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EFFECTS OF THE CARBOXYLESTERASE INHIBITOR BIS-(*p*-NITROPHENYL)-PHOSPHATE ON ELIMINATION OF HEXOBENDINE

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Abstract—A study was conducted of the effect of pretreatment of rats with the carboxylesterase inhibitor, bis-(*p*-nitrophenyl)-phosphate (BNPP) on the biliary and urinary excretion of intravenously-administered ^{14}C-hexobendine (HB) and its metabolites. Inhibition of HB ester cleavage by BNPP produced a marked decrease in the urinary excretion of HB hydrolysis products; this effect was accompanied by a definite increase in the biliary excretion of conjugates of *O*-demethylated HB metabolites. The results indicate the participation of complementary metabolic and excretory pathways in the elimination of HB; this hypothesis is further supported by the finding that BNPP did not alter the half-life of the plasma ^{14}C level.

Ester cleavage of HB, a coronary and cerebral vasodilatory substance [1–3], was markedly inhibited *in vitro* by the carboxylesterase inhibitor, BNPP [4]; however, BNPP did not significantly change the plasma or tissue levels of HB *in vivo* [5]. Yet, BNPP pretreatment reduced the concentration of HB hydrolysis products and simultaneously elevated the levels of demethylated metabolites in plasma, kidneys and liver. BNPP pretreatment was, thus, thought to produce a shift from ester cleavage towards the dealkylating pathway in HB metabolism [5]. In order to substantiate this hypothesis the influence of BNPP pretreatment on the elimination of HB was studied in rats by measuring the excretion of ^{14}C-labelled metabolites in bile and urine following ^{14}C-HB administration as influenced by BNPP pretreatment.

MATERIALS AND METHODS

Unless otherwise specified, the materials and methods have been described fully in the preceding paper [5].

Materials. Male Wistar rats weighing between 270–350 g were used in the experiments. Hexobendine-(carboxyl-^{14}C) with a spc.act. of 10 mCi/g and unlabelled reference substances of possible HB metabolites were kindly provided by Chemie-Linz AG, Austria. β-Glucuronidase (Escherichia coli), β-glucuronidase/arylsulphatase ('glusulase', Helix pomatia) and esterase (pig liver) were obtained from Boehringer Mannheim GmbH, Germany, and BNPP and all other chemicals from Merck AG, Germany.

Experimental procedure. Rats were pretreated with 50 mg/kg BNPP i.p. 18 hr and again 3 hr before ^{14}C-HB administration; the BNPP solution, 5 mg/ml saline (0.9% w/v), was neutralized by addition of the appropriate amount of 0.15 M NaOH. The control rats received an equivalent volume of saline.

Anaesthesia was then induced with 50 mg/kg pentobarbital i.p. After laparatomy a polyethylene cannula was inserted into the common bile duct and another cannula with a flanged end into the urinary bladder. One carotid artery was cannulated for blood sampling. Pentobarbital (1 mg/ml saline) was infused at a rate of 10–15 mg/kg/hr through a polyethylene tube placed into a jugular vein for the maintenance of anaesthesia and for fluid replacement. BNPP pretreatment (50 mg/kg via the jugular vein) was given for the third time half an hour before ^{14}C-HB (0.5 mg/kg i.v.) administration. Urine and bile were collected over 30 min periods. The volume of the individual samples was measured gravimetrically. Blood samples (0.2 ml) were withdrawn 0.25, 0.5, 1, 2, 3, 4, and 5 hr after ^{14}C-HB administration.

Analytical procedure. Radioactivity was measured in aliquots of bile, urine and plasma samples after addition of 9 ml dioxane scintillator [6], in a Packard liquid scintillation spectrometer, model 3330. Counting efficiency was determined by internal and external standardization techniques.

^{14}C-Labelled metabolites in bile and urine were separated by means of thin-layer chromatography using unlabelled reference substances as previously described [5].

