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METABOLISM AND DISPOSITION OF 2',3'-DI-*O*-NITRO-ADENOSINE-5'-(*N*-ETHYL-CARBOXAMIDE) IN DOGS*

HUBERT WIENER, PETER KRIVANEK and NORBERT KOLASSA
Pharmakologisches Institut der Universität Wien, Währinger Strasse 13a, A-1090 Wien, Austria

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Abstract—2',3'-Di-*O*-nitro-[8-^{3}H]-adenosine-5'-(*N*-ethyl-carboxamide) (20 μg/kg) was denitrated completely within 1–3 hr in perorally and intravenously dosed dogs. Extremely rapid disappearance of the unchanged drug in serum was parallelled by the instantaneous appearance of mononitrates with 3'-mononitrate levels exceeding those of 2'-mononitrate three-fold. The mononitrates were eliminated with a half-life of 30–70 min, giving rise to the completely denitrated product, adenosine-5'-(*N*-ethyl-carboxamide) (NECA). The latter product was not further metabolized and was eliminated with a half-life of about 4 hr. Urinary excretion averaged 50% of the administered dose within 4 days and was represented essentially by the completely denitrated drug. Volatile ^{3}H-label of the drug was found in serum and urine during *in vivo* experiments. Oral bioavailability of the drug was about 90%. *In vitro* studies indicated that thiols are involved in denitration and reactions are catalysed by glutathione *S*-transferases, which were partially purified from dog liver. Nitrate ester cleavage was more easily accomplished at the 2'-position than at the 3'-position of the drug and resulted in the liberation of inorganic nitrite. Comparison of *in vitro* denitration rates gave the following ranking order: 2',3'-di-*O*-nitro-NECA > isosorbide-2,5-dinitrate > 2'-nitro-NECA > 3'-nitro-NECA > isosorbide-2-mononitrate, while nitrate ester cleavage of isosorbide-5-mononitrate was not detectable.

Adenosine-5'-carboxamides are known to be considerably stronger and longer acting vasodilators than adenosine [1]. Within a series of substituted analogues, the most active compound was found to be adenosine-5'-(*N*-ethyl-carboxamide) (B-744-96)† [2]. Haemodynamic effects of the corresponding dinitrate, 2',3'-di-*O*-nitro-adenosine-5'-(*N*-ethyl-carboxamide) (B-744-99), in dogs were similar but delayed in onset and prolonged, suggesting metabolic activation of the drug [2]. In order to characterize the metabolic fate, dogs were given [^{3}H]B-744-99 and [^{3}H]B-744-96 by i.v. and p.o. route; the metabolite pattern in serum and urine is described in this report.

Studies on different organic nitrates have demonstrated that denitration catalysed by glutathione *S*-transferases [3] represents the major route of biotransformation [4]. Assuming that the dinitrate B-744-99 is a substrate of glutathione *S*-transferases, enzymes were isolated from dog liver and studied with respect to nitrate ester cleaving activity.

MATERIALS AND METHODS

Materials. [8-^{3}H]B-744-99 [2',3'-di-*O*-nitro-[8-^{3}H]adenosine-5'-(*N*-ethyl-carboxamide)], specific activity 1.53 Ci/mmole, molecular weight 398.3,[8-^{3}H]B-744-96 [[8-^{3}H]adenosine-5'-(*N*-ethyl-carboxamide)], specific activity 1.55 Ci/mmole, molecular weight 308.3, and unlabelled B-744-99, B-744-96, B-755-24 and B-744-100 were kindly provided by Byk Gulden Lomberg Chem. Fabr. (Konstanz, F.R.G.). Chemical and radiochemical purity was checked by TLC and distillation, and was found to be more than 95%. Stock solutions (pH 2) of radiochemicals were stored at −20° until used. [8-^{14}C]Hypoxanthine, specific activity 60 mCi/mmole, and [8-^{18}C]inosine, specific activity 60 mCi/mmole, were purchased from the Radiochemical Centre (Amersham, U.K.). Isosorbide-2,5-dinitrate, isosorbide-5-mononitrate and isosorbide-2-mononitrate were gifts of Sanol Schwarz-Monheim GmbH (Monheim, F.R.G.). 1-Chloro-2,4-dinitrobenzene was obtained from Merck (Darmstadt, F.R.G.); xanthine oxidase (EC 1.2.3.2) and purine-nucleoside phosphorylase (EC 2.4.2.1) from Boehringer (Mannheim, F.R.G.); and allopurinol, thiols, Mops and unlabelled purines from Sigma Chemical Co. (St. Louis, MO). All other commercially available chemicals were of analytical grade. The ion exchange celluloses (DE 52 and CM 52) were products of Whatman (Clifton, NJ).

All assay solutions and buffered saline (mM composition: NaCl 125, KCl 4.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, Tris–HCl buffer 50, glucose 5; pH 7.4) were prepared shortly before the experiments.

Analytical procedures. ^{3}H-Activity was extracted from biological samples by adding two volumes of acetone–HCl (0.1 N) (9:1, by volume). Radioactivity was measured by means of a TLC scanner (Berthold, Wildbad, F.R.G.) or a liquid scintillation spectrometer (Packard Tricarb 3330) with internal standards for quench correction; in the latter case,

* A preliminary report was presented at the 23rd Spring Meeting of the German Pharmacological Society, Mainz, 1982.

