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Pharmacokinetic Profile and Metabolism of *N*-Nitrosobutyl-(4-hydroxybutyl)amine in Rats[1]

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ABSTRACT

N-Nitrosodibutylamine and its ω-hydroxylated metabolite *N*-nitrosobutyl(4-hydroxybutyl)amine (NB4HBA) induce tumors in the urinary bladder of different animal species through their common urinary metabolite *N*-nitrosobutyl(3-carboxypropyl)amine (NB3CPA), resulting from the oxidation of the alcoholic group of NB4HBA to a carboxylic group.

NB4HBA disappearance from blood, the formation of its main metabolites, NB3CPA and NB4HBA-glucuronide (NB4HBA-G), and their urinary excretion, were investigated in rats after an i.v. dose of 1 mg/kg (5.7 μmol/kg).

NB3CPA and NB4HBA-G formation was readily detectable 2 min after treatment and levels were still measurable at 120 and 30 min, respectively. The parent compound disappeared from blood 90 min after injection. The NB4HBA blood concentration-time profile was adequately described by a one-compartmental linear model. NB4HBA half-life was 8 min, total body clearance and renal clearance were 86.1 and 0.22 ml/min/kg, respectively. The 0–96-h urinary excretion of NB4HBA was 0.3% of the administered dose. NB3CPA half-life was 15 min; NB3CPA and NB4HBA-G urinary excretion were 36 and 11.7%, respectively, urinary excretion of known compounds accounting for less than 50%. After i.v. injection of NB3CPA equimolar to the NB4HBA dose, only 50% of unchanged compound was recovered in the urine and after NB4HBA-G, 41% of the administered dose was excreted unchanged, NB3CPA accounting for 10%. Thus NB3CPA and NB4HBA-G might undergo further biotransformation, suggesting that NB3CPA may not be the ultimate carcinogen responsible for urinary bladder tumor induction.

INTRODUCTION

NDBA[3] and its ω-hydroxylated metabolite NB4HBA induce tumors of the urinary bladder in rats and mice (1). Because of NB4HBA's potent and selective organotropic carcinogenic activity, this compound has been used for developing models in laboratory animals (2, 3). The morphological characteristics of bladder cancers induced by NB4HBA in rodents resemble that observed in humans and there appear to be several close parallels between the human disease and rat models (4).

The carcinogenic activity of NDBA and NB4HBA is believed to depend on the formation of their principal and common urinary metabolite NB3CPA, resulting from oxidation of the alcoholic group of NB4HBA to a carboxylic group by the enzymatic system alcohol/aldehyde dehydrogenase (5, 6). NB3CPA reportedly is a selective bladder carcinogen in the rat, has direct carcinogenic activity when instilled into the bladder of female rats, is mutagenic without metabolic activation and produces neoplastic transformation of rat urothelial cells cultured *in vitro* (7–9), all these effects suggest a direct role of NB3CPA in the induction of urinary bladder cancer.

However recent studies have indicated that NB3CPA may not be the ultimate metabolite responsible for the induction of bladder cancer in rodents. Irving *et al.*, for instance, showed that disulfiram prevented the induction of bladder cancer in rats given NB3CPA, postulating that NB3CPA is not carcinogenic *per se*, and that further metabolism is required for its activation (10). Oyasu *et al.* reported that NB3CPA repeatedly instilled into a heterotopic transplanted rat bladder did not induce transitional cell carcinoma at the site of instillation but did in the natural bladder, thus suggesting that NB3CPA requires metabolic activation at some site other than bladder epithelium (2).

These observations have led to reconsideration of the mechanism of action of NB4HBA. Besides NB3CPA, several other minor metabolites are reportedly excreted in the urine of rats given NB4HBA orally, including the glucuronic acid conjugate of NB4HBA (NB4HBA-G), and transformation products of NB3CPA subsequent to its β-oxidation according to the Knoop mechanism (7). None of the metabolites tested was carcinogenic for the urinary bladder (8); NB4HBA-G was not tested.

Kinetic studies could help obtain a better understanding of the mechanism of action of chemicals, but despite the wide use of NB4HBA for inducing experimental bladder tumors in animals, no study has been reported of the disposition of this compound after i.v. injection.

