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STUDIES ON THE FORMATION OF CYCLOHEXYLAMINE AND N-METHYLCYCLOHEXYLAMINE FROM BROMHEXINE IN ANIMALS AND MAN, AND SIMULTANEOUS DETERMINATION OF CYCLOHEXYLAMINE AND N-METHYLCYCLOHEXYLAMINE BY GAS CHROMATOGRAPHY

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SUMMARY

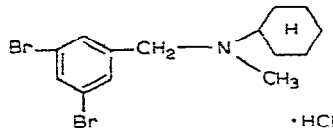
In order to detect cyclohexylamine and N-methylcyclohexylamine simultaneously in urine, a gas chromatographic method was developed in which Chromosorb 103 (porous polymer) was found to be suitable as the column packing. The detection limits were 0.1 μg/ml of cyclohexylamine and 0.4 μg/ml of N-methylcyclohexylamine in urine. In tests on animals administered bromhexine, which contains N-methylcyclohexylamine as part of its side-chain, neither cyclohexylamine nor N-methylcyclohexylamine was detected in the urine.

INTRODUCTION

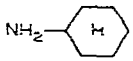
It has been found that sodium cyclamate, a sweetening agent, is converted in the body into cyclohexylamine as a metabolite[1,2], and sodium cyclamate has been prohibited from food additives in many countries because of the question of the toxicity of cyclohexylamine. Sodium cyclamate used to be added to foods in fairly large amounts, for example about 0.1 % in drinks, *e.g.*, 0.2 g per 200 ml in cider and aerated cider-like drinks, 0.26 g per 200 ml in fruit juice[3] and much more in syrups. Hence the amounts ingested by man could have been greater than 30 mg/kg per day.

Although the clinical dose of bromhexine (Bisolvon) is not more than 0.2 mg/kg per day, it contains N-methylcyclohexylamine in its structure, N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)amine hydrochloride, and the possibility of cyclohexylamine and N-methylcyclohexylamine being formed from bromhexine (Fig. 1) in the body has been considered.

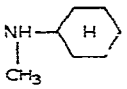
Schraven *et al.*[4] investigated the chemical nature of the metabolites of bromhexine in rabbits; 93 % of the substances recovered from urine after combined hydrolysis with β-glucuronidase and hydrochloric acid were identified as bromhexine (30.5 %) and its metabolites (62.5 %), and remaining 7 % being unidentified. They proved the chemical structures of the metabolites of bromhexine, but no examination



Bromhexine (Bisolvon)



Cyclohexylamine



N-methylcyclohexylamine

Fig. 1. Formulae of bromhexine, cyclohexylamine and N-methylcyclohexylamine.

to prove the existence of cyclohexylamine and N-methylcyclohexylamine in the metabolites has been carried out. However, one cannot ignore the possibility of the existence of amines in a fraction that was not recovered from urine and not identified by their method, because bromhexine may be oxidatively N-dealkylated by hepatic microsomal drug-metabolizing enzymes. Therefore, it is still possible that N-methylcyclohexylamine and cyclohexylamine may be formed from bromhexine in the body.

This investigation was undertaken to examine whether cyclohexylamine is formed during the breakdown of bromhexine in the body.

MATERIALS AND METHODS

Animals and volunteers

Male rats (Sprague Dawley strain) weighing 300–350 g and male Himalayan rabbits weighing 2.0–2.7 kg were used throughout the experiments and allowed free access to food (CE-2 and CR-1, Nihon Clea Co., Tokyo, Japan) and water.

In the experiments with humans, five healthy male volunteers aged 32–41 years were administered bromhexine tablets (three times a day, 12 mg each). The urine of patients who had been treated for a disease of the chest and bronchial tubes with bromhexine for 1–3 weeks (twice a day, 12 mg each) was also collected for 24 h and analyzed. Antibiotics (kanamycin or lincomycin) had also been administered to all of these patients, who comprised three males aged 55, 66 and 85 years and two females aged 71 and 72 years. During the experiments, they were allowed a free diet.

Drugs

Two kinds of dosage form of bromhexine were used: (1) tablets, one tablet containing 4 mg of N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)amine hydrochloride; and (2) injections containing 4 mg of bromhexine per 2 ml, Batch No. 2031 for clinical tests (Bisolvon, C. H. Boehringer Sohn GmbH, Ingelheim am Rhein, G.F.R.).

The following, all from Wako Pure Chemical Industries, Osaka, Japan, were also used: sodium cyclamate (sodium cyclohexylsulphamate, special grade), (2) N-methylcyclohexylamine and (3) cyclohexylamine hydrochloride.