† Abbreviations used: B-744-99, 2',3'-di-*O*-nitro-adenosine-5'-(*N*-ethyl-carboxamide); B-755-24, 2'-*O*-nitro-adenosine-5'-(*N*-ethyl-carboxamide); B-744-100, 3'-*O*-nitro-adenosine-5'-(*N*-ethyl-carboxamide); B-744-96 (NECA), adenosine-5'-(*N*-ethyl-carboxamide); GSH, glutathione; Mops, 3-(*N*-morpholino)propanesulfonic acid; DEAE-, diethylaminoethyl-; CM-, carboxymethyl-; TLC, thin-layer chromatography; i.v., intravenous; p.o., peroral.

0.1–0.2 ml samples were counted in 9 ml scintillation fluid (Atomlight, New England Nuclear).

Thin-layer chromatography (TLC) was performed on silica gel plates 60 F_{254} (Merck, Darmstadt, F.R.G.) using unlabelled reference substances visualized by fluorescence quenching. Solvent system A, consisting of ethyl acetate–methanol–H_2O (90:5:5, by volume), separated B-744-99 (R_f = 0.75), B-755-24 (R_f = 0.70), B-744-100 (R_f = 0.62), B=744-96 (R_f = 0.23) and adenine (R_f = 0.16) in a 2.5-hr development. Solvent system B, consisting of *n*-butanol–acetone–NH_3 (33%)–H_2O (50:40:3:15, by volume), was used for determining the radioactivity in nucleosides and nucleobases [5]. Solvent system C, consisting of *n*-propanol–methanol–NH_3 (33%)–H_2O (45:15:30:10, by volume) was used for the separation of nucleotides [5]. Development was performed over a distance of 18 cm in vapour-saturated chambers at 22°. The distribution of radioactivity was determined either by scanning or by liquid scintillation counting after transferring the silica gel of the individual spots into counting vials. Volatile ^{3}H-activity was assayed either by distilling samples at 50° to dryness *in vacuo* (Rotavapor; Büchi), followed by counting the radioactivity of the distillate, or by drying sample extracts in a stream of warm air; difference in radioactivity before and after drying was taken for volatile ^{3}H-activity.

Protein was determined by the method of Lowry *et al.* [6] using bovine serum albumin as standard. Spectral measurements were performed on a SP 6-550 UV/VIS spectrophotometer (Philips Pye Unicam, Cambridge, U.K.).

In vitro *drug stability testing*. Assays were performed with [^{3}H]B-744-99 and [^{3}H]B-744-96 at a final concentration of 10 μM; total radioactivity per assay tube was 15 μCi.

(i) *Stability at various chemical conditions*: The pH dependence of drug stability was determined during a 24-hr incubation period in Davies buffer [7], at pH 2–9 and 22°; analysis of the fate of ^{3}H-label by TLC (solvent system A) and distillation. An unknown radioactive product of [^{3}H]B-744-99 was formed at alkaline pH and co-chromatographed with adenine; when incubated with dog blood at 37° for 30 min, incorporation of ^{3}H-activity in nucleotides was determined by TLC (solvent system C).

Radiochemical stability at 37° was studied by incubating the drug in sterile-buffered saline, pH 7.4, for 48 hr; volatile ^{3}H-activity was estimated by distillation.

(ii) *Stability at various biological conditions*: The influence of enzymes on drug stability was assayed by incubation in the presence of either xanthine oxidase (0.2 U/ml) or purine-nucleoside phosphorylase (1 U/ml) in 0.1 M potassium phosphate buffer, pH 7, at 37° for 24 hr. Controls were run with [^{14}C]hypoxanthine and [^{14}C]inosine, respectively; analysis by TLC (solvent system B) and distillation.

Stability against monooxygenase action was determined in the 10,000 *g* supernatant fraction of dog liver homogenate prepared as described [8]. NADPH regeneration was ensured by adding a cofactor mixture [9]. The liver supernatant fraction (0.5 ml) was incubated with 0.1 M potassium phosphate buffer, pH 7.4, containing the cofactor mixture and [^{3}H]B-744-96; final volume 2 ml. After incubation for 4 hr at 37°, samples were extracted and the amount of volatile ^{3}H-activity was estimated by distillation. The stability in dog blood was conducted by drug incubation in 2-ml samples of a suspension of dog blood in buffered saline, pH 7.4, at 37° for 4 hr; final haematocrit 20%; samples were extracted and analysed by TLC (solvent system A) and distillation.

Animal studies. Experiments were carried out on two mongrel dogs of either sex, 12 and 14 kg in weight. Food was withheld from the animals for 12 hr before drug administration and for the ensuing 7-hr period. Drinking water was provided *ad lib.* In a cross-over design study, each of the dogs received on separate occasions separated by at least 4 weeks [^{3}H]B-744-99 and [^{3}H]B-744-96 i.v. as a bolus into a vena cephalica or [^{3}H]B-744-99 p.o. via a stomach tube (dosages are indicated in the figure legends). Drugs were dissolved in isotonic saline at 0.1 mg/ml. I.v. administration was followed immediately by injection of 5 ml saline to flush the cannula; oral dose was followed by 10 ml of water. Blood samples (1.5 ml) were taken at various time intervals after drug administration up to 14 days. A volume of saline equal to that of the blood sample was reinfused after sample collection. Blood was immediately centrifuged and the serum was separated. ^{3}H-activity of serum was extracted as stated above and analysed by liquid scintillation counting and TLC. During experiments each dog was placed in a metabolic cage and urine was collected in 20 ml of 0.1 N HCl during 24-hr intervals. The volumes were measured and aliquots were analysed. Pharmacokinetic parameters were calculated by standard procedures [10].

