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THE PHARMACOLOGY OF PENAMECILLIN

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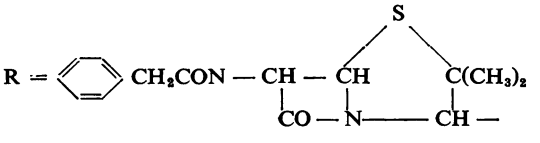
The methyl ester and other simple alkyl or aralkyl esters of benzypenicillin have little anti-bacterial activity *in vitro* or *in vivo* in the larger mammals. They are effective against bacteria in rats and mice because these species have an esterase of the necessary specificity to liberate the antibiotic (Richardson, Walker, Miller & Hanson, 1945). Esters with specific structural features which undergo spontaneous hydrolysis in aqueous media have been described (See Barnden, Evans, Hamlett, Jansen, Trevett & Webb, 1953 for references; McDuffie & Cooper, 1953), although only one, penethamate hydriodide (the dimethylamino ethyl ester), has been introduced into therapy.

With the acetoxymethyl ester of benzylpenicillin (penamecillin, Wy 20788), which is a double ester, a new principle of breakdown becomes possible (Jansen & Russell, 1964). Acetic and non-specific esterases are widespread in mammals and it is therefore to be expected that in mammalian species the acetic acid moiety of penamecillin (I) will be split off to leave the unstable mono-ester (II) which in turn will undergo rapid spontaneous hydrolysis to benzylpenicillin (III) and formaldehyde.

$$\underset{\text{(I)}}{R.COO.CH_2.OOC\text{–}CH_3} \xrightarrow[\text{esterase}]{\text{Non-specific}} \underset{\text{(II)}}{R.COO.CH_2OH} + CH_3.COOH$$

$$R.COO.CH_2OH \xrightarrow[\text{(III)}]{} R.COOH + HCHO$$



Since marked differences exist in stability, solubility and partition coefficient between benzylpenicillin anion (or its conjugate acid) and a neutral ester, such as penamecillin, we can expect to see these differences reflected in their effects on biological systems. The following report is a study of the absorption and excretion of penamecillin, after oral administration in mammals; comparison has been made with benzylpenicillin and phenoxymethylpenicillin.

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METHODS

Microbiological assay of penicillin in serum, etc. All assays were made by the cup-plate technique (Lightbown & Sulitzeanu, 1957) using *Sarcina lutea* (NCTC No. 8340) as a test organism and potassium benzylpenicillin as a reference standard in buffer solution or serum as appropriate. Blood samples were taken under aseptic conditions, allowed to clot and the serum removed for assay.

Detection of penamecillin in body fluids or tissues. To remove the proteins and to stop enzyme activity immediately following withdrawal body fluids were mixed with 6 volumes of acetone at room temperature and centrifuged. The precipitate was washed twice with fresh acetone and the washings combined with the original supernatant. Aliquots (10 or 20 μl.) of this solution or a suitable dilution of it, were applied to 19 cm strips of Whatman No. 1 chromatography paper previously treated with 0.1 M citrate buffer solution *p*H 5. The ascending chromatogram was developed at 21° C for about 40 min with the top phase of a mixture of petroleum ether (60–80° C): benzene : methanol : water (1 : 4 : 4 : 1, v/v). Under these conditions penamecillin had an R_F value of 0.8, whereas benzylpenicillin salt remained at the origin. The paper was dried and then run with wet ether for about 20 min until the solvent front had advanced to about 3/5 of the distance travelled by the first solvent mixture. The penamecillin spot was thus not shifted from its previous position but the benzylpenicillin spot moved to give an R_F value of 0.3 with this system.

Both the ester and its salt were revealed autobiographically by incubating the paper overnight on an agar plate seeded with *Sarcina lutea*, the method being similar to that described by Nicolaus, Coronelli & Binaghi (1961) for thin layer chromatograms. As little as 0.005 μg of penicillin per spot could be detected and the method could be used as a semi-quantitative assay.

For examination of tissues the following procedure was used:

2 hr after dosing the animals were anaesthetized with ether, and blood was withdrawn from the inferior vena cava and mixed with 4 volumes of chloroform.

A known weight of liver, from each animal, was homogenized in chloroform (4 ml./g wet tissue) using a Potter-Elvehjem-type homogenizer with a Teflon pestle. The kidneys were weighed and treated in a similar manner.

The chloroform extracts were then filtered and concentrated by evaporation at 37° C under reduced pressure. The resulting solutions were then subjected to the chromatographic procedure described above. The lower limit of detection in all these experiments with concentrated extracts was 0.005 μg/g wet tissue or/ml. whole blood.