Applications

Bromhexine injections and the sodium cyclamate solution were administered to rabbits intravenously via the auricular vein and to rats intraperitoneally. In the volunteers, bromhexine tablets were administered orally.

Collection of urine

Urine was collected for 24 h in man and rats, and for 48 h in rabbits. A large proportion of bromhexine and its metabolites are excreted into urine[4,5] and accordingly amines in the urine were analyzed in this investigation.

Extraction of amines

The amines in urine were extracted by a modification of the method described by Kojima and Ichibagase[2]. They used chloroform for the extraction of cyclohexylamine from the urine of animals administered sodium cyclamate. In this investigation, several extraction solvents were examined in order to find one suitable for the gas chromatographic analysis of the amines in urine. It was found that chloroform was not a suitable extraction solvent for the column packed with Chromosorb 103 because it produced considerable tailing that interfered with the detection of the peak of the amines. When using a Chromosorb 103 column, alcohols, aromatic solvents (except high-purity benzene) and halogenated hydrocarbons (except dichloromethane) were inadequate solvents. Ammonia also interfered in the determination of the amines. On the other hand, dichloromethane and *n*-hexane were suitable for the extraction of amines from urine and neither interfered in their gas chromatographic analysis using a Chromosorb 103 column.

Dichloromethane and *n*-hexane were therefore used for the extraction of cyclohexylamine, and salting-out with sodium chloride was used in order to increase the extraction capacity. The extraction scheme is shown in Fig. 2. First, 20 ml of urine plus 2 ml of concentrated hydrochloric acid were refluxed for 1.5 h in order to hydrolyze the conjugated amines. The solutions were then made alkaline with 5 *N* sodium hydroxide solution to pH 11.5 and extracted three times with 15 ml of dichloromethane. The dichloromethane extracts were combined and dried over sodium sulphate. The sample size was then reduced to *ca.* 1 ml on a rotary evaporator.

The amines in dichloromethane were extracted three times with 1 *N* hydrochloric acid (0.2, 0.1 and 0.1 ml). The hydrochloric acid solution containing the amines was made alkaline with 5 *N* sodium hydroxide solution and then saturated with sodium chloride. The amines in this solution were extracted three times with *n*-hexane (0.5, 0.3 and 0.2 ml), the extracts were combined and the amines in the *n*-hexane were transferred into 0.1 ml of 0.5 *N* hydrochloric acid. After the elimination of *n*-hexane, 0.02 ml of 5 *N* sodium hydroxide solution, *ca.* 0.04 g of sodium chloride and 0.1 ml of *n*-hexane were added to the hydrochloric acid solution. The mixture was shaken and centrifuged in order to transfer the amines into the *n*-hexane layer, which was subsequently used as the sample for gas chromatography.

Determination of amines

Hitachi 063 and Shimadzu 4BM gas chromatographs, both equipped with flame ionization detectors, were used for the analysis of the amines. The gas chromatographic assay method of cyclohexylamine in the urine of animals administered

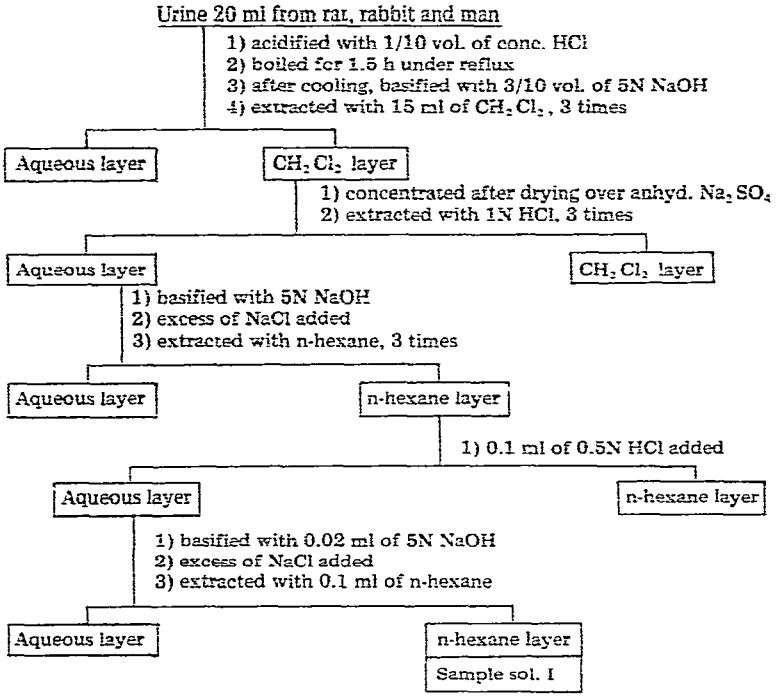


Fig. 2. Scheme of the method for preparing the sample solutions from urine.

cyclamate was described by Kojima and Ichibagase[2]. We tested whether their method was suitable for the determination of these amines, but the peaks of cyclohexylamine and N-methylcyclohexylamine were not separated clearly.

