PHTHALIDYL D-α-AMINOBENZYLPENICILLINATE HYDROCHLORIDE (PC-183), A NEW ORALLY ACTIVE AMPICILLIN ESTER

I. ABSORPTION, EXCRETION AND METABOLISM OF PC-183 AND AMPICILLIN

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(Received for publication January 29, 1974)

Absorption, excretion and metabolism of a new ampicillin derivative, phthalidyl D-α-aminobenzylpenicillinate hydrochloride (PC-183), were studied. In comparison with ampicillin, PC-183 showed the following characteristics.

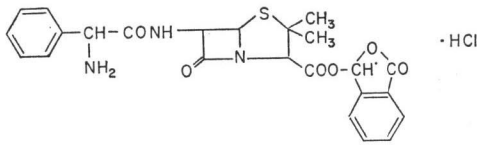
1. Upon oral administration in rats and humans, PC-183 was hydrolyzed and produced much higher plasma levels of ampicillin than did ampicillin itself. Likewise, urinary excretion of ampicillin was greater for PC-183 in both species. Concentration in tissues and bile of the rat was also higher for PC-183 than for ampicillin. These facts demonstrate superior intestinal absorption for PC-183 as compared with ampicillin.

2. The compound was easily hydrolyzed in the presence of rat tissue extracts, such as liver, kidney or small intestine. This hydrolysis was also catalyzed by blood plasma, the activity of which was highest in rats and mice, followed by humans and dogs in that order. The hydrolysis was so rapid that any attempt to detect intact PC-183 in blood, urine and tissues after oral administration to rats and humans was unsuccessful.

3. The hydrolysis products were ampicillin and phthalaldehydic acid. The latter was further metabolized to α-hydroxy-*o*-toluic acid and excreted into urine exclusively in this form. The metabolic pathway was the same for mice, rats, dogs and humans.

For the past several years, a number of penicillin derivatives have been synthesized and subjected to extensive screening programs in this laboratory. Among them, phthalidyl ester of ampicillin (phthalidyl D-α-aminobenzylpenicillinate hydrochloride, Fig. 1), which was coded PC-183, was found to be absorbed more readily than the parent compound ampicillin and to yield much higher levels in blood and tissues. This report deals with the absorption, excretion and metabolism of this new derivative as well as ampicillin which served as the reference compound.

Fig. 1. Chemical structure of PC-183 (phthalidyl D-α-aminobenzylpenicillinate hydrochloride)



Materials and Methods

1. Chemicals

PC-183 was prepared as the hydrochloride. ^{14}C-PC-183 was labelled at the two carbon atoms of the side chain in the phthalidyl radical and had a specific activity of 1.7 μCi/mg. Radiochemical purity, checked by thin-layer chromatography, was over 95 %. Ampicillin was our commercial preparation and was in the form of the trihydrate. α-Hydroxy-*o*-toluic acid was synthesized from commercially available phthalide and was free from contaminants on thin-

layer chromatography.

2. Animals and Administration of Drugs

Male Sprague-Dawley rats, aged 6~8 weeks, were fasted overnight and dosed by the oral route with the test compound either as a suspension (ampicillin) or as a solution (PC-183) in distilled water, which was always prepared freshly before use. Biliary excretion was studied in rats whose bile duct was cannulated. They were housed in a restraining cage during the collection of bile.

Mice were of *ddN* strain aged 7 weeks.

Studies in dogs were done with fasted beagles of both sexes weighing 8~14 kg. Drugs were administered in a capsule.

Studies in humans were carried out in 12 healthy male volunteers ranging in age from 30 to 45 years. After overnight fasting, they were given a capsule containing either compound, together with 100 ml of water, on two occasions at an interval of one week in a cross-over fashion.

Blood samples were taken in the presence of heparin and immediately centrifuged. Plasma, urine and bile were kept in the frozen state until assayed.

3. Bioassay of Ampicillin

Ampicillin in body fluids was determined by the usual cup-plate method using *Bacillus subtilis* ATCC 6633 as the test organism. Standard solution was prepared in the plasma for plasma assay and in 1/15 M phosphate buffer, pH 7.4, for urine assay.