Enzyme assays. Glutathione *S*-transferase activity was measured at 22° by following the conjugation of glutathione with the standard substrate 1-chloro-2.4-dinitrobenzene at 340 nm ($\Delta\varepsilon$ = 9.6 mM^{-1} cm^{-1}) [11]. Reaction mixtures (final volume 3 ml) contained 0.1 M potassium phosphate buffer, pH 6.5; 1 mM 1-chloro-2,4-dinitrobenzene; 3% (v/v) ethanol and 1 mM glutathione. Reactions were initiated by the addition of 2–20 μg protein. The change of absorbance was limited to less than 0.05 per min and was then a linear function of protein and of time for at least 3 min. Correction was applied for non-enzymatic reaction rate.

To minimize non-enzymatic denitration of B-744-99, enzyme assays of organic nitrate cleavage were conducted at 22° in a total volume of 0.5 ml containing 40 mM Mops/Tris buffer, pH 6.7; 0.2 mM glutathione; 0.1 mM test compound and an appropriate amount of protein (8–70 μg). Controls without enzyme or without organic nitrate were run simultaneously. Nitrite formed was assayed in a diazotization reaction with sulfanilamide [11]. The reaction was terminated by adding 2.5 ml of 1% (w/v) sulfanilamide in 20% (w/v) HCl to the incubation mixture. After mixing, 2.5 ml of aqueous 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride was added and the absorbance measured at 540 nm after 20 min. Absorbance was a linear function of nitrite concentration in the range of 1–10 nmole and was dependent on thiol concentration.

All assays were corrected for non-enzymatic nitrite release and for absorbance caused by the presence of protein. Enzyme activity was calculated by reference to a standard curve constructed from sodium nitrite incubated with the appropriate thiol as described above.

Xanthine oxidase activity in plasma was determined by a radiometric assay based on the formation of [^{14}C]xanthine from [^{14}C]hypoxanthine. Plasma (1 ml) was incubated with 1 ml buffered saline, pH 7.4, containing 0.12 μCi [^{14}C]hypoxanthine at a final concentration of 1 μM at 37° for 30 min. ^{14}C-Activity was extracted and analysed by TLC (solvent system B). Under the assay conditions selected, xanthine was the only metabolite formed at a rate of 0.6 nmole/30 min/per ml plasma.

Partial purification of enzyme. Glutathione *S*-transferases from dog liver were purified in a similar way to the method developed for the purification of rat liver enzyme [12]. All purification steps were performed at 0–4°. The compositions of buffers used were: buffer A, 10 mM Tris–HCl, pH 8; buffer B, 10 mM potassium phosphate, pH 6.7. The conjugation of 1-chloro-2,4-dinitrobenzene with glutathione was used to monitor the course of enzyme purification. Liver homogenates (25%, w/v) were prepared in distilled water using an Ultra-Turrax homogenizer (30 sec), and, thereafter, a Teflon–glass Potter–Elevehjem homogenizer (10 up-and-down strokes). After centrifugation at 100,000 *g* (Beckman, Ti 50.1 rotor) for 1 hr, floating lipid was removed by suction. Samples of supernatant were applied to a DEAE-cellulose (DE 52, Whatman) column (2.5 cm × 20 cm), which was equilibrated and eluted (72 ml/hr) with buffer A until no further enzyme activity emerged. Eluted fractions (7.2 ml) containing transferase activity were pooled and concentrated by $(NH_4)_2SO_4$ precipitation (90% saturation corresponding to 0.65 g of salt added to 1 ml of eluate). The precipitated proteins were collected by centrifugation at 10,000 *g* (Sorvall, SS 34 rotor) for 30 min, dissolved in 4 ml of buffer B and dialysed against 1 l. of buffer B, changed 3 times during 18 hr. Samples of the dialysed preparation were applied to a column (1.6 cm × 14 cm) of CM-cellulose (CM 52, Whatman) equilibrated with buffer B. The column was developed with a 580 ml linear gradient of 0–75 mM KCl in equilibrating buffer at a flow rate of 36 ml/hr. Fractions (3.7 ml) were collected and analysed for glutathione *S*-transferase activity with both 1-chloro-2,4-dinitrobenzene and B-744-99 as substrates.