After pilot experiments the scheme outlined below was routinely followed in order to split con-

Abbreviations—BNPP = bis-(*p*-nitrophenyl)-phosphate; HB = hexobendine; 3'-OH-HB = N,N'-dimethyl-*N*-(3-(3'-hydroxy-4',5'-dimethoxybenzoxy)-propyl)-N'-(3-(3',4',5'-trimethoxybenzoxy)-propyl)-ethylenediamine; 4'-OH-HB = N,N'-dimethyl-*N*-(3-(4'-hydroxy-3',5'-dimethoxybenzoxy)-propyl)-N'-(3-(3',4',5'-trimethoxybenzoxy)-propyl)-ethylenediamine; 3',3'-diOH-HB = N,N'-dimethyl-N,N'-bis-(3-(3'-hydroxy-4',5'-dimethoxybenzoxy)-propyl)-ethylenediamine; HBol = N,N'-dimethyl-*N*-(3-(3',4',5'-trimethoxybenzoxy)-propyl)-N'-3-hydroxypropyl-ethylenediamine; 3'-OH-HBol = N,N'-dimethyl-*N*-(3-(3'-hydroxy-4',5'-dimethoxybenzoxy)-propyl)-N'-3-hydroxypropyl-ethylenediamine; TMBA = 3,4,5-trimethoxybenzoic acid; OH-DMBAs = 3-hydroxy-4,5-dimethoxybenzoic acid and 4-hydroxy-3,5-dimethoxybenzoic acid; diOH-MBAs = 3,5-dihydroxy-4-methoxybenzoic acid and 3,4-dihydroxy-5-methoxybenzoic acid.

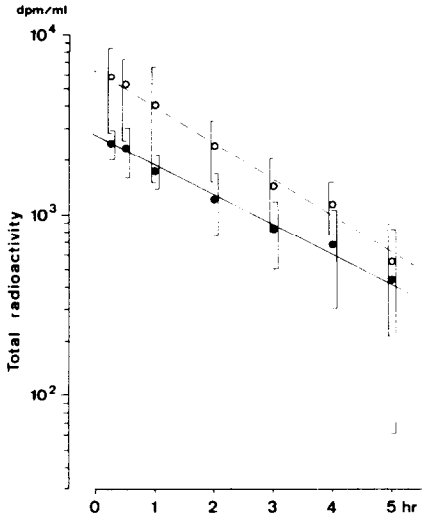

Fig. 1. Total ^{14}C concentrations in plasma as a function of time after i.v. ^{14}C-HB administration in controls (○) and in BNPP-pretreated rats (●). Each point and error bar represents the mean value of five experiments ± S.D.

jugates for the measurement of demethylated basic metabolites and to determine the 'total' amount of releasable TMBA and demethylated TMBA derivatives (OH-DMBAs, diOH-MBAs) after ester cleavage. One aliquot of the individual bile and urine samples was subjected to thin-layer chromatography, another aliquot was incubated in 0.2 M acetate buffer (pH = 5) with glusulase at 37° for 24 hr. Afterwards, one part was chromatographed and the other part was titrated with 5 M NaOH to attain pH 8 and mixed with 0.2 M borate buffer (pH = 8). After addition of esterase the sample was reincubated at 37° for 24 hr and, thereafter, the ^{14}C-labelled metabolites were separated chromatographically.

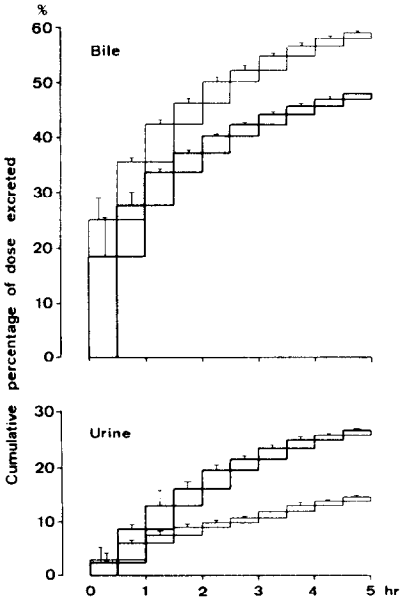

Fig. 2. ^{14}C excretion in bile (upper panel) and urine (lower panel) after i.v. ^{14}C-HB administration to control (empty columns) and BNPP-pretreated rats (hatched columns). The individual values ($\bar{x}$ ± S.D.; $n = 5$) were expressed as a percentage of the ^{14}C dose administered and depicted in a cumulative manner.

