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Note

Determination of saccharin in urine by electron-capture gas chromatography after extractive methylation

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The use of the sweetening agent saccharin (2,3-dihydro-3-oxobenzisosulphonazole) in food and cosmetics has increased during the last decade. Although only small amounts are added, questions have recently been raised about its safety[1,2].

The determination of trace amounts of saccharin in urine or blood samples requires selective and sensitive methods. Saccharin has been determined by gas chromatography with flame-ionization detection after methylation with diazomethane[3–7], methyl iodide[7,8], dimethyl sulphate[9] or dimethylformamide dimethylacetal[10] or after silylation[7,11]. Saccharin can also be detected with an electron-capture detector[8,10] after methylation.

Extractive alkylation is a flexible and convenient means of derivatizing organic acids before gas chromatographic analysis[12–15]. Recently, the direct determination of clioquinol in plasma and urine samples after extractive methylation was demonstrated[16].

This paper presents a sensitive and selective method for the determination of saccharin in urine by electron-capture gas chromatography after extractive methylation. The method consists in extraction of saccharin as an ion pair with tetrabutylammonium with methylene chloride containing methyl iodide. Excess of methyl iodide and methylene chloride are removed by evaporation and tetrabutylammonium iodide by extraction with silver sulphate solution.

EXPERIMENTAL

Apparatus

Gas chromatography. A Varian 1400 gas chromatograph with a tritium electron-capture detector operated in the d.c. mode was used. The glass column (150 × 0.2 cm I.D.) was filled with 3% OV-17 on Gas-Chrom Q (80–100 mesh) and operated at 180°. The injector and detector temperatures were 250° and 210°, respectively. The flow-rate of the carrier gas (nitrogen) was 30 ml/min.

Mass spectrometry. N-Methyl- and N-propylsaccharin were identified with an LKB 9000 mass spectrometer. The ionization energy was 70 eV.

Spectrophotometry. The photometric measurements were performed with a Zeiss PMQ II Spectralphotometer.

Reagents and chemicals

N-Methyl and N-propylsaccharin (internal standard) were synthesized from sodium saccharin and methyl or propyl iodide (Merck, Darmstadt, G.F.R.) in dimethyl sulphoxide[8]. Tetrabutylammonium hydrogen sulphate (AB Hässle, Mölndal, Sweden) was neutralized with sodium hydroxide and the solution was diluted to 0.5 *M* with phosphate buffer of pH 7.4 ($\mu = 0.5$). Ethyl acetate (Kebo AB, Solna, Sweden) was distilled before use.

Methods

Determination of extraction constant. The partition experiments were performed with equal phase volumes of methylene chloride and aqueous phosphate buffer of pH 11 ($\mu = 0.1$) using an equilibration time of 30 min in a thermostated waterbath ($25 \pm 0.1°$). The concentration of tetrabutylammonium in the aqueous phase was $5–50 \cdot 10^{-4}$ *M* and that of saccharin $5.5–55 \cdot 10^{-4}$ *M*. After separation of the phases, the concentration of saccharin as the anion was determined photometrically in the aqueous phase. The extraction constant was calculated as described elsewhere[17].

Determination of saccharin in urine. The urine sample (4.0 ml) is mixed with 1.0 ml of tetrabutylammonium (0.5 *M*) in buffer of pH 7.4. The solution is shaken for 1 h with a mixture of 0.2 ml of methyl iodide and 0.3 ml of methylene chloride containing N-propylsaccharin (10 μg/ml). An aliquot (10 μl) of the organic phase is transferred into a tube containing 100 μl of ethyl acetate. After evaporation to dryness by a stream of nitrogen, 1.0 ml of ethyl acetate is added and the solution is shaken with saturated silver sulphate solution for 10 min. A 1–4-μl portion of the organic phase is injected into the gas chromatograph.

RESULTS AND DISCUSSION

Extraction of saccharin

Saccharin can be extracted from acidic aqueous solutions into diethyl ether[8] or ethyl acetate[4,6]. The extracts were shown to contain many of the acids normally present in urine[4] and a purification step has to be included before quantitation by gas chromatography with flame-ionization detection.

Saccharin can also be extracted in anionic form as an ion pair with quaternary ammonium ions[18]. The extraction constant of saccharin with tetrabutylammonium as the counter ion was found to be $10^{3.1}$. Saccharin can thus be quantitatively extracted into methylene chloride from a 0.1 *M* solution of tetrabutylammonium using equal phase volumes[19].

Reaction conditions

The high electron-capture response of saccharin after methylation has been demonstrated previously[8,10]. In the extractive alkylation procedure, methyl iodide was used as alkylating reagent, as it has shown to react rapidly with sulphonamides[20]. The time course of the reaction with two different concentrations of methyl iodide is shown in Fig. 1. As the rate of reaction of saccharin is slow compared with most other sulphonamides[20] a high concentration of methyl iodide was used.