RESULTS

The metabolite pattern in serum over a 7-hr period after i.v. and p.o. administration of 20 μg/kg [^{3}H]B-744-99 to dogs is shown in Fig. 1. ^{3}H-Metabolite levels of 1 nmole/l. represent the lower limit of detection. Four labelled products were found in the serum extracts. Three non-volatile metabolites were identified by co-chromatography with unlabelled drug derivatives, namely, the 3'-mononitrate (B-744-100), the 2'-mononitrate (B-755-24) and the completely denitrated product (B-744-96). Exact chemical identification of the volatile metabolic

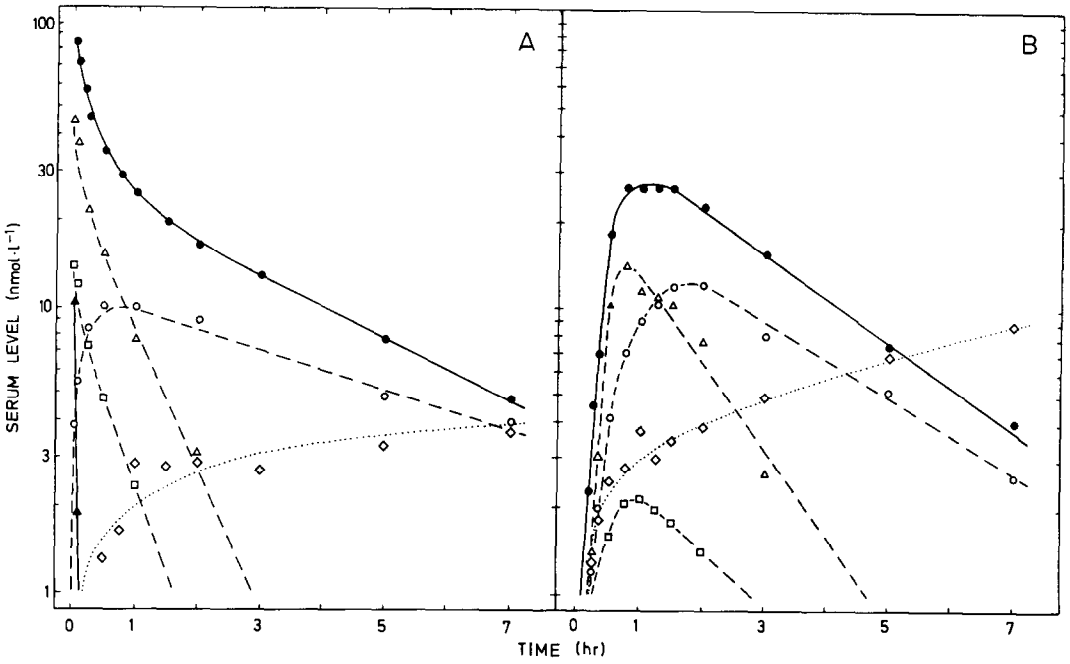

Fig. 1. Serum levels of ^{3}H-metabolites as a function of time after i.v. (panel A) and p.o. (panel B) administration of 20 μg/kg [^{3}H]B-744-99 at zero time. Serum ^{3}H-activity was analysed as described in Materials and Methods. Mean values of two experiments. (●—●) Non-volatile ^{3}H-activity, (◇···◇) volatile ^{3}H-activity, (▲—▲) [^{3}H]B-744-99, (□---□) [^{3}H]B-755-24, (△--△) [^{3}H]B-744-100, (○--○) [^{3}H]B-744-96.

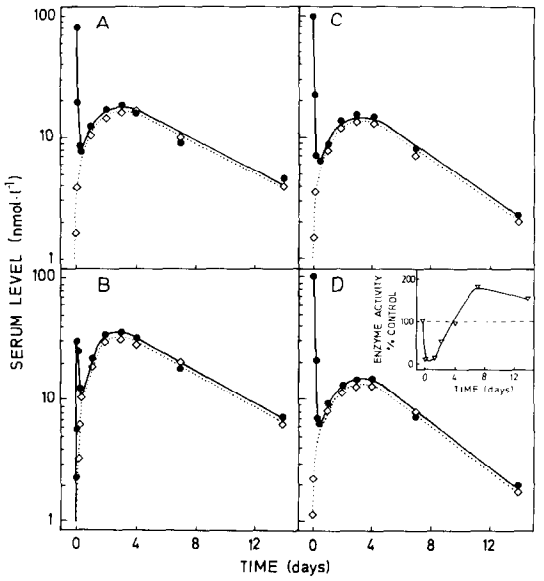

Fig. 2. Serum levels of total ^{3}H-activity and volatile ^{3}H-activity as a function of time. Administration of equimolar doses of (A) 20 μg/kg [^{3}H]B-744-99 i.v., (B) 20 μg/kg [^{3}H]B-744-99 p.o., (C) 15.5 μg/kg [^{3}H]B-744-96 i.v. and (D) 15.5 μg/kg [^{3}H]B-744-96 i.v. after pretreatment with 30 mg/kg allopurinol i.v. given 12 hr and again 0.5 hr before [^{3}H]B-744-96 administration. Inset: time course for the inhibition of xanthine oxidase activity in plasma as determined by a radiometric assay based on the formation of [^{14}C]xanthine from [^{14}C]hypoxanthine analysed by TLC. For details, see Materials and Methods. The enzyme activity is expressed relative to that found in plasma samples before administration of allopurinol. Mean values of two experiments. (●—●) Total ^{3}H-activity, (◇···◇) volatile ^{3}H-activity, (▽—▽) xanthine oxidase activity.

product was not attempted, but its distillation characteristic and elimination half-life suggest that it is tritiated water.