To demonstrate enzymic hydrolysis by serum or tissue esterases the following technique was used:

100 μg penamecillin were added in 10 μl. acetone to 10 ml. dilute rat serum at zero time. 0.5 ml. aliquots were taken at 0, 15 and 30 min at room temperature (21° C) and mixed with 3 ml. acetone to precipitate the serum proteins. The precipitate was washed twice with 2.5 ml. acetone and the supernatant and washings were made up to 10 ml. The penicillins were then estimated by chromatography followed by the autobiographic technique.

Dog serum and rabbit serum were used undiluted and homogenates of intestinal mucosa, gastric mucosa, liver and kidney in saline were used for the study of hydrolysis by tissue esterases.

RESULTS

Attempts to detect penamecillin in blood lymph and tissues. Experiments were carried out to try to detect the unhydrolysed compound in the blood of dogs which had received 50 mg/kg penamecillin administered as an aqueous suspension via a stomach tube. Blood samples were withdrawn from the cephalic vein and treated as previously described.

When penamecillin and benzylpenicillin were added to dog blood just before precipitation of the proteins with acetone, both compounds could be readily demonstrated by the

autobiographic technique. When chloroform was used to precipiate the proteins only penamecillin was extracted. These results showed that both compounds could be recovered from dog blood.

Using this technique, no unchanged penamecillin coud be demonstrated in blood samples taken during the first 2 hr after dosing, although the absorption of the drug was taking place, as indicated by the detection of the hydrolysis product, benzylpenicillin. Some of the extracts were evaporated to a small volume before chromatography to increase the sensitivity of the method. No penamecillin was detected.

In anaesthetized dogs blood was taken from the hepatic portal system at intervals after 50 mg/kg penamecillin administered as above but no unchanged compound was detected. In one animal the stomach was shown to contain penamecillin 1½ hr after dosing, although examination of blood from a stomach vein showed the presence of benzylpenicillin only.

The failure to demonstrate penamecillin in either the systemic or hepatic portal circulations seems to indicate that rapid breakdown takes place during the process of absorption, either in the lumen of the intestine or during its passage through the cells of the mucosa. The ease with which penamecillin is hydrolysed by both lipase and preparations of intestinal mucosa provides further evidence for this contention (see below).

As no penamecillin had been detected in blood from the hepatic portal circulation it was thought possible that some might be absorbed unchanged in solution in neutral fat and enter the lymphatic system. To put this to the test a dog was anaeshetized with sodium pentobarbitone and 50 mg/kg penamecillin was administered by stomach tube and the anterior end of the thoracic duct was then exposed. Two hr after dosing, lymph was withdrawn and examined for penamecillin by a similar technique to that used for blood samples. None was detected, indicating that no significant absorption takes place via this route.

Chloroform extracts of blood, liver and kidney were prepared from six male albino rats which had received 400 mg/kg penamecillin orally as an aqueous suspension. Two hr after dosing no penamecillin was detected in the tissues. If this dose level does not produce detectable tissue levels it seems unlikely that significant amounts of the substance would ever reach the tissues after a clinical dose.

Demonstration of hydrolysis by tissue and serum esterases. Penamecillin was readily hydrolysed by serum from the rat, rabbit and dog. In rat serum diluted with 4 volumes of 0.9% NaCl there was very rapid hydrolysis of the compound at room temperature (21° C). No penamecillin was detected even at 0, 15 and 30 min, indicating that very rapid breakdown had taken place. Eighty to one hundred per cent of the added activity was recovered as benzylpenicillin. When dilute serum was buffered to *p*H 5.0 and 7.0 with an equal volume of 0.1 M phosphate buffer there was no change in the pattern of breakdown. At *p*H 3.0 no breakdown was observed during 30 min and good recoveries were obtained. The recovery of penamecillin from the non-enzyme controls showed that no significant breakdown had taken place during the 30-min incubation period.

The esterases of rabbit and dog serum were less active than those of the rat. In these species undiluted serum took 20–30 min to bring about complete hydrolysis of

TABLE 1

COMPARISON OF SERUM CONCENTRATION OF PENAMECILLIN AND THE POTASSIUM SALTS OF BENZYLPENICILLIN AND PHENOXYMETHYLPENICILLIN IN TEN DOGS FOLLOWING A SINGLE ORAL DOSE OF 25 MG/KG BODY WEIGHT

