Journal of Chromatography, 383 (1986) 172—178
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3329

Note

N-Nitrosobutyl-N-(4-hydroxybutyl)amine glucuronide in vitro synthesis, characterization by mass spectrometry and determination by gas chromatography—thermal energy analysis

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(First received March 26th, 1986; revised manuscript received July 15th, 1986)

Since the discovery of the organ specificity of N-nitrosobutyl-N-(4-hydroxybutyl)amine (NBHBA-4) in the carcinogenic response [1], this compound has been used to induce selectively urinary bladder cancer in animals [2–4]. Studies on the metabolic fate of NBHBA-4 in rats suggest that the induction of bladder cancer can be ascribed to the formation of its major urinary metabolite N-nitrosobutyl-N-(3-carboxypropyl)amine (NBCPA) [5, 6]. Besides other minor metabolites, a percentage of the administered NBHBA-4 is reportedly excreted in the urine conjugated to glucuronic acid [5, 6].

Glucuronidation is usually considered to be a detoxifying pathway, leading to the formation of more hydrophilic derivatives that are easily eliminated from the body. However, as proposed for some urinary bladder carcinogenic aromatic amines, glucuronides transported from the liver to the kidneys and the urinary bladder may be hydrolysed in the urine, thus providing substrates for metabolic activation in the target cells [7, 8]. In order to assess whether the glucuronic acid conjugate of NBHBA-4 (NBHBA-4-G) plays a defined role in the induction of urinary bladder tumours in rats, standards and analytical methods are needed.

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0378-4347/86/$03.50

This paper reports the in vitro synthesis of NBHBA-4-G by rat hepatic microsomal preparations, its characterization by mass spectrometry (MS) and its quantitative determination by gas chromatography—thermal energy analysis (GC—TEA) in biological samples.

EXPERIMENTAL

Chemicals and reagents

NBHBA-4 was prepared according to the method of Okada et al. [9]; uridine-5′-diphosphoglucuronic acid (UDPGA), β-glucuronidase and β-glucuronidase/arylsulphatase were obtained from Boehringer (Mannheim, F.R.G.); N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and silylation-grade pyridine were obtained from Fluka (Buchs, Switzerland) and Pierce (Rockford, IL, U.S.A.), respectively. C_{18} Sep-Pak cartridges were from Waters (Milford, MA, U.S.A.).

In vitro NBHBA-4 glucuronidation

Male CD-COBS rats obtained from Charles River, Italy (body weight 180 ± 10 g) were used. Animals were decapitated after an overnight fast, the liver was rapidly removed and homogenized in four volumes of 0.05 *M* phosphate buffer (pH 7.4) containing 0.15 *M* potassium chloride and 0.005 *M* magnesium chloride. The homogenate was centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant was centrifuged at 105 000 *g* for 60 min in a Beckman L8-80 ultracentrifuge. The microsomal pellet was resuspended in the same buffer as for homogenization, and the samples were stored at −80°C until used. Protein levels were determined by the method of Bradford [10].

Incubations were carried out in crimp-seal vials at 37°C for 2 h in a Dubnoff metabolic shaker. The incubation medium containing 0.5 ml of microsomal suspension (10 mg of protein), 12 m*M* UDPGA and 1 m*M* NBHBA-4 in a final volume of 1 ml. The reaction was terminated by placing the samples in boiling water for 1 min. After cooling, the unconjugated NBHBA-4 was extracted with two 3-ml volumes of ethyl acetate.

The aqueous phase containing the glucuronide was adjusted to pH 3 with 1 *M* hydrochloric acid and centrifuged at 2000 *g* for 10 min to eliminate proteins, then processed as described by Feng et al. [11]. Briefly, the supernatant was applied to a C_{18} Sep-Pak cartridge prewashed with 2 ml of methanol and 5 ml of water. The cartridge was washed with two 5-ml volumes of water, and the glucuronide was eluted with 5 ml of methanol. The eluate was evaporated to dryness under reduced pressure. The dry residue was dissolved in a small volume of methanol and purified on preparative thin-layer chromatographic plates using the developing solvent system described by Suzuki and Okada [12], *n*-butanol—ethanol—water (4:1:1). The NBHBA-4-G band was scraped off and eluted from the silica gel with methanol; the sample was centrifuged and the supernatant was evaporated to dryness under reduced pressure. the dry residue was dissolved in water and the pH adjusted to 3, and the sample was passed again through a C_{18} Sep-Pak cartridge as described above for removal of silica gel.