In order to determine these amines simultaneously, several column packings were examined for their ability to give a clear separation. Chromosorb 103 was found to be the most suitable. A glass column (2.0 or 2.5 m long × 3 mm I.D.) was packed with Chromosorb 103, 60–80 mesh (Johns-Manville, Denver, Colo., U.S.A., porous polymer packing for analysis of amines).

The gas chromatographic determination was carried out under the following operating conditions. Nitrogen was used as the carrier gas at a flow-rate of 80–100 ml/min. The hydrogen flow-rate and air pressure were 30 ml/min and 1 kg/cm^2, respectively. The temperature of the injection port was maintained at 150°, and the column temperature was 175, 195 or 210°. The temperature in the flame ionization detector was 165 or 150°. The chart speed was 0.5 cm/min, the attenuation was ×1 and ×2 in the Hitachi instrument and the range was 1 (sensitivity 10^2 MΩ) in the Shimadzu instrument.

RESULTS

Calibration graph and detection limits

Aqueous solutions containing 4–20 μg of cyclohexylamine were prepared and then the amine was extracted quantitatively from these solutions with *n*-hexane. A standard amount of each of these extracts was carefully chromatographed under

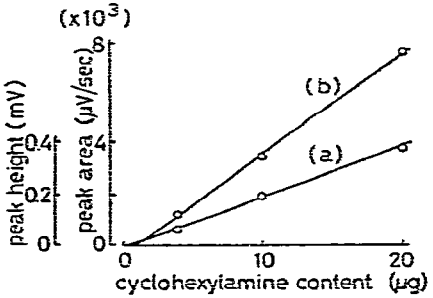


Fig. 3. Standard graphs of cyclohexylamine content *versus* (a) peak height and (b) peak area. The peak areas (μV/sec) and peak heights (mV) were measured with a digital integrator (Shimadzu ITG-3BX) and recorder (Shimadzu R-12M). Conditions: Shimadzu 4BM chromatograph with flame ionization detector; glass column, 2.5 m × 3 mm I.D., packed with Chromosorb 103, 60–80 mesh; inlet temperature, 150°; oven temperature, 210°; nitrogen flow-rate, 100 ml/min; hydrogen flow-rate, 30 ml/min; attenuation, ×1 ($10^8\ \Omega$); sample injected, 2 μl; chart speed, 5 mm/min.

identical conditions. Two calibration graphs were constructed of the concentration of amine in the reference solutions *versus* the response (peak height and peak area) obtained (Fig. 3). Further, the gas chromatogram of the aqueous solution containing 4 μg of cyclohexylamine is shown in Fig. 4 for reference.

In order to demonstrate the validity of the method described, the following experiment was carried out. Cyclohexylamine hydrochloride (12 μg) was dissolved in 20 ml of untreated rabbit urine, and then the amine was extracted and chromatographed. As shown in Fig. 5, the peak of cyclohexylamine was clearly observed.

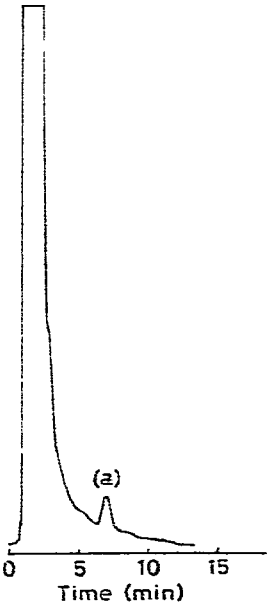
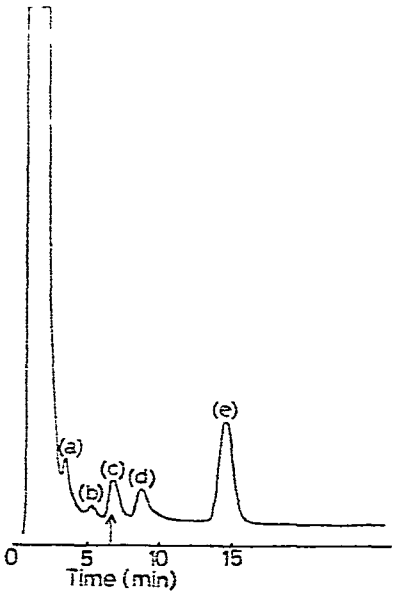


Fig. 4. Gas chromatogram of cyclohexylamine (a) extracted with *n*-hexane from an aqueous solution containing 4 μg of cyclohexylamine. Conditions as in Fig. 3.