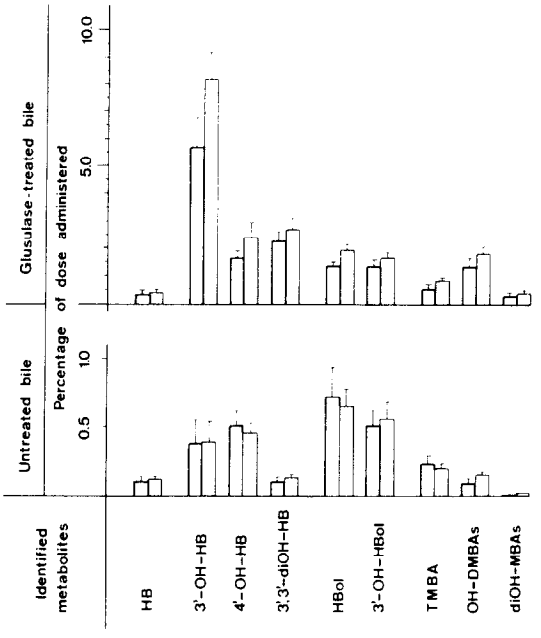

Fig. 3. ^{14}C-labelled HB and metabolites in bile collected during the first 30-min period after i.v. ^{14}C-HB administration to control (empty columns) and BNPP-pretreated rats (hatched columns). After thin-layer chromatographic analysis of untreated and glusulase-treated samples the amounts of the individual metabolites were expressed as a percentage of ^{14}C dose administered (± S.E.M.; $n = 5$).

Treatment of bile with glucuronidase instead of glusulase caused a smaller release of identifiable metabolites; this fact indicates the existence of not only glucuronic acid, but also of sulphuric acid conjugates. When ester cleavage by esterase was attempted in bile samples before glusulase treatment, the increase in free acids (TMBA, OH-DMBAs, diOH-MBAs) was small, probably because of the relative stability of conjugated metabolites, which constitute the main fraction in the bile, towards esterase attack.

All measured ^{14}C-values in bile and urine samples were expressed as a percentage of the radioactivity dose administered.

RESULTS

(1) *Total ^{14}C plasma levels and ^{14}C excretion in bile and urine.* The plasma levels of total ^{14}C exhibited a single exponential decline during the observation period from 15 min to 5 hr following i.v. ^{14}C-HB administration (Fig. 1). In BNPP-pretreated rats the ^{14}C plasma levels were significantly lower than in control rats by a factor of about 2, which confirms previous results and may be due to lower ^{14}C-TMBA and ^{14}C-HBol concentrations [5]. However, the half-lives for the decline of ^{14}C plasma levels were similar, about 1.5 hr in control and 1.8 hr in BNPP-pretreated rats.

The excretion of ^{14}C activity in the bile was higher in BNPP-pretreated than in control rats (Fig. 2).