For the assay of ampicillin in tissues, organs were removed and immediately homogenized in 3~20 volumes of ice-cold acetone and the homogenate was centrifuged at 3,000 rpm for 10 minutes. Ampicillin in the supernatant was determined by the disc method, where standard solution was prepared in acetone. The concentration was expressed in μg of anhydrous ampicillin per ml or g.

4. Identification of Antibiotic Activity

Antibiotic activity in blood, urine and tissues was identified by thin-layer chromatography, followed by bioautography. For the preparation of sample solution, blood after sampling was immediately mixed with an equal volume of cold 0.6 N $HClO_4$ and tissues were homogenized in 10 volumes of cold acetone. The mixtures were centrifuged and each supernatant was appropriately diluted with acetone. Urine was diluted 100- to 1,000-fold with acetone.

Ten μl of each sample solution was spotted on an Eastman chromatogram sheet No. 6061 (2×10 cm)[1] and the chromatogram was developed with chloroform-ethanol (9:1) until the solvent traveled 8 cm from the origin. Antibacterial activity was detected by placing the sheet on an agar plate seeded with *Sarcina lutea* ATCC 9341 for 15 minutes followed by overnight incubation. Under the above conditions, ampicillin produced a zone of growth inhibition near the origin, and PC-183 at an Rf value of 0.88. For the estimation of relative amounts of the two compounds, equimolar amounts (0.001~0.02μg) were spotted and developed as above and the standard curve was prepared by plotting the diameter of inhibition zones against the amount of each compound.

5. *In vitro* Hydrolysis of PC-183

To 5 ml of 0.2 M phosphate buffer (pH 7.4) were added 1 ml of a freshly prepared aqueous solution of PC-183 (2 mg/ml), 0.5 ml of blood plasma from different animal species or varying amounts of rat tissue extracts, and the solution was filled up to 10 ml with distilled water and incubated at 37°C. Tissue extracts were prepared by homogenizing rat liver, kidney and small intestine in 9 volumes of ice-cold 0.1 M phosphate buffer (pH 7.4), followed by centrifugation at 10,000 g for 10 minutes in the cold.

The hydrolysis was followed by measuring released phthalaldehydic acid colorimetrically as its 2, 4-dinitrophenylhydrazone. One ml aliquot of the reaction mixture was pipetted into

1 ml of ice-cold 0.6 N $HClO_4$ at intervals and, after centrifugation, 1 ml of the supernatant was added to an equal volume of 5 mM 2, 4-dinitrophenylhydrazine in 1 N HCl. The mixture was left at room temperature for 10 minutes; then 10 ml of 0.4 N NaOH was added. The optical density was read at 460 mμ against the control in which water was substituted for the supernatant. Under the above condition, phthalaldehydic acid added to the reaction mixture at concentrations ranging from 20 to 100 μg/ml was recovered in 96.6±0.8% yield (mean±S.E). PC-183 itself produced readings corresponding to 3~4% hydrolysis when subjected to the above procedure, for which correction was made.

6. Extraction of Metabolites of the Phthalidyl Moiety from Human and Dog Urine

Ten ml of urine was mixed with 1 ml of conc. HCl and left at room temperature for 30 minutes. The solution was then saturated with sodium chloride and extracted three times with an equal volume of ethylacetate. The ethylacetate layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 0.2 ml of anhydrous pyridine and trimethylsilylated with 0.2 ml of bis-trimethylsilylacetamide at room temperature overnight. Three μl were injected into a gas chromatograph column.

7. Gas Chromatography

Gas chromatography was used for the identification and quantitation of urinary metabolites of the ester moiety. The equipment was Hewlett-Packard model 402 gas chromatograph fitted with a flame ionization detector. Columns were either 3% SE-30, used for the identification of metabolites, or 3% OV-17, used for its quantitation. As will be shown later, the phthalidyl ester moiety is excreted into urine exclusively as α-hydroxy-*o*-toluic acid, which was quantitated after conversion into phthalide. Details of this procedure will be reported elsewhere as part of a paper dealing with comparative metabolic studies of *o*-, *m*-, and *p*-phthalaldehydic acid.

