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Note

Gas chromatographic determination of thiazine and thiazepine derivatives of biological interest

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The oxidative deamination of some diamino sulphur-containing amino acids produces the corresponding ketoacids which cyclize into unsaturated products called ketimine derivatives[1–4]. It has been shown that reduction with $NaBH_4$ of the L-thialysine, L-lanthionine and L-cystathionine ketimines produces thiomorpholine-2-carboxylic acid (TMC), thiomorpholine-2,6-dicarboxylic acid (TMDC) and perhydro-1,4-thiazepine-3,5-dicarboxylic acid respectively (HTZDC). The synthesis and some chemical properties of the cyclized ketimines and their reduction products have recently been described[5,6]. It has been also demonstrated[5] that oxidation with H_2O_2 of the cystathionine ketimine produces a cyclic compound with a lactamic bond, characterized as perhydro-3-oxo-1,4-thiazepine-5-carboxylic acid (oxo-HTZC).

It is likely that oxidation by H_2O_2 of the thialysine and lanthionine ketimines produces six-membered analogues of oxo-HTZC, which are 2-oxothiomorpholine (oxo-TM) and 2-oxothiomorpholine-6-carboxylic acid (oxo-TMC).

Although the biochemical importance of these compounds is not yet established, the presence of HTZDC and of the open form of oxo-HTZC has been reported in the urine of a cystathioninuric patient, as well as in the urine of rats with experimental cystathioninuria[7,8]. This suggests the occurrence of an unexpected additional metabolic path of cystathionine in mammals worthy of investigation. In this paper we describe a gas chromatographic (GC) procedure useful for the detection and quantitation of the products of reduction and of oxidation of cystathionine ketimine and of related ketimines.

MATERIALS AND METHODS

Chemicals

L-Thialysine ketimine (5,6-dihydro-2H-1,4-thiazine-3-carboxylic acid), L-lanthionine ketimine (5,6-dihydro-2H-1,4-thiazine-3,5-dicarboxylic acid), L-cystathionine ketimine (2,5,6,7-tetrahydro-1,4-thiazepine-3,5-dicarboxylic acid), thiomorpholine-2-carboxylic acid (TMC), thiomorpholine-2,6-dicarboxylic acid (TMDC), perhydro-1,4-thiazepine-3,5-dicarboxylic acid (HTZDC) and perhydro-3-oxo-1,4-thiazepine-5-carboxylic acid (oxo-HTZC) were prepared as previously described[1–6].

2-Oxothiomorpholine (oxo-TM) was prepared as follows. Cysteamine hydro-

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chloride (10 mmol, 1.14 g) was dissolved in 50 ml of water. Under a nitrogen stream, 10 mmol of iodoacetamide were added in portions, adjusting the pH to 8 with 1 *M* Na_2CO_3. The alkylation is complete in 10–15 min when the nitroprusside test for –SH groups is negative. The solution was then heated at 100°C for 2 h in sealed tubes. After cooling, the pH of the solution was lowered to 3.25 with 3 *M* HCl and the volume reduced to 5 ml in a rotary evaporator at 40°C. The precipitated NaCl was removed by centrifugation and the supernatant applied on a column (2 × 10 cm) of Dowex 50 (200–400 mesh) in hydrogen form. The compound was eluted with water, localized with iodoplatinate reagent[9] between 60 and 130 ml of effluent and dried under reduced pressure at 40°C. White crystals were obtained (80% of the theoretical yield) which melt at 85–87°C. The synthetic product does not react with ninhydrin. Thin-layer chromatography (TLC) on cellulose gave a single iodoplatinate-positive spot with R_F = 0.8 (butanol–acetic acid–water, 4:1:1). After hydrolysis in 2 *M* HCl at 100°C for 15 min, oxo-TM was quantitatively converted into S-carboxymethyl-cysteamine (CMCA) as demonstrated by co-chromatography in the amino acid analyzer with an authentic sample of CMCA prepared as described in ref. 10.

2-Oxothiomorpholine-6-carboxylic acid (oxo-TMC) was prepared by heating for 2 h at 100°C a solution of S-(carboxamidomethyl)cysteine prepared by alkylation of cysteine with iodoacetamide under the same conditions as described above for the alkylation of cysteamine. After cooling, the solution was acidified to pH 3.25 with 4 *M* HCl and evaporated to dryness under reduced pressure at 40°C. The dry residue was extracted three times with hot ethanol which upon cooling to room temperature gave the compound in 95% yield. After two recrystallizations from ethanol the compound melts at 206–208°C. The synthetic product does not react with ninhydrin and when subjected to cellulose TLC gives a single iodoplatinate-positive spot with R_F = 0.6 (butanol–acetic acid–water, 4:1:1). After hydrolysis in 2 *M* HCl at 100°C for 15 min, oxo-TMC was quantitatively converted into S-(carboxymethyl)cysteine (CMC) as demonstrated by co-chromatography in the amino acid analyzer with an authentic sample of CMC (Sigma).

All other compounds used were of analytical reagent grade.