By means of the method described, it was possible to detect down to 1 μg of cyclohexylamine in the aqueous solution; the detection limit of cyclohexylamine in urine, nevertheless, was 2 μg per 20 ml (0.1 μg/ml) in urine because of the interference of peaks attributed to unknown substances.

Separation of cyclohexylamine and N-methylcyclohexylamine

Fig. 6 shows a gas chromatogram of the extract from the mixed solution of cyclohexylamine and N-methylcyclohexylamine. The separation of the two amines in *n*-hexane extracted from the aqueous amine solution was good enough for one to be determined in the presence of the other by the procedures described. In the urine samples, however, the determination of N-methylcyclohexylamine was not completely satisfactory, because a peak attributed to an unknown substance appeared close to that of N-methylcyclohexylamine.



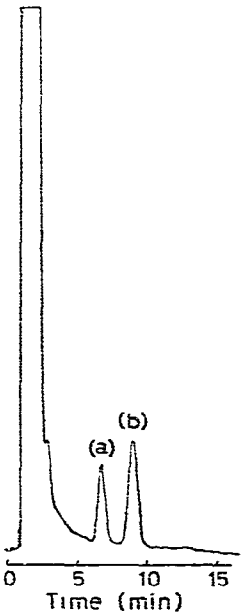


Fig. 5. Gas chromatogram of extract from rabbit urine containing cyclohexylamine. The retention time of cyclohexylamine is indicated by the arrow. (c) cyclohexylamine; (a), (b), (d) and (e) unknown substances (body components). Sample injection, 1 μl.

Fig. 6. Gas chromatographic separation of amines in an extract from a mixed solution. (a) Cyclohexylamine equivalent to 0.18 μg; (b) N-methylcyclohexylamine equivalent to 0.22 μg. Conditions as in Fig. 3.

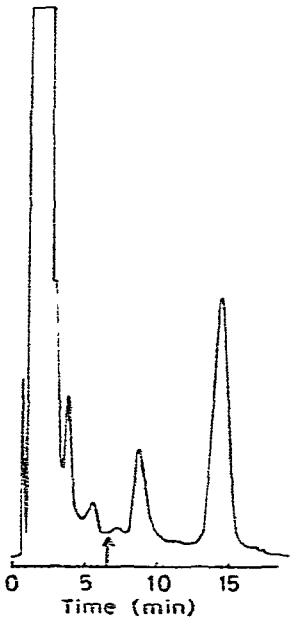
Detection of cyclohexylamine in urine

Doses of 10 mg/kg of bromhexine were administered intravenously to five rabbits and 20 mg/kg to three rabbits via the auricular vein. Urine was collected for 48 h after the injection and cyclohexylamine was examined according to the procedures described above. Cyclohexylamine was not detected in the urine from any of the rab-

bits. A typical gas chromatogram of the urine from rabbits administered 20 mg/kg of bromhexine is shown in Fig. 7. The retention time of cyclohexylamine was 6.7 min, but no peak was observed on the gas chromatogram at that time after sample injection. This was also verified by injection of samples to which cyclohexylamine had been added in amounts equivalent to the detection limit.

All of the eight rats also received 10 or 20 mg/kg of bromhexine intraperitoneally. No cyclohexylamine was detected in their urine.

The urine of the healthy men was examined before and after the intake of 36 mg/day of bromhexine and no cyclohexylamine was detected. A typical gas chromatogram of human urine taken 24 h after the intake of bromhexine is shown in Fig. 8. No peak of cyclohexylamine appeared at a retention time of 6.7 min.



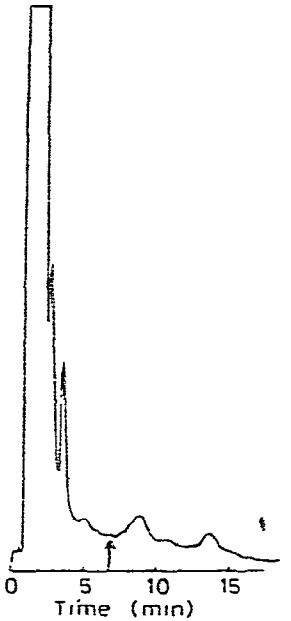


Fig. 7. Gas chromatogram of urine extract from a rabbit administered bromhexine. The retention time of cyclohexylamine is indicated by the arrow. Conditions as in Fig. 3.