8. Thin-layer Chromatography

Thin-layer chromatography was performed on commercially available silica gel plates containing a fluorescent indicator (E. Merck, silica gel F_{254}). The following solvent systems were used: (1) chloroform-ethanol (9:1), (2) chloroform-ethanol-acetic acid (9:1:0.2), (3) heptane-ethylacetate-acetic acid (10:10:0.2).

Spots were visualized with short-wave ultraviolet light. In the above systems, the Rf values of four phthalidyl radical related substances, phthalide, phthalaldehydic acid, α-hydroxy-*o*-toluic acid and phthalic acid, were respectively: (1) 0.81, 0.38, 0.21, 0.01; (2) 0.84, 0.56, 0.45, 0.06; and (3) 0.56, 0.36, 0.25, 0.03.

9. Measurement of Radioactivity

Radioactivity was measured with a Packard model 3380 liquid scintillation spectrometer in a dioxane based scintillation solution. Quenching was corrected by the external channel-ratio method. Radioactivity on thin-layer plates was detected with Aloka thin-layer chromatogram scanner model TLC-2B.

Results

Absorption, Distribution and Excretion of PC-183 and Ampicillin

1. Absorption and Excretion in Rats

Rats were fasted overnight and given ampicillin and PC-183 orally at a dose equivalent to 100 mg/kg of anhydrous ampicillin. Plasma and tissue concentrations of ampicillin were assayed at intervals until the 6th hour. The results are summarized in Table 1. PC-183 produced much higher and earlier peak levels of ampicillin in every tissue examined, indicating higher and faster absorption.

Table 1. Ampicillin concentrations in plasma and tissues after oral administration of PC-183 and ampicillin to fasting rats at a dose equivalent to 100 mg/kg of anhydrous ampicillin (mean of 3 animals ±SE)

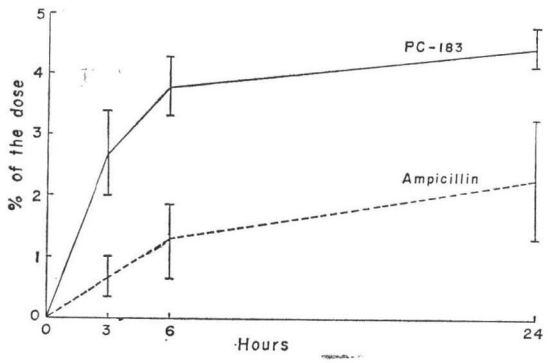
Antibiotics	Tissues	Concentrations of ampicillin (μg/g or ml)				
		0.5 hr	1 hr	2 hr	4 hr	6 hr
PC-183	Liver	106.0±12.9	59.8±25.6	39.7±6.6	6.8±1.2	4.3±0.7
	Kidney	53.2± 8.1	45.0±14.6	15.2±0.1	4.5±0.3	1.2±0.5
	Lung	7.9± 0.6	5.3± 1.3	3.1±0.4	1.0±0.1	0.7±0.4
	Spleen	3.7± 0.3	2.9± 0.9	1.5±0.2	0.8±0.1	N.D.
	Plasma	15.9± 3.1	8.3± 1.3	3.7±0.5	1.2±0.2	1.2±0.5
Ampicillin	Liver	23.3± 4.1	45.9± 9.6	35.7±9.0	12.3±1.8	N.D
	Kidney	12.6± 1.4	19.3± 2.5	11.1±2.3	7.8±0.7	3.0±1.2
	Lung	2.8± 0.5	3.7± 0.8	2.1±0.1	1.6±0.2	N.D.
	Spleen	1.2± 0.1	1.3± 0.1	1.1±0.1	0.8±0.1	N.D.
	Plasma	5.5± 1.9	5.0± 0.8	3.3±0.2	1.2±0.4	0.6±0.1

N.D.: not detectable

Excretion of ampicillin into urine for a 24-hour period was 21.8±3.9% for PC-183 and 5.0±1.0% for ampicillin; this also reflects better absorption for PC-183.