The presence of NBHBA-4-G in the incubation medium was also verified by

hydrolysing the glucuronic acid conjugated with β-glucuronidase at pH 7 at 37°C for 18 h after extraction of free NBHBA-4. The NBHBA-4 obtained after hydrolysis was quantitatively analysed as previously described [13].

Derivatization procedures

The trimethylsilylated methyl ester of NBHBA-4-G was obtained by treating a methanolic solution of glucuronide with diazomethane; the solvent and excess reagent were removed by evaporation. The residue was then treated with BSTFA (80 μl) and ethyl acetate (20 μl) for 30 min at room temperature. The reaction mixture was evaporated to dryness and the residue dissolved in ethyl acetate for MS analysis.

Alternatively, NBHBA-4-G was treated with 50 μl of BSTFA and 50 μl of pyridine at 60°C for 60 min in tightly stoppered tubes, to obtain the per(trimethylsilyl) derivative. The reaction mixture was evaporated to dryness and the residue dissolved in ethyl acetate before MS analysis.

Instrumentation

A VG 70-250 mass spectrometer operating in the electron-impact (EI) or chemical-ionization (CI) mode was used under the following conditions: electron energy, 50 eV; accelerating voltage, 6 kV. Ammonia was used as the reagent gas in the CI mode; the pressure in the ion source was 0.4 Torr. Samples were introduced by the direct inlet system.

GC analyses were performed on a DANI 3800 gas chromatograph coupled to a TEA 543 detector (thermal energy analyser, Thermo Electron, Waltham, MA, U.S.A.). A 1 m × 2 mm I.D. glass column packed with 1% SP-2100 on 100–120 mesh Supelcoport was used. For the analysis of NBHBA-4-G as its persilylated derivative, the oven temperature was kept at 200°C for 1 min, then raised to 240°C at a rate of 10°C min^{-1}. The carrier gas (helium) flow-rate was 30 ml min^{-1}; the GC—TEA interface and pyrolyser temperatures were 250°C and 500°C, respectively.

Calibration curves were constructed by analysing known amounts (10, 20, 40, 50 ng) of the persilylated NBHBA-4-G by GC—TEA.

Animal studies

In order to verify the applicability of the method to biological specimens, overnight fasted rats were injected with 5 mg kg^{-1} NBHBA-4 intraperitoneally, then immediately placed individually in metabolic cages. Urines were collected for 24 h and diluted to 16 ml with water; 3-ml portions were adjusted to pH 3 with hydrochloric acid and the glucuronide was extracted as described above. Parallel samples were analysed before and after hydrolysis with β-glucuronidase/arylsulphatase for NBHBA-4 content as previously described [13].

Extraction and clean-up efficiency were evaluated by adding known amounts of standard NBHBA-4-G to 3-ml samples of blank urine.

The same extraction procedure was applied to blood samples: 2 ml of heparinized blood from untreated animals were spiked with NBHBA-4-G (500 ng), diluted to 10 ml with water and adjusted to pH 3 with hydrochloric acid. After centrifugation at 2000 *g*, the supernatant was applied to a C_{18} Sep-Pak cartridge and extracted as described above.

RESULTS AND DISCUSSION

The NBHBA-4 glucuronic acid conjugate formed in the presence of rat liver microsomes and UDPGA was ca. 15% of the substrate added, determined by gravimetric analysis after extraction and purification. Hydrolysis with β-glucuronidase on the incubation medium after the extraction of free NBHBA-4 and the subsequent quantitative determination of NBHBA-4 released gave comparable yields.