Experimental procedures

Automatic amino acid analyses were performed on a Carlo Erba Model 3A29 amino acid analyzer. Melting points were determined by a Kofler block and are uncorrected.

Gas chromatographic analyses were carried out on a Perkin-Elmer 990 gas chromatograph fitted with a flame ionization detector. The methyl esters of ketimines and their reduction derivatives were separated on a glass column (120 cm × 2 mm I.D.) packed with 10% OV-17 on Chromosorb W HP DMCS, 100–120 mesh. Temperatures: column, 150°C; injector 200°C; detector, 220°C. Flow-rates: 25 ml/min for nitrogen carrier gas; 20 ml/min for hydrogen and 300 ml/min for air. The methyl esters of oxidized ketimines were separated on a glass column (200 cm × 2 mm I.D.) packed with 3% diethylene glycol succinate on Chromosorb W AW DMCS, 80–100 mesh. The column temperature was 210°C for 12 min, then programmed to 215°C at a rate of 0.5°C/min. The injector and detector temperatures were 220°C. Flow-rates: 20 ml/min for nitrogen carrier gas; 20 ml/min for hydrogen and 300 ml/min for air. Gas chromatographic–mass spectrometric (GC–MS) analyses were carried

out on a GCD Pye Unicam gas chromatograph connected to a low resolution mass spectrometer LKB 2091 fitted with a digital PDP 11 computer. Chromatographic separation was performed on a 25 m × 0.2 mm I.D. fused-silica capillary column with OV-101 as stationary phase. The flow-rate of helium carrier gas was 0.8 ml/min, and the column temperature was programmed from 100 to 250°C at a rate of 4°C/min. The injector temperature was 250°C and that of the molecular separator was 260°C. Mass spectrometer experimental conditions: ionization mode, E.I.; electron energy, 70 eV; ion source temperature, 250°C; ion source vacuum, $0.5 \cdot 10^{-6}$ mmHg.

RESULTS AND DISCUSSION

All the ketimines as well as the reduced and oxidized derivatives were analyzed by GC. The derivatization method was the same for all these compounds: they were solubilized in methanol and then treated with ethereal diazomethane. For the ketimines and their reduction products the best separations were obtained on a OV-17 column, while for the oxidized products on a DEGS one.

Fig. 1 illustrates the chromatographic separation of ketimines and their reduction products. It is notable that the reduction products of the three ketimines always exhibited two peaks. This unexpected behaviour was clarified by mass spec-

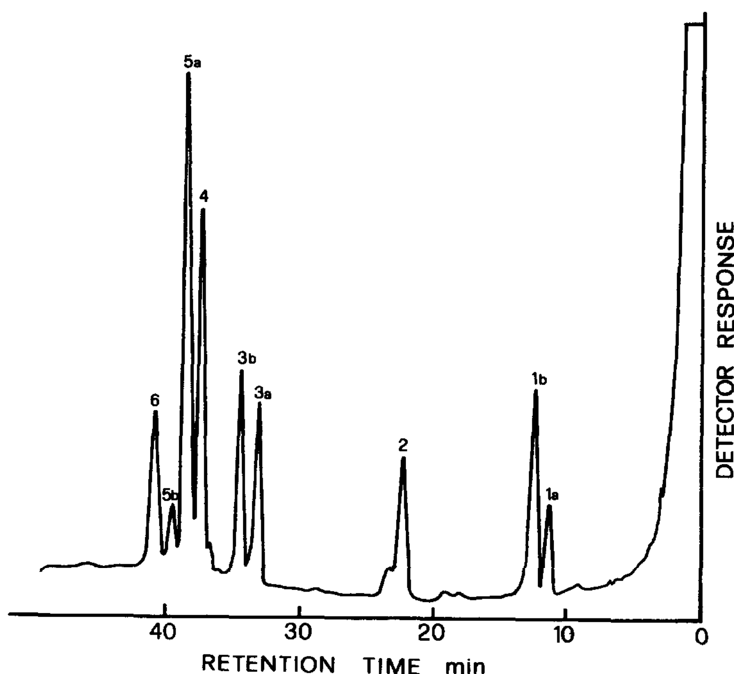

Fig. 1. Gas chromatographic separation of the methyl derivatives of the ketimines and their reduction products. For analysis conditions see text. Peaks: 1a and 1b = mono- and dimethyl TMC; 2 = L-thialysine ketimine; 3a and 3b = di- and trimethyl TMDC; 4 = L-lanthionine ketimine; 5a and 5b = di- and trimethyl HTZDC; 6 = L-cystathionine ketimine.