Over a 24-hour period, biliary excretion of ampicillin in bile duct-cannulated rats averaged 4.5±0.3% of PC-183 and 2.3±1.0% of ampicillin administered (Fig. 2).

Fig. 2. Biliary excretion of ampicillin in rats after oral administration of PC-183 and ampicillin at a dose equivalent to 100 mg/kg of anhydrous ampicillin (mean of 3 animals ±S.E)



Bioautographic examination of the liver, kidney and blood 30 minutes after PC-183 administration revealed that the antibiotic activity was due solely to ampicillin. This is understandable in view of the rapid hydrolysis of PC-183 catalyzed by blood and tissue extracts (next section).

2. Blood Levels and Urinary Excretion in Humans

Fig. 3 shows the plasma levels of ampicillin in 12 fasting volunteers given ampicillin and PC-183 orally at a dose equivalent to 250 mg anhydrous ampicillin. Peak levels, which were attained at 1 hour with both compounds, were respectively 2.2μg/ml after ampicillin and 5.7μg/ml after PC-183 administration. The latter gave significantly higher values at 0.5, 1 and 2 hours. No difference was observed thereafter.

Urinary excretion of ampicillin was examined in 6 subjects on two different occasions at 2-hour intervals until the 8th hour after dosage (Fig. 4). Total ampicillin excreted during the 8 hours was 35.1±4.7% after ampicillin and 53.7±6.3% after PC-183 administration. The majority of ampicillin was excreted during the first 4 hours with either compound. Excretion during the first 2 hours after PC-183 administration was 2.2 times the corresponding amount after ampicillin administration.

Fig. 3. Concentrations of ampicillin in plasma of fasting volunteers after oral administration of PC-183 and ampicillin at a dose equivalent to 250 mg of anhydrous ampicillin (mean of 12, ±S.E.)

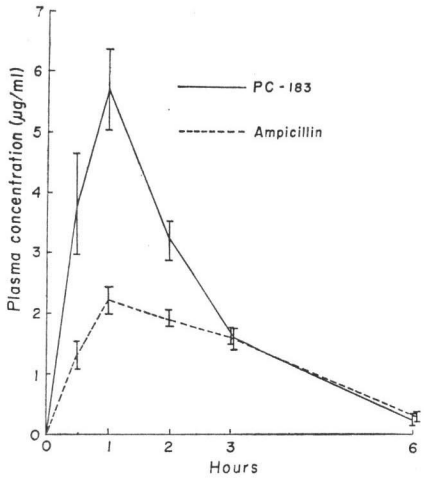
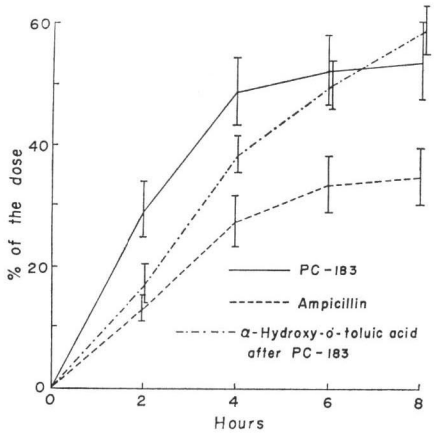


Fig. 4. Urinary excretion of ampicillin in fasting volunteers after oral administration of PC-183 and ampicillin at a dose equivalent to 250 mg of anhydrous ampicillin (mean of 6, ±S.E.)



Bioautographic study of the 1-hour blood samples and 0~2 hour urine samples after PC-183 administration proved that the antibiotic activity was due to ampicillin only. Intact PC-183 could not be detected.

The excretion of α-hydroxy-*o*-toluic acid, the principal metabolite of the phthalidyl moiety of PC-183, was slower than that of ampicillin and 9% of the dose could be detected in the 6~8-hour urine samples. Total excretion amounted to 59.4±4.2%.