All results are expressed in terms of potassium benzylpenicillin

	Males + females (10) μg/ml. serum (mean ± S.E.M.)						
Hours after dose	1	2	4	6	8	10	12
Drug:							
Penamecillin	0·378 ± 0·146	0·915 ± 0·254	0·771 ± 0·235	0·378 ± 0·111	0·207 ± 0·082 $0·05>P>0·02$	0·102 ± 0·033	0·062 ± 0·030
Potassium benzylpenicillin	5·52 ± 1·02	2·53 ± 1·02	0·369 ± 0·096	0·084 ± 0·028	0·014 ± 0·007	—	Nil
Potassium phenoxymethyl-penicillin	2·96 ± 0·60	0·721 ± 0·090	0·143 ± 0·034	0·027 ± 0·008	0·003	—	—

penamecillin. Preliminary tests using human serum esterases showed a pattern of breakdown similar to that obtained with dog and rabbit serum.

Attempts to inhibit the esterase activity of rat serum with neostigmine methyl sulphate (Prostigmin, Roche) at a final concentration of 10^{-4}M were unsuccessful and this suggests that non-specific esterases are concerned with the breakdown of penamecillin. It has been shown by a number of workers (Huggins & Lapides, 1947; Mounter & Whittaker, 1953) that the non-specific esterases of human serum are not sensitive to inhibitors at concentrations which will inhibit serum cholinesterases and it would appear that the non-specific esterases of the rat show similar properties. Similar experiments using homogenates of intestinal mucosa, gastric mucosa, liver and kidney showed that penamecillin was hydrolysed by the non-specific esterases from these organs. Ten per cent liver homogenates in 0.9% NaCl gave complete hydrolysis at 0 min, kidney homogenates were less active, taking 15 min for complete hydrolysis.

TABLE 2

COMPARISON OF THE NUMBER OF HOURS FOR WHICH A SERUM LEVEL OF 0·1 μG/ML. WAS MAINTAINED AFTER ORAL ADMINISTRATION OF PENAMECILLIN, POTASSIUM BENZYLPENICILLIN OR POTASSIUM PHENOXYMETHYLPENICILLIN (25 MG/KG BODY WT.) IN THE DOG

Dog no. (sex)	Penamecillin	Time taken for Penamecillin to reach this level (min.)	Potassium benzyl-penicillin	Potassium phenoxymethyl-penicillin
1 (♂)	2·5	75	5·5	3·5
3 (♂)	6·0	<30	7·0	4·5
10 (♂)	7·5	<30	4·5	5·0
11 (♂)	9·0	75	4·0	5·5
12 (♂)	12·0	135	5·5	4·0
2 (♀)	10·0	75	5·5	3·5
5 (♀)	12·0	30	5·0	5·5
6 (♀)	4·5	<30	7·0	4·0
8 (♀)	10·5	<30	5·0	5·0
9 (♀)	6·5	75	7·0	4·0
Mean ± S.E.M. (10) (males and females)	8·1±1·0	(58·5)	5·6±0·3	4·5±0·2

Blood level studies in dogs. As demonstrated above, no unchanged penamecillin appears in blood after oral administration in dogs and the rate of hydrolysis of the ester is very rapid. In considering blood levels in dogs it was therefore necessary only to assay the serum for antibacterial activity as potassium benzylpenicillin.

A cross-over study was performed in beagles in order to compare the time course of the blood levels achieved after the oral administration of 25 mg/kg penamecillin, potassium benzylpenicillin and potassium phenoxymethylpenicillin given after food. Blood samples were taken for serum penicillin assay at intervals during the first twelve hours. The results of the serum assays are given in Table I. The Student t-test on the 8 hr values was carried out using the modified test for paired data (Mapes, 1963). Peak serum concentrations occurred 1 hr after dosing with potassium benzylpenicillin and potassium phenoxymethylpenicillin. With penamecillin the peak was less marked and usually occurred 2–3 hr after the dose. It was felt that the prolonged duration of a therapeutic blood level was of greater importance than the time of occurrence or height of the peak level, and therefore the results are expressed in terms of the time taken for penamecillin to reach a serum level of 0.1 μg/ml., together with the time for which this level was maintained. Table II gives the results of this comparison for penamecillin, potassium benzylpenicillin, and potassium phenoxymethylpenicillin. Although potassium benzylpenicillin and potassium phenoxymethylpenicillin give therapeutic levels within 30 min of the dose these are maintained for only 5–6 hr. Penamecillin takes, on average, about an hour to reach a level of 0.1 μg/ml. in the serum and this is maintained for 8 hr. The slow hydrolysis of the ester leads to a marked increase in the time for which an effective serum penicillin level can be maintained.

TOXICOLOGY

Acute Toxicity

A suspension of penamecillin in 0.5% carboxymethyl cellulose was administered orally to groups (5 males and 5 females) of mice and rats. No animal died over a 14-day period after a single dose of 5 g/kg body weight.