A low pH in extractive alkylation procedures helps to prevent hydrolysis of the alkylating reagent[21] and the derivative formed. The acid dissociation constant of

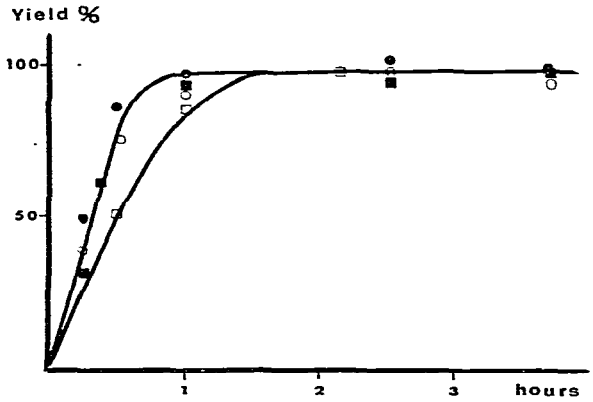

Fig. 1. Time course of the methylation of saccharin. Organic phase (1.0 ml): methylene chloride. Aqueous phase (5.0 ml): 0.1 *M* tetrabutylammonium in buffer ($\mu = 0.1$). ●, pH 7.4, 6 *M* methyl iodide; ○, pH 7.4, 6 *M* methyl iodide, urine present; ■, pH 12, 6 *M* methyl iodide, urine present; □, pH 12, 3 *M* methyl iodide present. Urine constituted 80% of the organic phase.

saccharin ($pK_a = 2.5$) created possibilities for mild conditions in the derivatization step. The rate of methylation of saccharin was the same at pH 7 and 12, as can be seen from Fig. 1. The influence of urine was insignificant. The methyl derivative of saccharin was stable for at least 6 h under the reaction conditions used.

Identity of formed derivatives

Methylation of saccharin with diazomethane has been shown to give a mixture of N- and O-methylsaccharin[6,8,22]. A small amount of 2-methoxycarbonylbenzenesulphonamide, formed through ring fission, has also been found in the reaction mixture[22]. On the other hand, methylation with methyl iodide in dimethyl sulphoxide gave only the N-methyl derivative[8].

Extractive methylation of saccharin gave the N-methyl derivative. The mass spectrum was in good accordance with that obtained by Couch *et al.*[4]. Fragments from an O-methyl derivative could not be detected in the mass spectrum.

Electron-capture response

In a separate study, the sulphonamide moiety was shown to have inherent electrophoretic properties[23]. The minimum detectable concentrations[24] of N-methyl- and N-propylsaccharin were 2.5×10^{-16} and 2.8×10^{-16} mole/sec, respectively. These levels correspond to about 2 pg of derivative injected on a column with 1600 theoretical plates and with a retention of 5 min.

Purification of the reaction mixture

Injection of the organic phase from the methylation reaction directly into the gas chromatograph was not possible as methyl iodide and methylene chloride seriously affected the electron-capture detector. Evaporation was used to remove methyl iodide and methylene chloride.

Tetrabutylammonium iodide is formed as a side-product in the reaction and its degradation products formed in the injector caused long tailing fronts in the chro-

matogram. It was easily removed by extraction with aqueous silver sulphate solution[25].

N-methylsaccharin is quantitatively retained in the organic phase.

Determination of saccharin in urine

The present method was used in the determination of saccharin in urine. Investigations of the fate of saccharin in different animals have been undertaken[6,26,27]. Only small fractions are metabolized, the main fraction being the hydrolysis product 2-sulphamoylbenzoic acid[27]. Its methylation product does not have the same retention as N-methylsaccharin and its electron-capture response is low.

N-Propylsaccharin added directly to methylene chloride was found to be satisfactory as an internal standard as no structurally related compounds could be found that reacted similarily (*e.g.*, phthalimide).

The absolute recovery and precision of the method was 97 $\pm$ 6% ($n = 6$) at the 0.5 μg/ml level as compared to a dilution of crystalline N-methylsaccharin.

A chromatogram from an analysis of 1.2 μg/ml of saccharin in urine is shown in Fig. 2. Saccharin has been detected at concentrations down to 10 ng/ml of urine with this method.

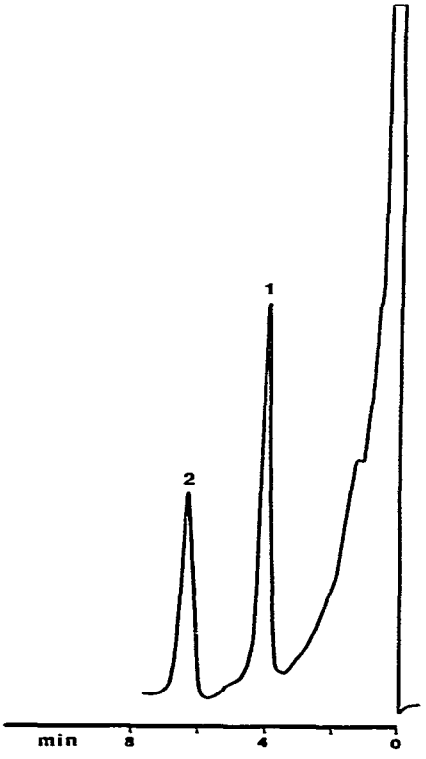

Fig. 2. Gas chromatogram from an analysis of 1.2 μg/ml of saccharin in urine. 1, Saccharin as N-methyl derivative (95 pg injected); 2, internal standard (N-propylsaccharin, 60 pg injecteed).

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