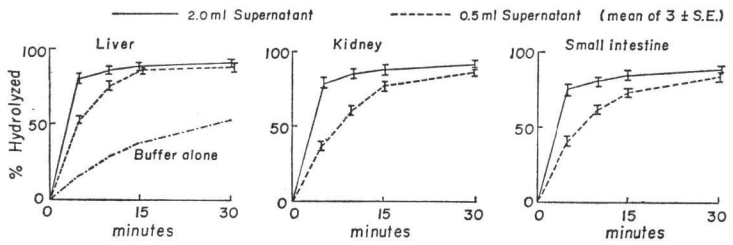
Interestingly, PC-183 does not seem to give higher blood levels than ampicillin in dogs; the reason is not clear.

Hydrolysis of the Phthalidyl Ester of PC-183

1. *In vitro* Hydrolysis in the Presence of Tissue Extracts

Time course of hydrolysis catalyzed by rat tissue extracts is shown in Fig. 5. In the absence of tissue extracts, PC-183 hydrolyzed moderately at neutral pH, and about 50% was cleaved after 30 minutes. This hydrolysis was greatly accelerated by tissue extracts. The presence of homogenate supernatant corresponding to 200 mg of liver, kidney or small intestine

Fig. 5. Hydrolysis of PC-183 catalyzed by rat tissue extracts
Two mg of PC-183 was incubated at 37°C in 10 ml of phosphate buffer (pH 7.4) containing 0.5 and 2.0 ml of the supernatant of a 10% homogenate (mean of 3, ±S.E.)



tissues was sufficient to hydrolyze within 5 minutes 2 mg of PC-183 dissolved in 10 ml. Blood plasma from different species also catalyzed the hydrolysis, but to a different extent (Fig. 6). The activity was highest for mice and rats; the addition of 5 % plasma resulted in quantitative hydrolysis of 2 mg PC-183 within 2 minutes. Activity of human and dog plasma decreased in that order.

The hydrolysis of PC-183 was compared with that of pivampicillin, a recently introduced ampicillin ester which resembles PC-183 structurally. The two compounds were incubated in whole blood of dogs and humans at a concentration of 100 μg/ml; the hydrolysis was followed by quantitative bioautography described under Methods. The results are shown in Fig. 7. It

Fig. 6. Hydrolysis of PC-183 catalyzed by blood plasma of different animal species
A solution of PC-183, 200μg/ml in phosphate buffer (pH 7.4) containing 5 % plasma was incubated at 37°C

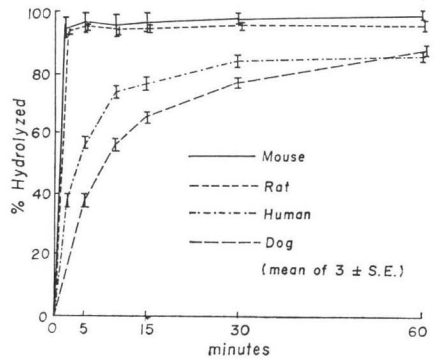
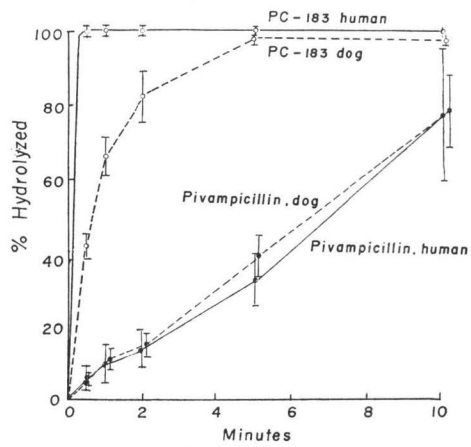


Fig. 7. Hydrolysis of PC-183 and pivampicillin in whole blood of dogs and humans
A solution of both compounds in whole blood (100μg/ml) was incubated at 37°C (mean of 3, ±S.E.)



is evident that PC-183 was hydrolyzed much more readily than pivampicillin in both species. Preliminary study on the pH-dependence of spontaneous hydrolysis revealed that PC-183 is much more stable in acidic than neutral media. Under the condition where about 50 % was hydrolyzed during the first 30 minutes at pH 7.4, the hydrolysis rates at pH 5.0, 3.0 and 1.2 were 6.6, 3.7 and 2.5 %, respectively.

