5 mmol), 2-chloro-*N,N*-dimethylacetamide (0·8 g, 6·5 mmol) and sodium iodide (75 mg) in *N,N*-dimethylformamide (10 mL) at room temperature (20 °C) for 18 h. Water (60 mL) was added and the mixture extracted with ethyl acetate (2 × 50 mL). The extracts were washed with aqueous sodium bicarbonate and water, dried and evaporated under reduced pressure to give the title compound which crystallized from ethanol–water, mp 71–72°C. The *N,N*-diethylglycolamide ester (IV) was prepared in a similar way using 2-chloro-*N,N*-diethylacetamide, mp 60–61 °C. Ampicillin *N,N*-diethylglycolamide ester (VII) was prepared by reacting potassium *N*-(1-methoxycarbonylpropen-2-yl)-D-α-aminobenzylpenicillinate (Baltzer et al 1980) with 2-chloro-*N,N*-diethylacetamide in a similar way. The enamine-protecting group was removed as described by Clayton et al (1976), yielding VII as a fine white amorphous solid.

Elemental analyses (C, H and N) were in all cases within ±0·4% of the theoretical values. The ^{1}H-NMR spectra of the esters were consistent with the structures.

Kinetic measurements

All studies were performed at 37·0 ± 0·2 °C. The reactions were initiated by adding 100 μL of a stock solution of the compounds in water or acetonitrile to 10 mL of preheated buffer or plasma solution, the final concentrations of the compounds being about 10^{-4} M. The solutions were kept in a water-bath at 37 °C and at appropriate intervals samples were taken and immediately analysed by HPLC. In the case of plasma solutions, the samples (250 μL) taken were added to 1000 μL of methanol to deproteinize the plasma. After immediate mixing and centrifugation for 3 min at 10 000 rev min^{-1}, 20 μL of the clear supernatant was analysed by HPLC.

For the analysis of benzylpenicillin (I) and its esters (II-IV) a mobile phase system of methanol—acetonitrile–0·2 M phosphate buffer pH 2·5 (1:3:6 v/v) was used. The column effluent was monitored at 215 nm, the flow rate was 1·0 mL min^{-1} and the compounds were quantified by measuring the peak heights in relation to those of standards chromatographed under the same conditions. Under these conditions the compounds showed the following retention times: I, 2·1 min; II, 4·6 min; III, 2·6 min; IV, 5·9 min.

The hydrolysis of the ampicillin esters VI and VII was followed using a mobile phase system of 0·02 M phosphate pH 7·0–methanol (4:6 v/v) whereas for product analysis of ampicillin formed, a mobile phase system consisting of 0·02 M phosphate pH 7·0–methanol (4:1 v/v) was used.

Table 1. Half-lives for the degradation of benzylpenicillin and various pencillin esters at 37 °C.

Compound	$t_{\frac{1}{2}}$ (min)	
	0·01 M phosphate buffer pH 7·4	80% human plasma (pH 7·4)
I	3500	1560
II	1190	5·6
III	740	27
IV	705	21
VI	110	50
VII	650	70

Results and discussion

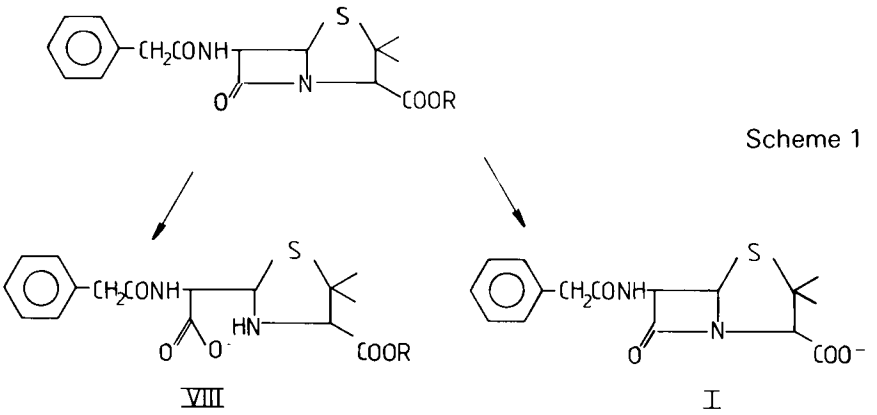
The rate of degradation of various esters of benzylpenicillin and ampicillin in aqueous buffer solutions and in 80% human plasma solutions followed good first-order kinetics over several half-lives. The half-lives observed are listed in Table 1. As can be seen human plasma markedly catalysed the rate of degradation of the esters but in no cases except for pivampicillin (VI) was the parent penicillin produced in any measurable amounts as evidenced by HPLC analysis of the reaction solutions. By this method less than 1% free penicillin formed from the esters could be detected in the solutions. For pivampicillin the amounts of ampicillin formed in plasma solutions corresponded to 50%, in a agreement with previous findings (Bundgaard & Klixbüll 1985). Although only 50% of ampicillin is released the hydrolysis of pivampicillin does involve a quantitative hydrolysis, the reason for the decreased yield being a secondary reversible reaction between the initial hydrolysis products, ampicillin and formaldehyde, to yield a 4-imidazolidinone derivative (Bundgaard & Klixbüll 1985).