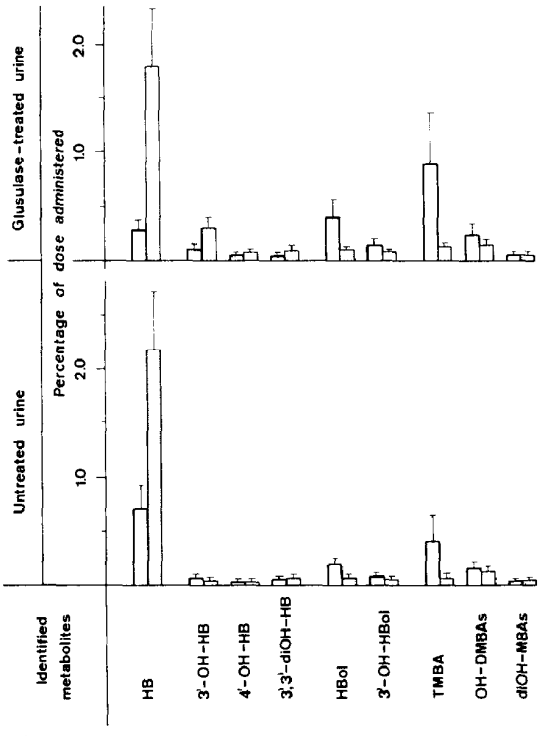

Fig. 4. ^{14}C-labelled HB and metabolites in urine collected during the first 30-min period after i.v. ^{14}C-HB administration to control (empty columns) and BNPP-pretreated rats (hatched columns). After thin-layer chromatographic analysis of untreated and glusulase-treated samples the amounts of the individual metabolites were expressed as a percentage of ^{14}C dose administered (± S.E.M.; n = 5).

This difference was more marked during the early sampling periods after drug administration, yet all mean values of biliary ^{14}C excretion in BNPP-pretreated animals during each sampling period exceeded the corresponding control values. The mean cumulative ^{14}C excretion in bile amounted to 48 per cent of the ^{14}C dose in control and to 59 per cent in BNPP-pretreated rats 5 hr after ^{14}C-HB administration.

The excretion of ^{14}C activity in the urine was significantly lower by a factor of about 2 in BNPP-pretreated than in control rats (Fig. 2) except for the first 30-min period after drug administration, during which similar ^{14}C excretion rates were observed. The mean cumulative ^{14}C excretion in urine amounted to 27 per cent of the ^{14}C dose in control and to 15 per cent in BNPP-pretreated rats 5 hr after ^{14}C-HB administration.

(2) ^{14}C-*Labelled metabolites in bile.* The bile collected during the first 30-min period after ^{14}C-HB administration was subjected to thin-layer chromatographic analysis (Fig. 3), since these samples showed the greatest difference in biliary ^{14}C excretion between control and BNPP-pretreated rats. The proportion of ^{14}C-labelled HB and unconjugated metabolites identified in bile was small (about one tenth of total ^{14}C label in these samples) and the absolute amounts of the metabolites were not changed by BNPP-pretreatment.

The treatment with glusulase revealed that in both, control and BNPP-pretreated rats about one half of the ^{14}C label was excreted in the form of conjugates of demethylated HB derivatives, mainly as m-monodemethylated HB (3'-OH-HB). More-