After i.v. administration, unchanged B-744-99 disappeared rapidly within 5 min and maximum levels of the mononitrates B-744-100 and B-755-24 were measured at 2 min post-dose. After p.o. drug administration, ^{3}H-levels were detected in serum within 10 min and reached a maximum after 45 min; however, unchanged B-744-99 was not detectable in serum. Both mononitrates B-744-100 and B-755-24 peaked at 45–60 min post-dose. The elimination of B-744-100 proceeded with an average half-life of 70 min after p.o., and of 30 min after i.v. drug administration. In parallel, B-755-24 levels were observed at 1/3–1/4 the concentrations of B-744-100. Both mononitrates were detectable in serum up to 1–3 hr after drug administration. In i.v. dosed dogs the completely denitrated metabolite B-744-96 reached a peak level at 45 min and declined thereafter with a half-life of about 4 hr. Following p.o. application, the peak level was between 1.5 and 2 hr and an elimination half-life of 2.3 hr. At 7 hr B-744-96 could still be detected in serum with either route of administration.

Oral bioavailability of the drug was about 90% as derived from the ratio of the area under the serum concentration curve of total ^{3}H-activity up to 7 hr (calculated by the trapezoidal rule) after oral and after i.v. drug administration.

Increasing amounts of volatile ^{3}H-activity were found in serum. Figure 2, A and B, shows that peak levels were reached on the third to fourth day after i.v. and p.o. [^{3}H]B-744-99 administration; ^{3}H-activity declined slowly thereafter with a half-life of 4–5 days. The application of the denitrated congener [^{3}H]B-744-96 (15.5 μg/kg i.v.) resulted in similar formation of volatile ^{3}H-activity (Fig. 2C); non-volatile ^{3}H was identified as unchanged drug and no other ^{3}H-labelled metabolic product was detected. To examine hydroxylation at the tritiated C-8 of the purine ring by xanthine oxidase as a source of tritium water formation, dogs received the same dose of [^{3}H]B-744-96 after pretreatment with allopurinol. Allopurinol (30 mg/kg i.v.), given 12 and 0.5 hr before application of ^{3}H-labelled drug, resulted in almost complete inhibition of plasma xanthine oxidase for 1 day (inset in Fig. 2D). Thereafter, a slow recovery of enzyme activity followed. The initial rate was reached at the fourth day post-dose. However, as seen in Fig. 2, C and D, the serum time course of ^{3}H-activity was superimposable on the data obtained without allopurinol indicating that inhibition of plasma xanthine oxidase did not influence the formation of volatile ^{3}H-activity.

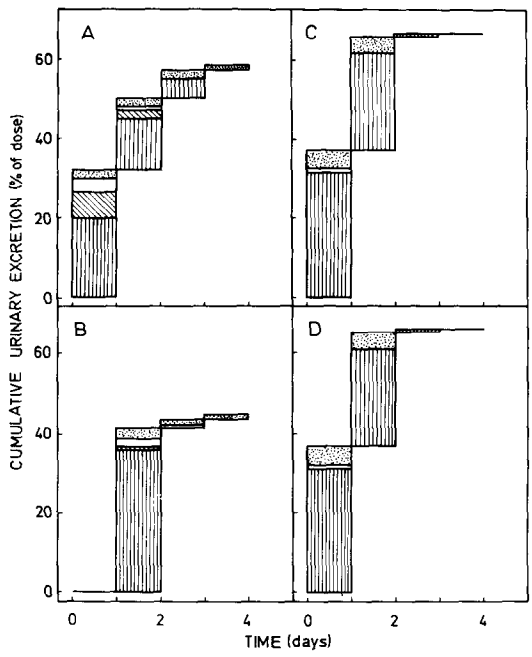

Fig. 3. Cumulative urinary excretion of ^{3}H-metabolites during 4 days. Drug treatment of dogs as described in Fig. 2: (A) 20 μg/kg [^{3}H]B-744-99 i.v., (B) 20 μg/kg [^{3}H]B-744-99 p.o., (C) 15.5 μg/kg [^{3}H]B-744-96 i.v., (D) 15.5 μg/kg [^{3}H]B-744-96 i.v. after pretreatment with 30 mg/kg allopurinol i.v. 12 hr and 0.5 hr before drug administration. Analysis of ^{3}H-metabolites by TLC and distillation. For details, see Materials and Methods. Mean values of two experiments expressed as a percentage of ^{3}H-dose administered and depicted in a cumulative manner. Whole columns represent total ^{3}H-activity; ▩ volatile ^{3}H-activity; ▨ [^{3}H]B-744-100; ▥ [^{3}H]B-744-96; □ unidentified.

The urinary excretion of radioactivity over 4-day periods is detailed in Fig. 3. After [^{3}H]B-744-99 administration (panels A and B), cumulative urinary excretion of ^{3}H-metabolites was delayed and slightly lower in p.o. than i.v. dosed dogs, amounting to 45 and 57% of the dose, respectively. As determined by TLC, most of urinary radioactivity was attributed to [^{3}H]B-744-96.* Unchanged drug was not observed in any urine sample. Mononitrates were not detectable except for small amounts of [^{3}H]B-744-100 in experiments with i.v. dosed dogs. The fraction of volatile ^{3}H increased with time up to 3/4 of the daily ^{3}H-excretion on the fourth day post-dose. As derived from serum profile and urinary excretion data, formation of volatile ^{3}H-activity amounted to about 1/3 of the administered ^{3}H-dose. Urinary excretion of ^{3}H-activity in [^{3}H]B-744-96 treated dogs (panels C and D) was somewhat higher than in [^{3}H]B-744-99 dosed dogs and averaged 67% of dose. No difference in metabolite pattern was seen between allopurinol treated dogs and controls without allopurinol.