The present paper reports the disposition profile of NB4HBA given i.v. to rats, and the urinary excretion of its metabolites NB3CPA and NB4HBA-G at i.v. doses equimolar to NB4HBA.

MATERIALS AND METHODS

Chemicals. NB4HBA and NB3CPA were synthesized according to the methods of Okada *et al.* (11, 12); NB4HBA-G was synthesized according to a procedure previously described (13); *N,O*-bis-(trimethylsilyl)trifluoroacetamide was obtained from Fluka (Buchs, Switzerland); C_{18} Sep-Pak cartridges were from Waters (Milford, MA). All other reagents were of purest grade available.

Animals and Treatments. Male CD-COBS rats (Charles River, Calco, Como, Italy), weighing 200 ± 10 g were used and maintained under constant conditions with a 12/12 h light/dark cycle. Kinetic studies were done in animals fasted overnight before the injection of 1 mg/kg (5.7 μmol/kg) NB4HBA into the vena saphena under slight ether anesthesia. Groups of four rats were killed by decapitation 2, 5, 10, 15, 20, 30, 40, 60, 90, 120, and 1440 min after treatment. Blood samples were collected in heparinized tubes and stored at 4°C. NB4HBA, NB3CPA, and NB4HBA-G were extracted within 2 h of collection.

Urinary excretion of NB4HBA and its metabolites was studied in four animals individually housed in metabolic cages with free access to food and water, urine was collected at room temperature 12, 24, 48, 72, and 96 h after i.v. dosing. At each collection time, the cages were rinsed with 5 ml of distilled water, the rinses were combined with the corresponding urine sample and centrifuged at 3000 × *g* for 10 min. The supernatants were stored at −20°C until analyzed for NB4HBA, NB3CPA, and NB4HBA-G content.

Two different groups of animals were injected i.v. with NB3CPA and NB4HBA-G at doses equimolar to NB4HBA and their urinary excretion was followed at 24-h intervals up to 96 h.

NB4HBA, NB3CPA, and NB4HBA-G were given as a 1:1 sa-

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[3] The abbreviations used are: NDBA, *N*-nitrosodibutylamine; NB4HBA, *N*-nitrosobutyl(4-hydroxybutyl)amine; NB4HBA-G, *N*-nitrosobutyl(4-hydroxybutyl)amine-glucuronide; NB3CPA, *N*-nitrosobutyl(3-carboxypropyl)amine; NDMA, *N*-nitrosodimethylamine.

line:ethanol solution, doses/kg being dissolved in 1 ml. Control animals received vehicle alone.

NB4HBA, NB3CPA, and NB4HBA-G Extraction and Analysis. Blood samples (2 ml) and urine samples (1–5 ml) were diluted to 10 ml with distilled water, the pH was adjusted to 3 and the samples were centrifuged at 3000 × *g* for 10 min. The supernatant was applied to a C_{18} Sep-Pak cartridge, washed with 2 × 5 ml water and eluted with 5 ml methanol. The eluate was evaporated to dryness and the compounds were derivatized following the procedure described previously (13, 14). Extraction efficiency was evaluated by adding known amounts of standards NB4HBA, NB3CPA, NB4HBA-G to blank blood and urine samples.

The trimethylsilyl derivatives of NB4HBA and its metabolites were quantitatively analyzed on a Dani 3800 gas chromatograph equipped with a TEA 543 detector (Thermo Electron, Waltham, MA). A wall-coated, fused-silica, wide-bore column (CP Sil 19 CB, 10 m x 0.53 mm i.d.; film thickness, 2.02 μ; Chrompack, Cernusco sul Naviglio, Milano, Italy) was used. For analysis of NB4HBA-G the oven temperature was kept at 240°C for 1 min, then raised to 260°C at the rate of 5°C/min. When NB4HBA and NB3CPA were measured the oven temperature program was as follows: 140°C for 2 min, then from 140 to 165°C at a rate of 15°C/min. The carrier gas (helium) head pressure was 0.2 atm. The gas chromatograph-thermal energy analyzer interface and pyrolyzer temperatures were 250 and 500°C, respectively.