In contrast to the behaviour in human plasma solutions, the alkyl and glycolamide esters were partly hydrolysed to yield the parent penicillin in aqueous buffer solutions of pH 7·4–10. Thus, at pH 7·40 and 37 °C benzylpenicillin was formed in amounts of 17, 34 and 30% from the esters II, III and IV, respectively, whereas the ester VII was converted into ampicillin in a yield of 20%.

In 80% rat plasma, some benzylpenicillin was also formed. The esters investigated here, II and IV, were degraded very rapidly, the half-lives being 1·2 and 0·3 min, respectively, and the amounts of benzylpenicillin formed being 16% for compound II and 37% for compound IV. The data for compound II are in accord with the earlier work by Richardson et al (1945), showing some formation of benzylpenicillin from the methyl ester given parenterally to rats.

The degradation of the penicillin esters is suggested to take place by two parallel reactions: hydrolysis of the ester group to produce free penicillin and cleavage of the β-lactam bond to yield a penicilloic acid ester (Scheme 1). The β-lactam moiety in

Scheme 1



VIII I

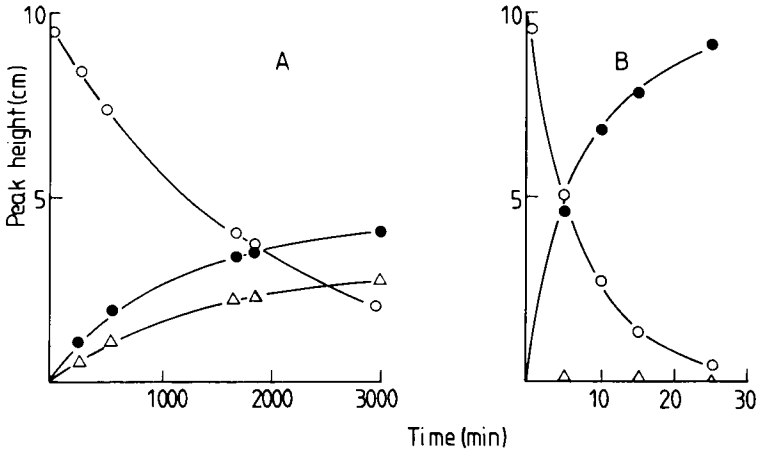


FIG. 1. Plots showing the rate of degradation of benzylpenicillin methyl ester (II) in 0·01 M phosphate buffer solution of pH 7·4 (A) and 80% human plasma solution (B) at 37 °C and the formation of products. Key: ○, HPLC peak due to compound II; △, peak due to benzylpenicillin; ●, peak presumed to be benzylpenicilloic acid methyl ester.

penicillin esters is known to exhibit a markedly higher reactivity than that in penicillin towards nucleophiles as amines and hydroxide ions (Bundgaard 1979; Proctor et al 1982) because of the better leaving ability of the thiazolidine nitrogen in the esters. This is also apparent from the stability data in Table 1. Evidence for the reaction scheme was obtained from monitoring the reaction solutions by HPLC. For all the benzylpenicillin esters II-IV a peak with a retention time less than that of the esters but different for each ester appeared in the chromatograms during the degradation in buffer solutions concurrently with the formation of benzylpenicillin (Fig. 1). This peak which most likely is due to the corresponding benzylpenicilloic acid ester (VIII) was also formed in human plasma solutions but now to a greater extent and at the same time, a peak due to benzylpenicillin did not appear (Fig. 1).

These observations along with the kinetic data strongly indicate that human plasma rapidly inactivates the penicillin esters through hydrolytic cleavage of their β-lactam bonds with the formation of the corresponding penicilloic acid esters. Thus, contrary to the presently held opinion (Hamilton-Miller 1967; Ferres 1980, 1983), the failure of simple alkyl esters of penicillins to function as prodrugs is not directly due to a high enzymatic stability but rather to a pronounced susceptibility to undergo hydrolytic cleavage of their β-lactam ring in plasma or at other sites in-vivo. In the case of rat plasma both routes shown in Scheme 1 are important in the rapid loss of penicillin esters, the major reaction being β-lactam hydrolysis.

The component in human plasma (or rat plasma) apparently catalysing the hydrolysis of the β-lactam moiety in penicillin alkyl esters but not that in penicillin itself is not known but it may be an ester-specific β-lactamase enzyme. Plasma proteins such as albumin appear not to be involved since the half-life of degradation of the esters II-IV in a solution of 4% human serum albumin at pH 7·4 was found to be only a little shorter than those observed in buffer solutions of the same pH. When 10% human plasma solutions were used, the rate of ester degradation decreased considerably compared with the 80% solutions. Thus, the half-life for ester IV was 135 min in 10% plasma solutions and 21 min in 80% solutions. A metal ion catalytic effect was ruled out from experiments showing no effect on the rate of degradation by the addition of EDTA to the plasma solutions. Four different batches of human plasma have been investigated but in all no penicillin was formed from the alkyl or glycolamide esters. Similar degradation rates (within ± 10%) of the esters were observed in the various plasma samples.