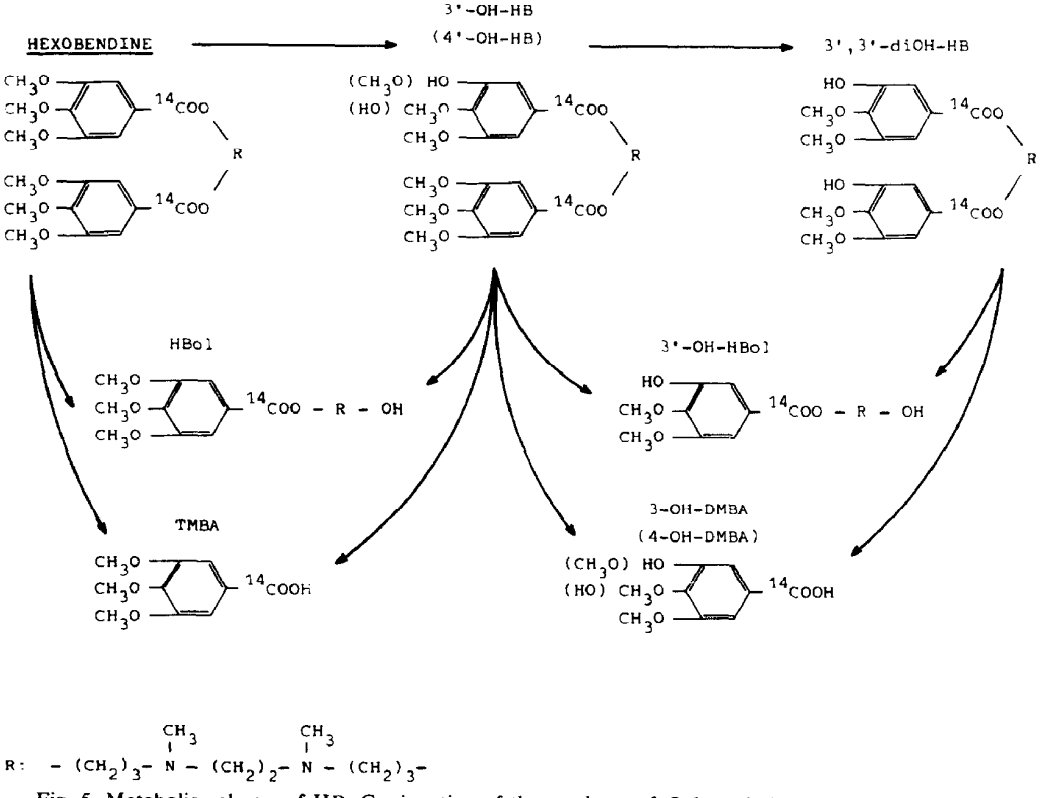

Fig. 5. Metabolic scheme of HB. Conjugation of the products of *O*-demethylation and/or hydrolysis is not shown, but this reaction is of greatest importance especially in the excretion of *O*-demethylated HB derivatives (3'-OH-HB, 4'-OH-HB and 3',3'-diOH-HB).

over, an increase in products of partial or total hydrolysis of HB and demethylated HB derivatives was observed after incubation with glusulase. However, it remains uncertain whether this change was caused by esterase activity of glusulase (see below), or truly represents cleavage of conjugates.

Whilst the relative ^{14}C distribution among the individual metabolites did not differ significantly between control and BNPP-pretreated animals, the absolute amounts of ^{14}C-labelled metabolites deconjugated by glusulase treatment were definitely higher after BNPP pretreatment. Consequently, the increase in biliary ^{14}C excretion after ^{14}C-HB administration under the influence of BNPP was brought about mainly by an increase in the excretion of conjugates of *O*-dealkylated HB derivatives.

(3) ^{14}C-*Labelled metabolites in urine.* Corresponding to the results obtained with bile the excretion pattern of ^{14}C-labelled HB and metabolites in the urine collected during the first 30-min period after ^{14}C-HB administration is presented in Fig. 4. In contrast to the low proportion of unconjugated excretion products in untreated bile, about two thirds of ^{14}C label were identified in untreated urine of control animals, namely 27 per cent as unchanged HB, 7 per cent as partially-hydrolyzed HB (HBol), 15 per cent as TMBA and 6 per cent as OH-DMBAs. BNPP-pretreatment significantly increased the excretion of HB and decreased that of the hydrolysis products (HBol and TMBA).

The analysis of conjugated metabolites by means of glusulase was hampered by esterase activity of the enzyme preparation used, as deduced from the cleavage of HB by glusulase treatment in control samples. However, no change was observed in the amount of HB detected after incubation of urine of BNPP-pretreated rats with glusulase, which may be explained by inhibition of esterase activity by renally-excreted BNPP [7]. Therefore, the considerable increase in m-demethylated HB (3′-OH-HB) observed after glusulase treatment points to a moderate renal excretion of conjugates of *O*-dealkylated HB derivatives.

In contrast to the first sampling period ^{14}C excretion in the urine during the second 30-min period following ^{14}C-HB administration was significantly reduced by BNPP pretreatment (Fig. 2). Chromatographic analysis (results not shown) revealed that this effect was due to a significant decrease in the excretion of HB hydrolysis products (HBol) and TMBA). However, the absolute amount of unchanged HB was equal in both groups, in contrast to the results obtained for the first sampling period. Again, excretion of conjugates of *O*-dealkylated HB derivatives in urine was apparent from the marked increase in 3′-OH-HB after glusulase treatment in BNPP-pretreated rats.