Prolonged administration

Rats. Groups of 20 rats (10 males and 10 females) were given the drug in the diet at a concentration of 0.4%, 0.8%, or 1.6% for a period of 6½ to 7 weeks. A control group received diet alone. At the highest dose the calculated drug intake, on the basis of body weight and food uptake during the 3rd week of the test, was 1.5–1.6 g/kg/day in both males and females. Food intake and growth was not adversely affected apart from a temporary depression in weight gain of females on the highest dose during the first week.

The microhaematocrit, haemoglobin concentration, erythrocyte and leucocyte counts and differential leucocyte count were determined twice before treatment and on the 2nd, 3rd, and 6th weeks thereafter there were no statistically significant drug-related changes.

At autopsy organ weights (per 100 g body weight) were determined for heart, lungs, liver, left and right kidney, left and right testis (or ovary), spleen, thymus, pancreas, left and right adrenal, thyroid, pituitary, brain, seminal vesicle and ventral prostate (or uterus). The only statistically significant drug-related change occurred in the pituitaries

of females which had received the highest dose. These were significantly smaller than in the controls ($P<0.05$) both on an absolute and relative basis. Histopathological examination of all tissues revealed no evidence of organic injury which could be related to drug treatment.

Dogs. Groups of 4 dogs (2 male and 2 female) were given the drug orally in capsules at 400, 800 or 1,600 mg/kg/day for 5½ weeks. A control group was given starch in capsules. All animals survived and the only untoward effect, observed in 3 of the dogs on the highest dose, was vomiting during the first 3 or 4 days of treatment. The micro-haematocrit, haemoglobin concentration, erythrocyte and leucocyte count, differential leucocyte count and sedimentation rate were determined twice before and on the 3rd and 5th week after treatment. No significant changes between the values in pre- and post-treatment groups were found.

Bromsulphthalein retention, serum alkaline phosphatase, glutamic-oxalo-acetic transaminase, glutamic-pyruvic transaminase, bilirubin, cholesterol, albumin and globulin, blood urea and blood sugar were also determined at the same time intervals.

In dogs on the highest dose level, alkaline phosphatase and glumatic-oxalo-acetic transminase values were higher than in controls but the differences were not considered to be of biological importance. In the same animals the serum cholesterol values were lower than in the controls.

Urine analysis revealed no changes attributable to drug action in the following: *p*H, specific gravity, albumin, sugar, ketone bodies, bilirubin, occult blood and deposit.

At autopsy organ weights were determined for heart, lungs, liver, left and right kidney, spleen, thymus, pancreas, left and right adrenal, thyroid, pituitary, brain. No changes of biological significance were detected.

Histopathological examination of all tissues revealed only one change attributable to drug action, in one dog on the highest dose which showed moderate hydropic vacuolisation within liver cells.

DISCUSSION

The animal studies described above suggest that penamecillin may find therapeutic application as an oral benzylpenicillin preparation, for it produces a better sustained antibiotic serum level than benzylpencillin itself. The precise mechanism of absorption of penamecillin is not known with certainty. There is a considerable amount of experimental evidence to indicate that no significant amount of the unchanged compound ever reaches the tissues. The ester combination has some protective effect on the benzylpenicillin molecule in the acid conditions of the stomach and it is possible that some unchanged penamecillin is absorbed in this region of the gut. However, this would be readily hydrolysed by the non-specific esterases of the mucosal cells or by serum esterases on passing into the circulation. The amount of penamecillin passing in via this mechanism would tend to be limited by the low solubility of the unchanged compound. Once the compound reaches the duodenum rapid hydrolysis can take place in the lumen of the intestine by the action of pancreatic lipase and it is probable that the variation in the peak observed with penamecillin is largely a measure of the rate and extent of this hydrolysis. Any unchanged penamecillin which passes into the mucosal cells will

be hydrolysed there by tissue esterases or broken down in the serum. The slow hydrolysis of penamecillin by non-specific esterases results in a gradual release of its metabolite, benzylpenicillin, and enables therapeutic blood levels to be maintained for a longer period than is possible with conventional benzylpenicillin preparations.

SUMMARY

1. The biological properties are described of a new, relatively stable, ester of benzylpenicillin which releases the antibiotic under the action of non-specific esterases.

2. Administered orally to dogs it produces better sustained blood levels than salts of benzylpenicillin or phenoxymethylpenicillin do.

3. Sub-acute (6 weeks) toxicity studies in rats and dogs revealed no evidence of organic injury which could be related to drug treatment.

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