Data Analysis. NB4HBA blood concentrations *versus* time were analyzed following a one-compartmental open model system. Data were fitted by a nonlinear regression iterative program (15) on an HP-85 desk computer (Hewlett-Packard). The adequacy of the kinetic model was confirmed by examining the plots of residuals (for systematic deviations) and the precision of parameter estimates. Total blood drug clearance (*CL*) was calculated as the ratio between the injected dose (*D*) and *AUC*, where *AUC* is the area under the blood concentration-time curve determined by the trapezoidal rule starting from C_0 (estimated blood concentration at time 0) and extrapolated to infinity, dividing the last blood concentration by the apparent first-order elimination rate constant (*k*). The apparent volume of distribution (V_{area}) and the steady-state volume of distribution (V_{ss}) were calculated as CL/k and $AUMC/AUC$,[2] respectively, where *AUMC* is the area under moment curve. Renal clearance (CL_R) was calculated by dividing the total amount of NB4HBA excreted in urine by the *AUC*.

According to classical texts (16, 17), the apparent first-order rate constant for elimination of NB3CPA (the only metabolite measurable over a long time) was determined from the terminal linear segment of the log blood concentration *versus* time curve.

In the light of the urinary excretion, levels of the parent compound and the detected metabolites in the urine cannot be employed for a detailed and accurate kinetic description.

RESULTS

Recovery values for NB4HBA, NB3CPA, and NB4HBA-G from blood and urine were 76 ± 9, 88 ± 8, and 98 ± 1.8% (mean ±SE), respectively. Experimental data were corrected for recovery values.

A semilogarithmic plot of the time course of mean blood concentrations of NB4HBA (and its measured metabolites) is shown in Fig. 1 and estimated pharmacokinetic parameters are reported in Table 1.

The parent compound was detectable up to 60 min, NB3CPA up to 120 min, while the glucuronide conjugate of NB4HBA disappeared from blood 40 min after treatment. The limits of sensitivity of the methods were 0.02, 0.03, and 0.125 nmol/ml for NB4HBA, NB3CPA, and NB4HBA-G, respectively. NB3CPA blood levels declined more slowly than the levels of unchanged compound and a half-life of 15 *versus* 8 min was estimated.

As shown in Table 1, NB4HBA total body and renal clearances were 86.1 and 0.22 ml/min/kg, respectively. The *AUC* was 66.2 nmol min/ml, V_{area} and V_{ss} were 990 and 980 ml/kg, respectively.

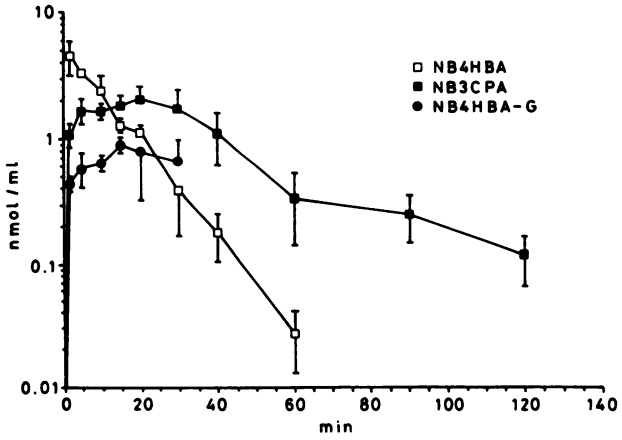

Fig. 1. Time-dependent NB4HBA disappearance from blood (□) and simultaneous formation of NB4HBA-G (●) and NB3CPA (■) after 1 mg/kg (5.7 μmol/kg) NB4HBA i.v. *Bars*, SE of the mean.

Table 1 *Pharmacokinetic parameters of NB4HBA in rat after i.v. injection of 5.7 μmol/kg*

Parameter	Unit	Value
C_0	nmol/ml	5.57
k	min^{-1}	0.087
$t_{½}$	min	8.006
V_{area}	ml/kg	990
V_{ss}	ml/kg	980
AUC	nmol min/ml	66.2
CL	ml/min/kg	86.1
CL_R	ml/min/kg	0.22

Table 2 *0–96-h urinary profile of NB4HBA, NB4HBA-G, and NB3CPA administered i.v. at the dose of 5.7 μmol/kg to rats*

Values are means ± SE of at least four animals. Limits of sensitivity of the methods: NB4HBA, 0.04 nmol; NB4HBA-G, 0.25 nmol; NB3CPA, 0.06 nmol excreted in 24 h.

