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Qualitative Analysis of Halothane Metabolites in Man

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Twenty three healthy volunteers received ^{14}C-labeled halothane intravenously. The radioactivity in their urine was characterized by the following techniques: liquid–liquid extraction, thin-layer chromatography, paper chromatography, column chromatography, inverse isotope dilution, lyophilization, and hydrolysis. The only radioactive metabolite that could be detected was trifluoroacetic acid, which appears in urine in a nonvolatile salt form. (Key words: Halothane; Metabolism; Biotransformation; Toxicity; Qualitative analysis.)

In a previous publication, we showed that volunteers excrete a nonvolatile radioactive substance in their urine after injection of ^{14}C-labeled halothane.[1] Stier[2,3] isolated trifluoroacetic acid from the urine of several animal species and man after administration of halothane.

The importance of the identification of the metabolites of halothane in man relates to their possible toxicologic significance. As previously postulated,[1] a breakdown product of halothane rather than the unchanged molecule may be the cause of hepatic damage in the susceptible population. There is growing evidence that "halothane hepatitis" is a sensitivity reaction[4–6] that may involve hapten formation which results in an allergenic response.

Two likely products of halothane biotransformation are trifluoroacetic acid and trifluoroethanol, the latter probably as a glucuronide.[7,8] This investigation was designed to search for these two compounds in the urine of man after administration of ^{14}C-halothane.

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Method

Each of 23 healthy male volunteers received an intravenous injection of 25 or 50 μCi ^{14}C-halothane (labeled on the fluorinated carbon), as previously described.[1]

Total urine was collected for five days from all volunteers, and for as long as 21 days from some. Each voided specimen was frozen as soon as possible and kept at 40 C until analysis.

Liquid–Liquid Extraction

About 1,500 ml of iced urine was acidified to $pH < 1$ by addition of concentrated sulfuric acid, placed in a liquid–liquid extraction apparatus (Ace Glass No. 6440), and extracted for 24 to 48 hours with diethyl ether. The ether was separated from the water phase and the radioactivity quantitatively back-extracted into 2 ml of concentrated ammonia hydroxide.

Thin-Layer Chromatography

Extracts prepared as described above were spotted on silica gel-G plates 500 μ thick and developed in a system of ammonia–isopropyl alcohol (1:4). The developed plates were dried and scraped in ½-cm sections and radioassayed by liquid scintillation counting.

Paper Chromatography

Extracts and untreated urine concentrates were spotted on 46 × 57-cm Whatman #1 paper and developed downwards in a chamber using the solvent system described above. The developed papers were sprayed with bromcresol green (0.3 per cent in methanol–water, 8:2, and 8 drops 30 per cent sodium hydroxide) for the visualization of acidic compounds. Unsprayed chromatograms were cut in strips and 1-cm sections placed in liquid scintillation vials for radioassay.

Column Chromatography

Glass columns (1 × 30 cm) of Dowex-1-formate were packed and back-washed until even distribution of the resin had been

achieved. Urine extract, 1–2 ml, as described above, was placed on the column. Elution was performed with a continuous gradient of ammonium formate (0 to 2 N). Collection was either by a fraction collection apparatus or by changing collection vessels every 100 ml.

Inverse Isotope Dilution

Urine, 100 ml, was refluxed with 10 ml of concentrated sulfuric acid for an hour. The *p*H was then adjusted to about 9 with 10 per cent sodium hydroxide. Three milliliters of unlabeled trifluoroethanol § were added and the mixture distilled at 78 to 83 C. A sample of the trifluoroethanol distillate was radioassayed by liquid scintillation counting. The N-alpha-naphthylcarbamate ester of trifluoroethanol (mp 79 to 80 C) was prepared by heating alpha-naphthylisocyanate with the distilled trifluoroethanol in the presence of pyridine and recrystallizing from hot ligroin to constant specific activity.[8]

The benzyl-thiouronium salt of trifluoroacetic acid (mp 179 to 180 C) was prepared by adding a saturated solution of 2-benzyl-2-thiopseudourea hydrochloride to urine samples after the addition of carrier trifluoroacetic acid.¶ Upon standing, a white crystalline material separated; this was filtered, decolorized with charcoal, and recrystallized from hot water.

The derivatives were identified by elemental analysis and infrared spectra and were radioassayed by liquid scintillation counting.

Lyophilization

As soon as it had been obtained from the volunteer, 1 ml of untreated urine was shell-frozen in a liquid scintillation fluid. A second 1-ml sample was added to a vial containing scintillation fluid. The shell-frozen vial was lyophilized to dryness, the lyophilizate dissolved in 1 ml of water, and liquid scintillation fluid added to this system. Both vials were assayed by liquid scintillation counting with an internal standard for the calculation of efficiency.