The identity of NBHBA-4-G was confirmed by MS analysis. Fig. 1 shows the 50-eV EI mass spectra of the silylated methyl ester and the persilylated derivative of NBHBA-4-G (a and b, respectively). The fragmentation pattern is in agreement with previous studies [14, 15]. The $[M]^+$ ion in the mass spectrum of the silylated methyl ester of NBHBA-4-G is of very low intensity, but the presence of the ion at $[M - 15]^+$ due to the loss of a methyl group can be used to deduce the molecular weight (Fig. 1a). The peak at *m/z* 550 is due to the loss of NO and is characteristic for N-nitrosamines [16]. The ion at *m/z* 317

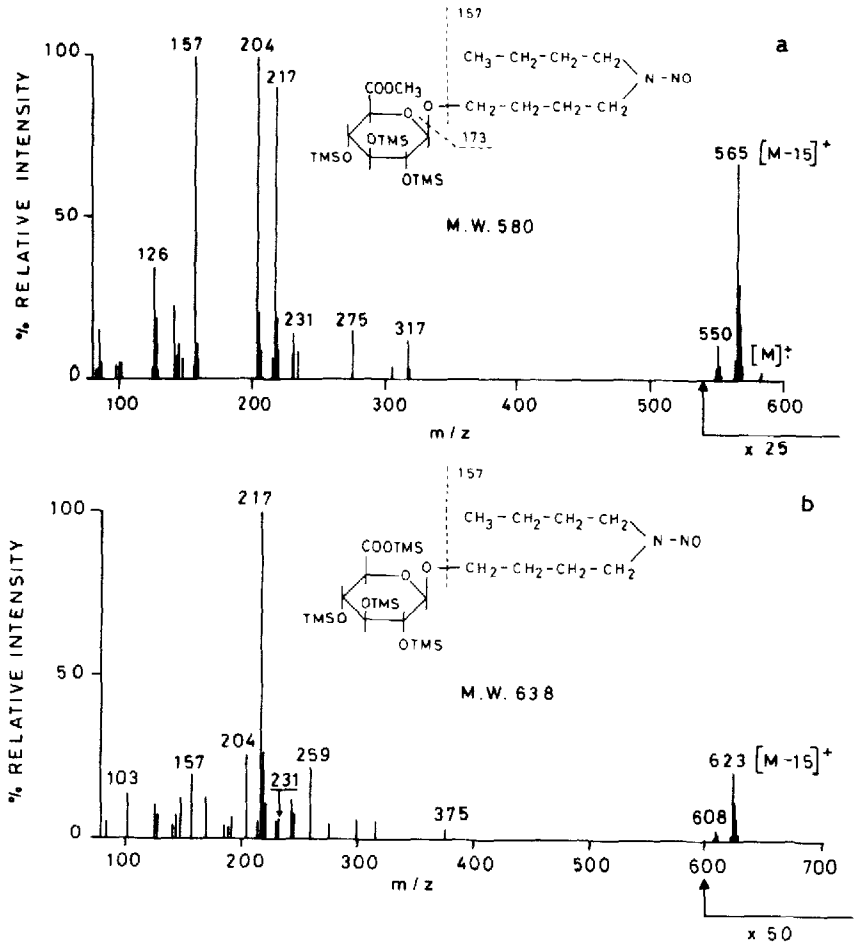

Fig. 1. EI mass spectra of (a) the silylated methyl ester and (b) persilylated derivative of NBHBA-4-G.

is due to the elimination of a trimethylsilanol group from the silylated methyl ester of the glucuronic acid moiety and can be used in determining the presence of the glucuronide [14]. Some of the fragment ions are common to most silylated carbohydrates (i.e. ions of masses 217 and 204). Ions at *m/z* 231, 173, 157 and 126 identify the aglycone moiety and represent M − 334 − 15, M − 407, M − 423 and M − 423 − NOH, respectively. The loss of 334 mass units is due to cleavage of the carbohydrate moiety and transfer of a trimethylsilyl group to the aglycone [14].

As shown in Fig. 1b, the fragmentation pattern of the mass spectrum of persilylated NBHBA-4-G is very similar to the silylated methyl ester derivative, being the result of the elimination of groups carrying the trimethylsilyl ester instead of the methyl ester.

The identity of the glucuronide of NBHBA-4 was confirmed by CI-MS. As shown in Fig. 2, the ammonia Cl mass spectrum of the silylated methyl ester of NBHBA-4-G indicates the presence of the ion $[M + H]^+$, which results from the elimination of NH_3 from the molecular species $[M + NH_4]^+$. The base peak is represented by the fragment at *m/z* 552 due to the loss of CH_3 and NOH from the molecular species. Several ions present in the EI mass spectrum are also formed in the Cl mode.