Compound administered	Compound recovered % of administered dose			% of compound not recovered
	NB4HBA	NB4HBA-G	NB3CPA	
NB4HBA	0.26 ± 0.16	11.7 ± 2.4	36.0 ± 2.5	52.2 ± 2.3
NB4HBA-G	ND[a]	40.9 ± 1.8	9.9 ± 0.01	49.1 ± 1.8
NB3CPA	ND	ND	49.3 ± 4.8	50.6 ± 4.7

[a] ND, not detectable.

The 0–96-h urinary excretion of NB4HBA and its metabolites after i.v. NB4HBA is reported in Table 2. Less than 0.3% of the administered NB4HBA was excreted unchanged in the urine, NB4HBA-G and NB3CPA were, respectively, 11.7 and 36%. For all three compounds urinary excretion was almost complete in the 12-h sample (1st urine collection), NB4HBA and its glucuronide were undetectable at 24 h, NB3CPA was less than 1% at 72 h but still detectable at 96 h (data not shown). Thus, the total urinary recovery of identified compounds was calculated as about 48%.

In order to assess whether NB4HBA-G and NB3CPA were further metabolized, these were injected i.v. at doses equimolar to 1 mg/kg (5.7 μmol/kg) NB4HBA. Their urinary excretion is also reported in Table 2; about 41% of the administered NB4HBA-G was excreted unchanged and about 10% as NB3CPA. When the latter compound was injected i.v. about 50% of the dose was recovered unchanged in the 0–96-h urine.

Since urine was collected at room temperature, the stability of NB4HBA and its metabolites was checked over a 24-h period. No loss of the compounds was detected.

A possible NB4HBA pathway in the rat, drawn on the basis of urinary findings, is shown in Fig. 2.

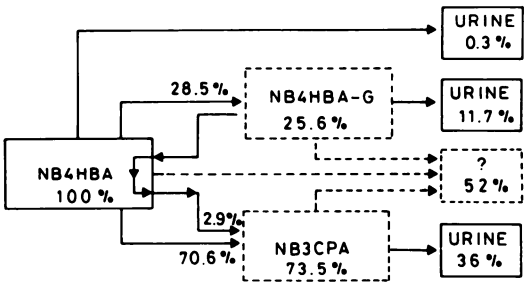

Fig. 2. NB4HBA urinary metabolic pathway; proposed model.

DISCUSSION

Only a limited number of *in vivo* studies describe the kinetic profile of nitrosamines in animals and they deal mostly with *N*-nitrosodimethylamine (18, 19). Moreover, previous studies in which NB4HBA metabolism was characterized and quantitated were conducted on urine samples after high oral doses (20); only one study reported the levels of NB3CPA in organs and body fluids of rats given NB4HBA, though blood was not considered (21).

Described here for the first time is the metabolic pattern of NB4HBA in the rat, which mainly metabolizes the compound to NB3CPA and NB4HBA-G, although the fate of about 50% of the administered dose was again not defined through the known metabolites.

Though the linear kinetic properties of the compound need to be confirmed by a series of single different doses and data analyzed according to the superposition principle (16, 17), we assumed that after a low i.v. dose (5.7 μmol/kg) of NB4HBA, the compound distributed rapidly in body tissues and the rate of disappearance from blood was adequately described by first-order kinetics. NB4HBA elimination from blood was rapid, the half-life being about 8 min. Soon after injection NB4HBA was metabolized to NB3CPA and NB4HBA-G which were already present at 2 min. NB4HBA, NB4HBA-G, and NB3CPA disappeared rapidly from the blood, being below the limit of detection of the methods at times longer than 120 min.

Keefer *et al.* (22) reported that NDMA metabolism was impaired by ether anesthesia as shown by a 9-fold decrease of NDMA clearance in rats exposed to ether for 2 min. In our experimental condition this effect was not observed, NBH4BA clearance being twice that reported for NDMA in unanesthetized rats. However, metabolic switching due to inhibition of one or more metabolic pathways may have occurred.