In order to estimate the fraction of tritiated water formed by self-radiolysis of the ^{3}H-labelled drug, radiochemical stability was assayed at different temperatures (22 and 37°) and pHs (2–9). Twenty-four hour incubation of [^{3}H]B-744-99 and [^{3}H]B-744-96 resulted in no more than 3% of the tritium being liberated. No increase in formation of volatile ^{3}H was detected during incubation with xanthine oxidase, purine-nucleoside phosphorylase, dog blood or liver 10,000 *g* supernatant fraction. However, at alkaline pH considerable amounts of [^{3}H]B-744-99 decomposed to [^{3}H]adenine, which was identified by co-chromatography with unlabelled adenine in two different solvent systems (A and B), and by incorporation of ^{3}H in nucleotides after incubation with dog blood (data not shown); after incubation at pH 7.4 only traces of [^{3}H]adenine were detected. In contrast, [^{3}H]B-744-96 was stable under alkaline conditions.

Figure 4 illustrates the metabolism of [^{3}H]B-744-99 by dog blood *in vitro*. The pattern of metabolite formation was quite similar to that observed *in vivo*. The data again showed rapid appearance of both mononitrates with predominance of the 3'-mononitrate followed by formation of the completely denitrated product [^{3}H]B-744-96. Incorporation of ^{3}H in nucleotides was not observed.

Incubation of B-744-99 with several thiols resulted in the non-enzymatic formation of inorganic nitrite (Table 1). Cysteamine was found to be the most effective catalyst followed by cysteine and GSH. Only traces of nitrite formation were seen with CoA.

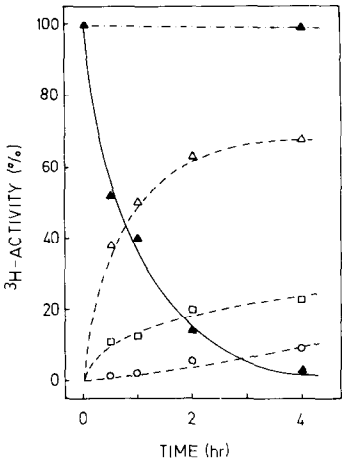

Fig. 4. Metabolism of [^{3}H]B-744-99 by dog blood *in vitro*. Incubation of 10 μM [^{3}H]B-744-99 in a suspension of dog blood in buffered saline, pH 7.4, at 37°; final haematocrit 20%. At the time indicated, samples were extracted and analysed for ^{3}H-metabolites as described in Materials and Methods. Mean values of two experiments. (▲—▲) [^{3}H]B-744-99, (□--□) [^{3}H]B-755-24, (△--△) [^{3}H]B-744-100, (○--○) [^{3}H]B-744-96, (- -·-) controls run without blood.

No measurable amount of nitrite was formed after incubation with glutathione disulfide.

Table 2 summarizes the results of partial purification of dog liver glutathione *S*-transferases assayed with 1-chloro-2,4-dinitrobenzene as substrate. Three peaks of enzyme activity were eluted from the CM-cellulose column and were designated as D (for dog) I–III by their order of elution.† Transferase D II represented the major component of 1-chloro-2,4-dinitrobenzene-conjugating activity and exhibited a specific activity of 5.7 μmole/min per mg averaging a 10-fold purification of the enzyme at an

Table 1. Non-enzymatic and enzymatic formation of nitrite from B-744-99 and various thiols*

Thiol	Nitrite formed (nmoles/min per ml)	
	Non-enzymatic reaction	Enzymatic reaction
GSH	0.25	0.55
Cysteine	0.40	0.43
Cysteamine	0.76	0.76
CoA	0.05	0.05

* Reaction mixtures (0.5 ml) containing 0.1 mM B-744-99 and 1 mM thiol in 40 mM Mops/Tris buffer, pH 7.4, were incubated at 22° for 20 min in the absence (non-enzymatic reaction) and presence (enzymatic reaction) of 2.2 μg partially purified glutathione *S*-transferase D II (see Table 2 and Fig. 5). Thiol was added last to initiate the reaction. Nitrite formed was determined as described in Materials and Methods. Blank incubation values (without thiol) were subtracted from the corresponding total rates. Data represent the means of duplicate determinations from three separate experiments conducted with one batch of enzyme. Results varied by no more than 10% of the respective mean.

* The apparently delayed recovery of B-744-96 in urine as compared to serum level decline may be explained by concealment of a more prolonged elimination phase, when serum levels had fallen below the level of detection, and by retention of urine in spontaneously urinating dogs from one collecting period to the next.

† Further glutathione *S*-transferase activity was adsorbed by DEAE-cellulose and eluted with increasing salt concentrations. It was active with 1-chloro-2,4-dinitrobenzene, but showed no detectable nitrate ester cleaving activity with B-744-99.