2. Identification of Hydrolysis Products

Hydrolysis products of PC-183 were identified as ampicillin and phthalaldehydic acid. After 30-minute incubation, the reaction mixture was made acidic and was extracted with an equal volume of ethylacetate. The ethylacetate layer was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was examined by thin-layer chromatography in solvent systems 1, 2 and 3. In all three solvent systems, phthalaldehydic acid was the only product detected. Another aliquot of the reaction mixture was mixed with two volumes of acetone and, after centrifugation, the mixture subjected to bioautography. From the aliquot taken at 5 minutes, inhibitory zones were observed at Rf values corresponding to both PC-183 and ampicillin; at 30 minutes only ampicillin was detected.

Metabolites of the Phthalidyl Moiety

1. Metabolites in Human and Dog Urine

Possible metabolites were extracted from acidified urine with ethylacetate and, after silylation, examined by gas chromatography using the column 3% SE-30 operated at 100~180°C. As normal urine is known to contain various acidic compounds[2], many peaks were observed on the chromatogram. On close examination, however, it was revealed that the peaks from the urine of dosed animals of both species corresponded to those of respective control urine in every detail with the exception of one peak which appeared at retention times of 3.5 and 1.7 minutes at 100° and 120°C, respectively (Fig. 8). These retention times and the mass spectrum of this peak were identical with those of phthalide. However, detailed examination established that the original metabolite was, indeed, α-hydroxy-*o*-toluic acid. This conclusion is based on the following observations.

(1) When the urine was directly extracted with ethylacetate at neutral pH and subjected to gas chromatography or thin-layer chromatography, phthalide, which would have been detected if present as such in the urine, could not be detected.

(2) When extraction was made at pH 7.4 after the urine had been kept acidic for 30 minutes at room temperature, phthalide was detected as the only peak on the gas chromatogram. Thin-layer chromatography with solvent systems 1~3 also proved the presence of phthalide.

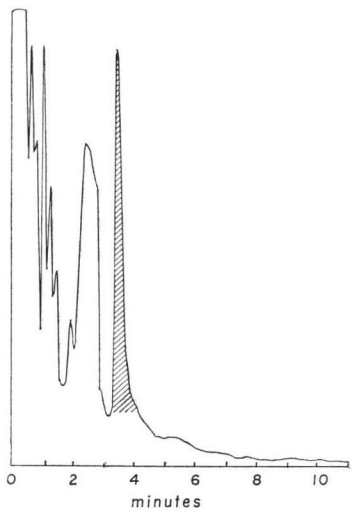
(3) It is well-known that α-hydroxy-*o*-toluic acid undergoes intramolecular dehydration and is easily converted into phthalide in acidic media.

Fig. 8. Gas chromatogram of human urinary extracts prepared after oral administration of PC-183

Acidified 6-hour urine was extracted with ethylacetate and extracts were trimethylsilylated.

Shaded peak, absent in control urine, was identified as phthalide, which was produced from α-hydroxy-*o*-toluic acid during extraction.

Operating conditions: column, 3% SE-30, 3 mm×90 cm, column temperature, 100°C flow rate of carrier gas, He 60 ml/min.



These facts can be explained by assuming that the original metabolite is α-hydroxy-*o*-toluic acid. Peaks corresponding to silylated phthalaldehydic acid or phthalic acid, other likely metabolites of phthalidyl moiety, were not detected in either species, nor did prior treatment of the urine with β-glucuronidase or arylsulfatase reveal any new metabolites.