The suggested implication of an ester-specific β-lactamase is supported by an earlier study by Snow (1962) who reported that supernatant fractions of liver homogenates from various animals contained an enzyme which converted the methyl ester of benzylpenicillin into benzylpenicilloic acid methyl ester. Furthermore, benzylpenicillin itself was found not to be hydrolysed by the liver enzyme. Apparently, such an enzyme is also found in human plasma as well as in plasma from rats as indicated by the results reported here.

Although the present study has shown that alkyl esters of benzylpenicillin are rapidly hydrolysed at their β-lactam bond by plasma, most probably by an ester-specific β-lactamase, the opposite behaviour of the double ester prodrugs remains unexplained. Thus, the acetoxymethyl ester of benzylpenicillin is hydrolysed quantitatively to benzylpenicillin in human serum (Agersborg et al 1966). Similarly, although the ampicillin ester VI (pivampicillin) is only slowly hydrolysed in 80% human plasma solutions, the half-life being 50 min (Table 1), it is not attacked by a plasma enzyme at its β-lactam moiety. Other ampicillin double ester prodrugs as talampicillin and bacampicillin behave similarly (Clayton et al 1976; Shiobara et al 1974; Bodin et al 1975). In contrast, the glycolamide ester of ampicillin (VII) is totally hydrolysed at its β-lactam bond by the same plasma which does not attack pivampicillin at its β-lactam bond. Apparently, the plasma enzyme attacking the β-lactam bond of penicillin esters has a high degree of specificity as regards the esters structure.

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J. Pharm. Pharmacol. 1988, 40: 509–511
Communicated January 4, 1988

Sustained anti-adherence activity of taurolidine (Taurolin) and noxythiolin (Noxyflex S) solutions

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Abstract—Taurolidine (2% w/v) and noxythiolin (1% w/v and 2·5% w/v) solutions inhibit the adherence in-vitro of *Escherichia coli* and *Staphylococcus aureus* to human epithelial and fibroblast cells. This effect, demonstrable after 30 min exposure of cells to test drugs, persists after removal of the active compound. Significantly reduced adherence of bacteria is apparent for 5 h after taurolidine treatment and for 6 h after treatment with 2·5% noxythiolin. Anti-adherence activity of taurolidine and noxythiolin may contribute to the observed clinical efficacy of these agents.

Taurolidine (Taurolin, bis-(1,1-dioxoperhydro-1,2,4-methylene thiadiazinyl-4) methane) is a novel antimicrobial for local or parenteral use. It has a unique spectrum of antimicrobial activity including Gram-positive and Gram-negative bacteria and fungi (Reeves & Schweitzer 1974; Browne et al 1977). Clinical experience suggests that the compound has useful activity in-vivo when administered by the intravenous (Nitsche et al 1985) or intraperitoneal (Browne et al 1978) routes. Of particular interest are observations of significant neutralizing activity against endotoxin in-vitro (Thomas et al 1985; Blenkharn 1987b) and against endotoxaemia in animals (Pfirrmann & Leslie 1979) and man (Nitsche et al 1985).

Recent reports have shown that taurolidine (Gorman et al 1987), and the related compound noxythiolin (Gorman et al 1986), exhibit marked anti-adherence properties in-vitro. The clinical significance of these observations remains uncertain. However, the ability to interfere with adherence of pathogenic micro-organisms to host cells may contribute significantly to the clinical efficacy of these agents. With both compounds, pretreatment of urinary or buccal epithelia, or test organisms (*Staphylococcus saprophyticus*, *Escherichia coli* and *Candida albicans*), significantly inhibits organism/cell adherence when compared with water or 0·015% *N*-methylthiourea. The extent of this apparent anti-adhesive activity, particularly with regard to the treatment intervals for these drugs, has not previously been defined and is the purpose of this report.

Materials and methods

Cell harvest and preparation. Exfoliate epithelial cells were harvested from the pooled 12 h bile drainage of two patients having external biliary decompression following surgery for choledocholithiasis. Urine from three healthy adult females was similarly pooled for cell harvest. Samples of bile (1700 mL) and urine (2400 mL) were centrifuged at 700 *g* for 60 min. The resultant cell pellets were washed six times in RPMI 1640 medium.

Cells were trypsinized by the addition of 2 mL 0·05% trypsin and 0·02% disodium EDTA in Hanks' balanced salts solution. Suspensions were shaken gently and after 15 min incubation at 37 °C diluted with an excess of RPMI 1640. After centrifugation at 500 *g* for 10 min, cell pellets were washed three times with, and finally suspended in, Eagle's minimal essential medium (MEM) containing 10% newborn calf serum.

The established HEp2 line of epithelial cells, derived from a human laryngeal carcinoma, and the MRC 5 line of human embryonic lung fibroblasts were also examined. HEp2 cells were grown in Earle's MEM supplemented with Hanks' balanced salts solution (BSS), 15% calf serum and 2 mM glutamate. For MRC 5 cells, monolayers were grown in Eagle's MEM supplemented with Eagle's BSS, 10% foetal bovine serum, 2 mM glutamate and 1% non-essential amino acids. Monolayers of