Table 2. Partial purification of glutathione *S*-transferases from dog liver*

	Step	Volume (ml)	Protein (mg)	Activity (μmoles/min)	Specific activity (μmoles/min per mg)
(1)	100,000 × *g* Supernatant	42	1533	920	0.6
(2)	DEAE-cellulose	97	112	146	1.3
(3)	$(NH_4)_2SO_4$ (90%)	6	65	104	1.6
(4)	CM-cellulose				
	Peak D I	21	3.4	7.1	2.1
	Peak D II	25	10.8	62	5.7
	Peak D III	21	3.2	1.6	0.5

* Dog liver (11 g) was homogenized and fractionated as described in Materials and Methods. Glutathione *S*-transferase activity was assayed with 1-chloro-2,4-dinitrobenzene as substrate. Protein was measured according to Lowry *et al.* [6].

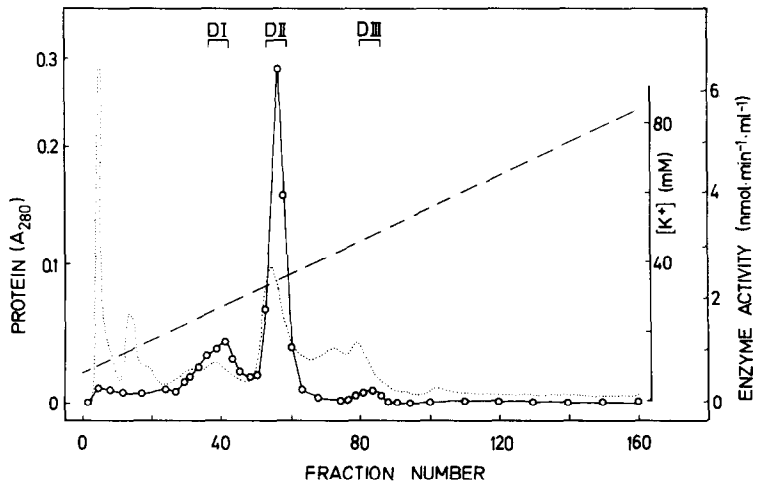

Fig. 5. Elution pattern of protein and glutathione *S*-transferases from CM-cellulose column. Enzyme activity (○—○) was assayed in the presence of 1 mM GSH with 0.1 mM B-744-99. For details see Materials and Methods. Protein was estimated from absorbance changes at 280 nm (A_{280}.....); ⌐¬ indicates the fractions that were pooled as partially purified enzyme D I, D II and D III. Results of a typical purification experiment.

Table 3. Specific activities of dog liver glutathione *S*-transferases*

Substrate	Transferase		
	D I	D II	D III
1-Chloro-2,4-dinitrobenzene	2.06	5.67	0.45
B-744-99	0.052	0.125	0.007
B-755-24	0.001	0.002	<0.0003†
B-744-100	0.0002	0.0004	<0.0001†
Isosorbide-2,5-dinitrate	0.006	0.024	<0.0001†
Isosorbide-2-mononitrate	<0.0001†	0.0002	<0.0001†

* Specific activities in μmoles/min per mg of protein determined under standard assay conditions as described in Materials and Methods.

† No enzyme activity was observed with certainty and the value given represents the lower limit of the assay at the highest enzyme concentration used.

8%-yield of enzyme activity of the initial extract. Nitrate reductase activities with B-744-99 as substrate always coincided with the peaks of enzyme activity observed with 1-chloro-2,4-dinitrobenzene. The final step of enzyme purification by elution from CM-cellulose is shown in Fig. 5. As seen in Table 1, the purified transferase D II required GSH as the second substrate. The other thiols tested were unable to replace GSH in enzymatic denitration of B-744-99. Similarly, the enzyme was inactive with glutathione disulfide.

Specific activity of the separated transferases for several organic nitrates is presented in Table 3. With all substrates tested, the same order in enzymatic activity was found, i.e. transferase D II was the most active species and D III the least active. Cleavage of organic nitrate proceeded most rapidly with B-744-99 and at lower rates with isosorbide-2,5-dinitrate > B-755-24 > B-744-100 > isosorbide-2-mononitrate. None of the transferases was active on isosorbide-5-mononitrate. The date for 1-chloro-2,4-dinitrobenzene are included for comparison.

DISCUSSION

The metabolite pattern in serum after administration of B-744-99 indicates step-wise cleavage of the two nitrate groups transforming the drug to its mononitrates and finally to its completely denitrated congener B-744-96. The latter product circulated in the dog for at least 7 hr and was cleared into urine.

During chromatographic analysis of serum and urine samples, we observed that decreasing portions of radioactivity were recovered from TLC plates at increasing time intervals after drug administration. By distilling the samples, considerable amounts of radioactivity were transferred into the distillate suggesting the presence of volatile ^{3}H-activity, presumably in the form of tritiated water. The occurrence of a long elimination half-life of volatile ^{3}H-label in serum resembling that of tritiated water [13] supports this view.