2. Metabolites in Rat and Mouse Urine

Six-hour urine of rats and 24-hour urine of mice collected after oral administration of 100 mg equivalent of ^{14}C-PC-183 per kg were subjected to thin-layer chromatography with solvent systems 1~3. One major peak was observed, at an Rf value corresponding to α-hydroxy-*o*-toluic acid. Identification was confirmed by the observation that the Rf value

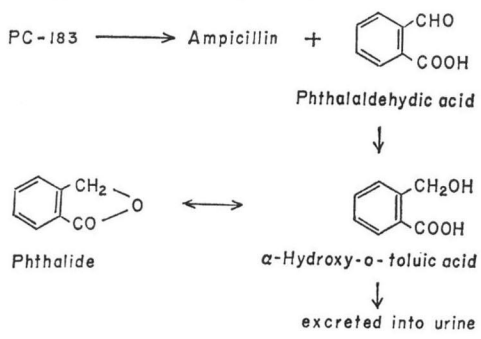
changed to that of phthalide when the urine was kept acidic before chromatography, an indication of the pH-dependent interconversion of α-hydroxy-*o*-toluic acid and phthalide.

Measurement of the radioactivity following elution of the peak area with methanol indicated that α-hydroxy-*o*-toluic acid accounted for 92.1 % and 75.8 % of the total radioactivity in rat and mouse urine, respectively; the remaining radioactivity was detected at the origin. The latter radioactive fraction failed to move after β-glucuronidase, arylsulfatase treatment, acid or alkaline hydrolysis and could not be extracted with ethylacetate. Nor was it identical with phthalaldehydic acid or phthalic acid.

3. Metabolites in Rat Organs

The liver, kidney and plasma of rats were excised after oral administration of ^{14}C-PC-183, homogenized in 3 volumes of acetone, and the extracts evaporated to dryness. Examination of the residue by thin-layer chromatography revealed that both α-hydroxy-*o*-toluic acid and phthalide were present in various tissues in different ratios. At 30 minutes, the percentage of radioactivity accounted for by α-hydroxy-*o*-toluic acid and phthalide in the liver was 35.7 % and 59.7 %, respectively; it was 77.0 % and 20.2 % in the kidney and 88.1 % and 10.4 % in the plasma. No appreciable difference was found at 60 minutes. The results of the *in vitro* and *in vivo* studies described above suggest the metabolic pathway of PC-183 depicted in Fig. 9.

Fig. 9. Metabolic pathway of PC-183



Discussion

Ampicillin is the most widely used semi-synthetic penicillin. Ampicillin is orally active but its absorption from the gastrointestinal tract is limited. Two lines of efforts have been made to improve the oral bioavailability of this important penicillin: manipulation of the physicochemical properties of ampicillin formulation and structural modification of its molecule. Thus, the anhydrous form of ampicillin was claimed to produce higher and earlier peak levels than the ordinary trihydrate form.[3] However, the degree of improvement, if any, seems to be very limited.

Metampicillin[4] and hetacillin,[5] which are the condensation products of ampicillin with formaldehyde and acetone, respectively, were once reported to give superior blood levels of ampicillin, but this was later questioned.[6,7] Recently, two derivatives have appeared which succeeded in largely eliminating this drawback. They are amoxycillin and pivampicillin. Their advantages over ampicillin with respect to oral absorption were amply demonstrated.[8~13] PC-183 resembles pivampicillin in that it is rapidly hydrolyzed in the presence of blood or tissues. Because esters of penicillins are generally known to be devoid of antibacterial activity as such, the marked activity detected in blood and organs following their administration can be attributed to their unusual hydrolyzability. Although PC-183 is quatitatively similar to pivampicillin in this respect, it is different quantitatively in being far more easily hydrolyzed. Whether this difference is of clinical significance is not clear.

Toxicity of PC-183 and phthalaldehydic acid has been examined in detail. The LD_{50} values of PC-183 for mice and rats were over 4,000 mg/kg for both oral and subcutaneous route. Oral administration of PC-183 for 26 weeks at daily doses up to 1,000 mg/kg caused no

hematological or histopathological abnormalities in rats.

Phthalaldehydic acid was found to be a compound of low toxicity. The LD_{50} values for mice were 4,480 mg/kg for oral and 1,860 mg/kg for subcutaneous route. The values for rats were 7,500 mg/kg for oral and 2,430 mg/kg for subcutaneous route. Oral administration for 5 weeks caused no abnormalities in rats at daily doses up to 300 mg/kg. These results will be published elsewhere.

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