Acid Hydrolysis

Four milliliters of urine were placed in glass ampules and frozen at 60 C. Three drops of concentrated hydrochloric acid were added. The ampules were fire-sealed and boiled overnight in a water bath, cooled, and then frozen. The tips of the ampules were opened and three drops of sodium hydroxide added to half of the samples. The ampules were resealed and all of them brought to room temperature. Then they were chilled to about 0 C and 1-ml samples were assayed directly and also after lyophilization by liquid scintillation counting.

Incubation with Glucuronidase

Four milliliters of urine were mixed with 10 ml of Ketodase ** and 10 ml of acetate buffer, *p*H 5. This mixture was incubated at 37 C for 24 hours in an open flask, lyophilized, and radioassayed before and after incubation by liquid scintillation.

Results

Liquid–Liquid Extraction

When daily urine samples were acidified and extracted by this procedure, 85 to 90 per cent of the radioactivity partitioned into the ether phase. Without acidification of urine, no radioactivity was extractable. The ether-soluble radioactivity was quantitatively back-extracted into concentrated ammonium hydroxide. There was no observable difference among volunteers or days after administration of ^{14}C-halothane.

Thin-Layer and Paper Chromatography

Chromatograms of ether-derived extracts of urine revealed a single radioactivity spot which had the same mobility as authentic sodium trifluoroacetate ($R_f = 0.77$). This area of the chromatogram gave an acid reaction with bromcresol green. Unextracted but concentrated urine samples produced similar chromatograms.

Inverse Isotope Dilution

Analysis for trifluoroethanol and trifluoroacetate was performed on 0–24-hour urine

§ Kindly supplied by Halocarbon Laboratory, Hackensack, New Jersey.

¶ Catalog No. 19328, K and K Laboratories, Plainview, New York.

** Brand of beef liver beta-glucuronidase, Warner-Chilcott, Morris Plains, New York.

specimens from two volunteers. There was no detectable radioactivity in the trifluoroethanol derivative; however, the trifluoroacetate derivative contained at least 90 per cent of that in the urine.

Lyophilization and Acid Hydrolysis

No significant radioactivity was lost from neutral or alkaline urine samples during lyophilization. However, after acidification virtually all of the radioactivity was removed by lyophilization.

Glucuronidase Treatment

None of the urinary radioactivity was rendered volatile during incubation with this enzyme.

Discussion and Conclusions

Trifluoroacetic acid was the only detectable radioactive metabolite of halothane excreted in the urine of human volunteers using five analytical criteria. The urinary radioactivity was ether-extractable only at low *p*H, which would be expected for an organic acid. The 85–90 per cent extractability is not surprising for the method employed. The fact that the ether-soluble radioactivity was completely partitioned into ammonium hydroxide is further evidence of the presence of an organic acid. Chromatographic similarity to the authentic compound and the isolation of a radioactive derivative of this acid provide confirming evidence that trifluoroacetate is the urinary metabolite of halothane in man. The volatility of this acid under acidic conditions was also demonstrated.

Trifluoroethanol or its glucuronide conjugate was not present in these urine samples, as indicated by the absence of radioactivity in the derivative and the finding that no volatile radioactivity was produced by treatment with glucuronidase. Moreover, no evidence of other radioactive metabolites was found. Only one radioactive spot was detected on chromatograms by a procedure sufficiently sensitive to detect any metabolite that represented 2 per cent of the urinary radioactivity.

Cohen and Trudell [9] have found evidence of "macrometabolites" of ^{14}C-halothane in mice and squirrel monkeys. Using their elegant methods, they have also identified trifluoroacetyl-ethanolamine as a secondary metabolite in the same human urine samples described in this report.[10] They confirmed that trifluoroacetate was the major metabolite. It is possible that the ethanolamine conjugate is not ether-extractable and thus did not appear on our chromatograms.

Thus, it would appear that trifluoroacetate is the major end-product of halothane biotransformation in man. While not demonstrable with the procedures described, it is possible that other metabolites may exist in trace quantities. Considering the intrinsic chemical reactivity of trifluoroacetic acid, it is a likely candidate for binding covalently to hepatic macromolecules and thus becoming a hapten antigen. Our previous toxicologic studies with this acid in animals [11] indicated that while it is nontoxic when administered directly, its formation appears to be at least partially responsible for the toxicity of trifluoroethanol in animals.

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