However, studies on the radiochemical stability of B-744-99 and B-744-96 indicate that the ^{3}H-label is stable under various conditions *in vitro*. Hydroxylation at the tritiated C-8 of the purine ring by xanthine oxidase as a source of tritium water formation seems unlikely, because neither inhibition of xanthine oxidase *in vivo* nor incubation of drug with xanthine oxidase or liver 10,000 *g* supernatant *in vitro* influenced formation of volatile ^{3}H. Significant formation of [^{3}H]adenine was seen neither during incubation with purine-nucleoside phosphorylase, nor with dog blood nor with liver 10,000 *g* supernatant. No enhancement of ^3H_2O formation was caused by nitrate ester cleavage reaction as evidenced, on the one hand, by the absence of volatile ^{3}H after *in vitro* conversion of B-744-99 and, on the other, by the demonstration of significant amounts of volatile ^{3}H after *in vivo* administration of the already denitrated drug B-744-96 similar to those seen after B-744-99. Yet the low rate of hydrogen–tritium exchange observed in a physiological salt solution cannot explain the quite high level of volatile ^{3}H-label measured in the *in vivo* experiment. Since a specific enzymatic process catalysing ^3H_2O formation has not been identified in the present study, we tend to the assumption that 'unspecific' interaction of B-744-96 with body elements catalyses the exchange reaction without transforming the drug into a metabolite.

In view of the delay in circulatory effects seen after administration of the nitrate esters of B-744-96 [2], the hypothesis of metabolic activation of these compounds is supported by our results. B-744-96 appears as the final metabolite and is known as an efficient adenosine agonist [14, 15], while B-744-99 itself does not react with putative adenosine receptors coupled to adenylate cyclase in rat liver cell membranes (W. Schütz, personal communication). Thus, B-744-96 represents a stable adenosine derivative, assuming that the tritium loss observed in the present study is the expression not of true metabolism but rather of a hydrogen–tritium exchange. The *N*-ethyl-carboxamide substitution at the 5′-position of adenosine prevents phosphorylation and blocks deamination [16, 17].

The results presented here indicate that thiols promote denitration of B-744-99. Quantitative differences in the non-enzymatic reaction may reflect differences in the reactivity of the thiol function, which seems to be determined especially by nearby amino and carboxyl groups [18]. Because of denitration of B-744-99 in the presence of thiols, we expected it to be a substrate in the glutathione *S*-transferase-catalysed reaction with GSH, known to be the major route of biotransformation of organic nitrates [3, 4]. In fact, each of the transferases isolated from dog liver catalysed denitration with selectivity for GSH as a nucleophile; GSH could not be replaced by cysteine, cysteamine or CoA. Free inorganic nitrite is formed and the reaction probably proceeds via the enzymatic formation of an unstable glutathione sulfenyl nitrite that is attacked non-enzymatically by a second molecule of thiol [3].

All transferases isolated are active with the standard substrate 1-chloro-2,4-dinitrobenzene, but transferase D II accounts for most of the enzyme activity. As evidenced by substrate specificity testing, the reaction rate with B-744-99 was about 5-fold higher than that observed with isosorbide-2,5-dinitrate as substrate. Specific activities with isosorbide-2,5-dinitrate were in the same order of magnitude as in rat and human liver [3]. The corresponding mononitrates were rather poor substrates. Fast glutathione *S*-transferase reaction with B-744-99 and rather low activities with the corresponding mononitrates reflect *in vivo* metabolism characterized by rapid disappearance of unchanged drug and rather slow denitration of the mononitrates. Similar to the stereospecificity found with nitrate cleavage of isosorbide-2,5-dinitrate and isosorbide mononitrates [19], biotransformation of B-744-99 is stereospecific with preferential hydrolysis of the nitrate group in the 2′-position leading to a predominance of the 3′-mononitrate of B-744-96 *in vivo* and *in vitro*.* The postulated metabolic scheme for

* Alternatively, the prevalence of the 3′-mononitrate may not depend on stereospecific enzymatic hydrolysis of B-744-99 but on rapid isomerization of the monoesters formed.

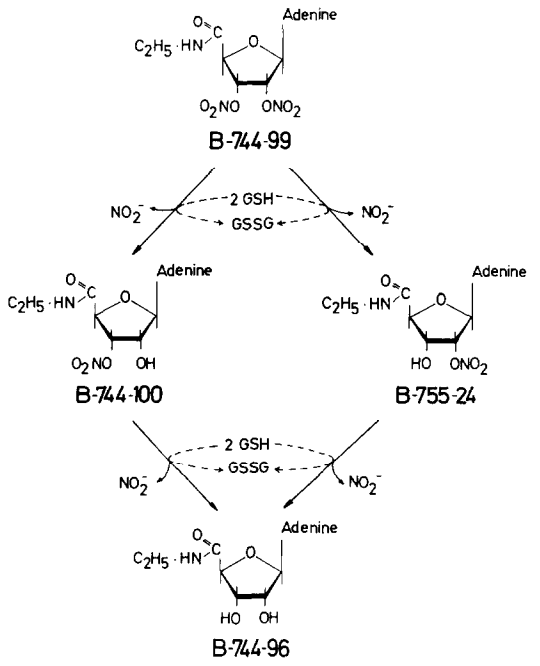

Fig. 6. Metabolic scheme of B-744-99.

B-744-99 biotransformation reactions is illustrated in Fig. 6.

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