Fig. 8. Gas chromatogram of a urine extract from a healthy man administered bromhexine. The retention time of cyclohexylamine is indicated by the arrow. Conditions as in Fig. 3.

The urine from the five patients taking bromhexine continuously was also analyzed, and no peak of cyclohexylamine was observed on the gas chromatogram (Fig. 9).

The N-methylcyclohexylamine peak appeared close to that of an unknown substance in urine, and the two peaks were not separated completely. However, we could not observe a peak originating from N-methylcyclohexylamine in the urine of any of the animals and humans treated with bromhexine.

An investigation on the extraction and detection of cyclohexanone and cyclohexanol in rabbit urine was also carried out, but neither of these compounds was detected under the experimental conditions used.

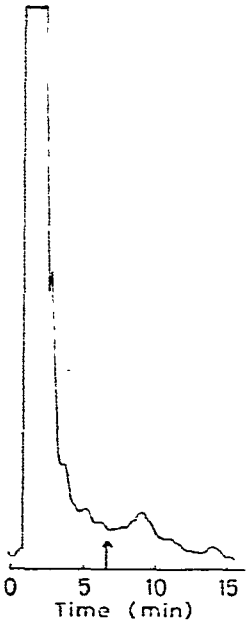


Fig. 9. Gas chromatogram of a urine extract from a patient treated continuously with bromhexine. The retention time of cyclohexylamine is indicated by the arrow. Conditions as in Fig. 3.

DISCUSSION

Kojima and Ichibagase[2] established a gas chromatographic method for detecting cyclohexylamine in the urine of animals administered sodium cyclamate. The separation of cyclohexylamine from N-methylcyclohexylamine by their method, however, was not good enough. Before the metabolic studies of bromhexine in animals, microquantitative gas chromatographic procedures for detecting amines were developed, including the procedures for extraction from biological materials used in this study. It became possible to determine cyclohexylamine in urine at levels of 100 ng/ml.

The excretion of bromhexine and its metabolites into urine after the oral application has been reported as follows. The excretion was most rapid in man, *viz.*, 88% of the administered drug was excreted by the kidneys within 96 h (ref. 5) and 70% within 24 h (unpublished work); 48% was excreted by the kidneys within 24 h and 65% within 48 h in rabbits[4], and 35% within 24 h in rats[6]. Based on these reports, urine was collected for 24 h in man and 48 h in rabbits in our experiments. The rat urine was collected for 24 h in this experiment to keep the level of the amine in urine high, instead of the absolute amount of the amine in urine, in order to avoid a reduction in the detection limits which might be affected by the unknown substance in urine.

A comparison of the metabolic patterns of bromhexine in the rat, mouse, rabbit, dog and man showed that the pattern observed in rabbits was closest to that in man[5]. Consequently, rabbits were mainly used in our work.

Schraven *et al.*[4] reported that it is possible to extract up to 95% of the ^{14}C in the metabolites from the urine of rabbits administered [^{14}C]bromhexine; 93% of this represented compounds that could not be accompanied by the production of cyclohexylamine, and the remaining 7% represented compounds that were not developed on a thin-layer chromatographic plate by either ethyl acetate or chloroform. These results suggest that if cyclohexylamine is produced from bromhexine in the body, it must be in extremely small amounts.

In this investigation, cyclohexylamine was not detected in the urine of humans, rabbits and rats administered bromhexine. For a rabbit weighing 2.5 kg and administered 20 mg/kg of bromhexine, the total amount of bromhexine administered is 50 mg. If it is assumed that half of it is metabolized and excreted into the urine within 48 h, then the amount of bromhexine excreted should be over 20 mg, even if the recovery rate is considered. On the other hand, the detection limit of cyclohexylamine was 2 μg per 20 ml (0.1 μg/ml) in urine, and the volume of urine collected from a rabbit in 48 h was 100–150 ml, so that the detection limit of the amine in urine is 10–15 μg from the total amount present. Consequently, it can be said that the amount of cyclohexylamine in the metabolites of bromhexine must be very small, if it exists at all, and that the ratio of the amount of cyclohexylamine produced in the metabolites of bromhexine to the total amount of bromhexine excreted is less than 1:1300. These calculations support the earlier experimental results[5], indicating that virtually all of the metabolites detected in the urine of both humans and rabbits after the application of bromhexine are not connected with the formation of cyclohexylamine or N-methylcyclohexylamine.

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