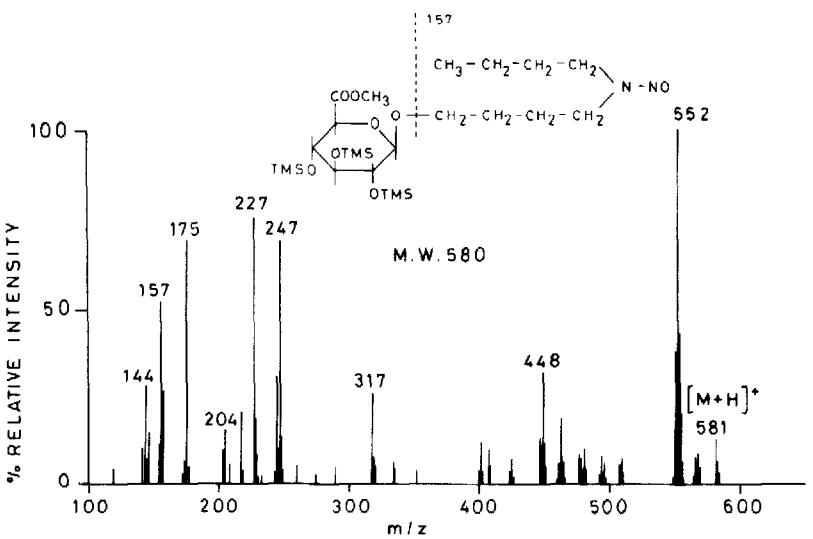

Fig. 2. CI mass spectrum of the silylated methyl ester of NBHBA-4-G.

Fig. 3 shows a typical GC—TEA profile of a blank urine sample and a urine sample spiked with extracts containing 500 ng of NBHBA-4-G as described above and analysed after derivatization with BSTFA. Persilylation gave more volatile derivatives and better GC peaks. A linear relationship was observed between the amount of persilylated NBHBA-4-G injected (10—50 ng) and the GC—TEA peak area, the correlation coefficient being 0.9968. The recovery of known amounts of NBHBA-4-G added to blank urine samples was 96 ± 1.3% (mean ± S.E.).

The 24-h NBHBA-4-G urinary excretion in rats given 5 mg kg^{-1} NBHBA-4 intraperitoneally was 42.8 ± 6.49 nmol (mean ± S.E.). These values are similar

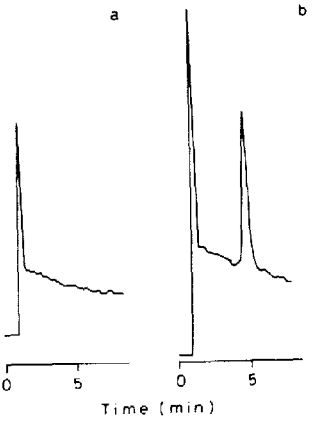

Fig. 3. Typical GC—TEA profiles of (a) a blank urine sample and (b) a sample spiked with 500 ng of NBHBA-4-G, extracted as described in the text and analysed after persilylation.

to those obtained by measuring the amount of NBHBA-4 released after hydrolysis of the same urine samples with β-glucuronidase/arylsulphatase (33.8 ± 11.17 nmol, mean ± S.E.).

However, direct measurement of the glucuronide rather than the aglycone released by β-glucuronidase overcomes all the difficulties introduced by the presence of inhibitors in biological samples. Moreover, using β-glucuronidase/arylsulphatase does not clarify whether only glucuronides or other conjugates are determined.

Preliminary results indicate that this method is applicable to blood samples, the recovery of the compound being 98 ± 1.8% (mean ± S.E.). The limit of sensitivity of the method was 100 ng ml^{-1} of blood and 60 ng ml^{-1} of urine, corresponding to a 24-h urinary excretion of ca. 1 μg by the rat. This analytical method could be useful for monitoring glucuronic acid-conjugated NBHBA-4 in biological fluids.

ACKNOWLEDGEMENT

This work was supported by the Italian National Council for Research, Special Project "Oncology" Contract No. 85.02556.44.

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