DISCUSSION

The present results demonstrate the role of the liver as primary site of HB metabolism. About 50 per cent of the ^{14}C dose administered were excreted in the bile of control rats during the 5-hr observation period in accordance with the value of 65 per cent measured during 12 hr [8]. Only minute amounts of unchanged HB were detectable. *O*-Demethylation of the drug prevailed, with subsequent conjugation of the phenolic hydroxyl groups; biliary excretion of hydrolysis products was of minor importance only.

A considerable smaller portion of the ^{14}C dose administered (27 per cent) was excreted by the kidneys. Apart from unchanged HB, the products of HB ester cleavage (HBol and TMBA) contributed mainly to urinary ^{14}C excretion during the first hour of sampling.

The following scheme of HB metabolism in rats can be outlined on the basis of the present data (Fig. 5): HB is either hydrolyzed, with formation of HBol and TMBA, or *O*-demethylated in either the meta or, to a lesser extent, in the para position of the TMBA residue with formation of 3′-OH-HB, 4′-OH-HB and 3′,3′-diOH-HB; subsequent ester cleavage of these demethylated HB derivatives yields 3′-OH-HBol and OH-DMBAs. Di-demethylation of one TMBA residue does not appear to play a significant role, since only minute amounts of di-OH-MBAs were found after total hydrolyzation. Extensive conjugation with glucuronic and/or sulphuric acid takes place in the case of demethylated HB derivatives and, possibly, to a minor degree also in the case of partially-hydrolyzed (HBol and 3′-OH-HBol) and acid metabolites.

BNPP pretreatment enhanced biliary ^{14}C excretion following ^{14}C-HB administration by increasing the excretion of conjugates of dealkylated HB derivatives. In this context not only a shift in HB metabolism from BNPP-inhibited ester cleavage towards *O*-dealkylation may be reasonably proposed but, moreover, a true activation of the conjugation reaction by BNPP or its metabolites, since the BNPP-degradation product *p*-nitrophenol has been shown to cause non-specific activation of UDP-glucuronyltransferase *in vitro* [9]. However, it is not possible to rule out a choleretic action of BNPP or its metabolites as contributory factor, since the mean bile volume of 5.2 ml in BNPP-pretreated animals was certainly higher than 4.1 ml in controls during the observation period (mean body weight = 310 g in both groups).

BNPP pretreatment decreased the amounts of total ^{14}C label in the urine, affecting mainly the excretion of TMBA and HBol, whose plasma levels were also decreased by BNPP [5]. The amount of unchanged HB was higher only during the first sampling period in BNPP-pretreated rats, at a time when BNPP-inhibited plasma esterase may be of greater importance with respect to HB metabolism during distribution of the drug. Thereafter the amount of non-metabolized HB excreted in the urine during the second sampling period was not changed by BNPP pretreatment, which corresponds to the observation of unaltered plasma levels of HB over the equivalent period [5].

The observation that BNPP failed to elicit a rise in plasma and tissue levels of HB [5], may be plausibly explained by direct ester hydrolysis of HB being only one possible phase-1 reaction in inactivation of the drug; inhibition of this reaction by BNPP can easily be compensated for by a higher rate of *O*-dealkylation. The consequence is a reduc-

tion in urinary excretion of the TMBA residue and an increase in biliary excretion of dealkylated HB metabolites (Fig. 2), in agreement with the concept that urine and bile are complementary pathways of drug excretion [10]. This concept of complementary partial clearances is in accordance with the observation that neither the half-life of the total ^{14}C level in plasma (Fig. 1) nor the plasma level of unchanged HB [5], was influenced by BNPP pretreatment. BNPP can prolong plasma levels of the drug in question only if the esterase reaction is the sole or main phase-1 reaction as was shown recently in the case of the anaesthetic agent propanidid [11].

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