In agreement with previous data (20), NB3CPA represented the major NB4HBA urinary metabolite, accounting for about 40% of the administered parent compound; NB4HBA-G was about 12% and only a small fraction of the administered NB4HBA was recovered unchanged in the urine. In keeping with the low NB4HBA urinary excretion, the CL_R/CL ratio suggested that NB4HBA was rapidly eliminated through nonrenal routes.

The total urinary excretion of NB4HBA and its metabolites showed that about 50% of the nitrosamine remained unrecovered. Moreover, the large NB4HBA clearance compared to blood flow rate to the liver suggests that extrahepatic metabolism might be important.

In order to verify whether NB4HBA-G and NB3CPA were further metabolized, these compounds were administered i.v. to rats at doses equimolar to NB4HBA.

The presence of NB3CPA in the urine of rats given NB4HBA-G indicates that this compound was first hydrolyzed by β-glucuronidase and the released aglycon was then oxidized to NB3CPA. Most of NB4HBA-G and NB3CPA was excreted within 24 h, but measurable amounts were still present 72 h after treatment (data not shown), suggesting an enterohepatic circulation for NB4HBA-G.

When NB3CPA was administered to rats, only 49% of the dose was detected in the 0–96-h urine, suggesting that it may be further metabolized or is eliminated by nonrenal routes.

The scheme depicted in Fig. 2 was drawn bearing in mind all the urinary excretion data and assuming first-order elimination for NB4HBA. Knowing that NB3CPA excreted in the urine amounts to 49% of the administered dose, we can assume that NB3CPA excreted after the administration of NB4HBA represents 49% of that formed, hence the theoretical urinary NB3CPA would be 73.5%. Moreover, assuming that the amount of NB4HBA-G excreted in the urine after NB4HBA represents 41% of the glucuronide formed, the theoretical NB4HBA-G formation from NB4HBA was 28.5%, 10% of which was hydrolyzed back to NB4HBA and then oxidized to NB3CPA, thus 2.9% enters the NB3CPA pool. Since the actual urinary excretion of NB4HBA-G was 11.7%, then part of NB4HBA-G formed has probably been metabolized to unidentified products, or excreted through nonrenal routes.

Similarly, the actual urinary NB3CPA was 36% of the NB4HBA dose. Thus a large proportion of the administered dose has probably been metabolized to unknown compounds or excreted nonrenally.

Several compounds retaining the nitroso moiety have been identified in the urine of rats given NB3CPA or NB4HBA. They represented metabolic products resulting from further oxidation of NB3CPA, *N*-nitrosobutyl(2-hydroxy-3-carboxypropyl)amine, *N*-nitrosobutyl(carboxymethyl)amine, and *N*-nitrosobutyl(2-oxopropyl)amine.Ofthese,*N*-nitrosobutyl(carboxymethyl)amine was not mutagenic or carcinogenic in the rat, *N*-nitrosobutyl(2-oxopropyl)amine was mutagenic and induced liver but not bladder cancer in rats and *N*-nitrosobutyl(2-hydroxy-3-carboxypropyl)amine was mutagenic but no data on its carcinogenicity is available (8).

The gas chromatography-thermal energy analysis chromatograms of our urine samples contained four more peaks, besides NB4HBA, NB3CPA, or NB4HBA-G; two of them were identified by gas chromatography-mass spectrometry as *N*-nitrosobutyl(2-hydroxy-3-carboxypropyl)amine and *N*-nitrosobutyl(carboxymethyl)amine (data not shown). However no quantitative analysis were performed, because no reference compound was available.

In this and previous studies only NB4HBA metabolites retaining the nitroso moiety were considered (5, 7, 20), but nitrosamines reportedly follow other metabolic pathways such as α-hydroxylation or denitrosation (23, 24). As shown in Fig. 2, the pool of unknown compounds deriving directly from NB4HBA or NB4HBA-G or NB3CPA could amount to as much as 52% of the administered dose. This percentage might include part of NB4HBA and its metabolites distributed in organs and tissues, as shown by Wada *et al.* (21). NB4HBA and its metabolites may also be excreted by fecal or other nonrenal routes.

The overall results lead to the conclusion that all the compounds considered, including NB3CPA, might undergo further metabolization, thus supporting the hypothesis previously put forward (2, 10) that NB3CPA may not be the only metabolite responsible for urinary bladder tumor induction.

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