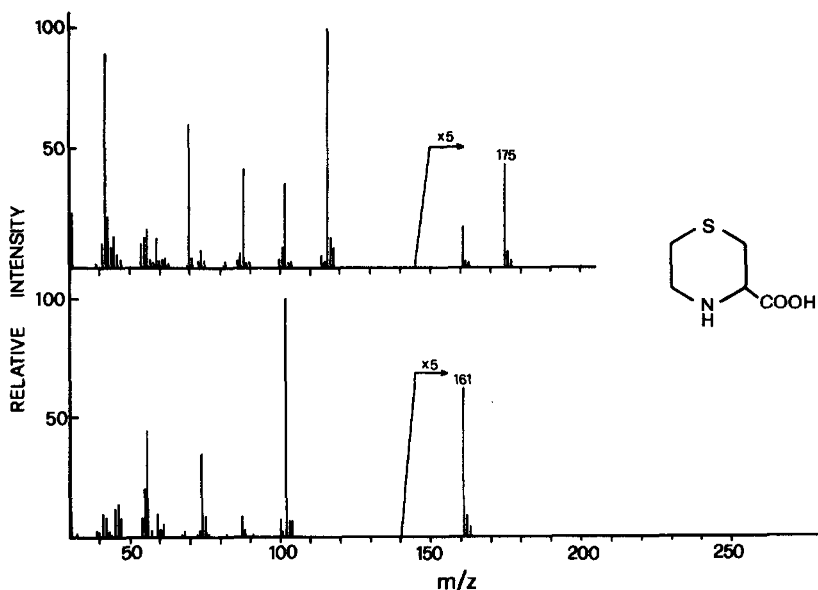

Fig. 2. Structured formula of TMC and mass spectra of its mono- and dimethyl derivatives. Fragments with relative intensity (R.I.) > 1% are reported.

trometric analysis. The mass spectra of each peak showed the occurrence of two derivatives of the same molecule methylated in various degrees. TMC has two functional groups that can be methylated. The mass spectra of each peak showed that a

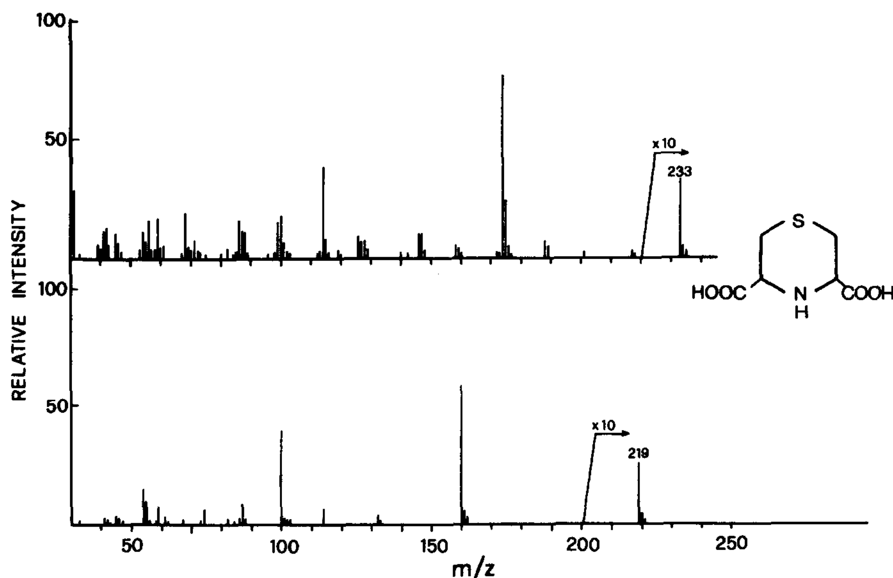

Fig. 3. Structured formula of TMDC and mass spectra of its di- and trimethyl derivatives. Fragments with R.I. > 1% are reported.

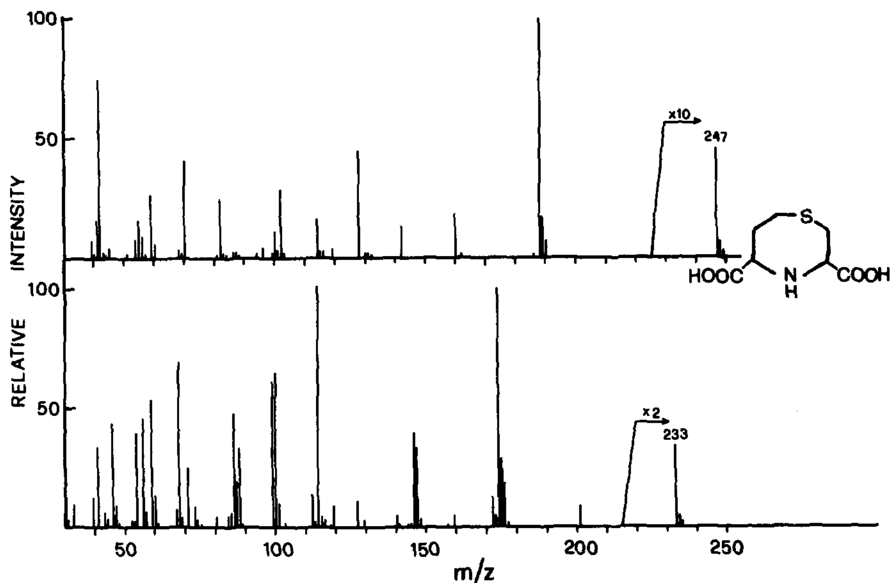

Fig. 4. Structural formula of HTZDC and mass spectra of its di- and trimethyl derivatives. Fragments with R.I. > 1% are reported.

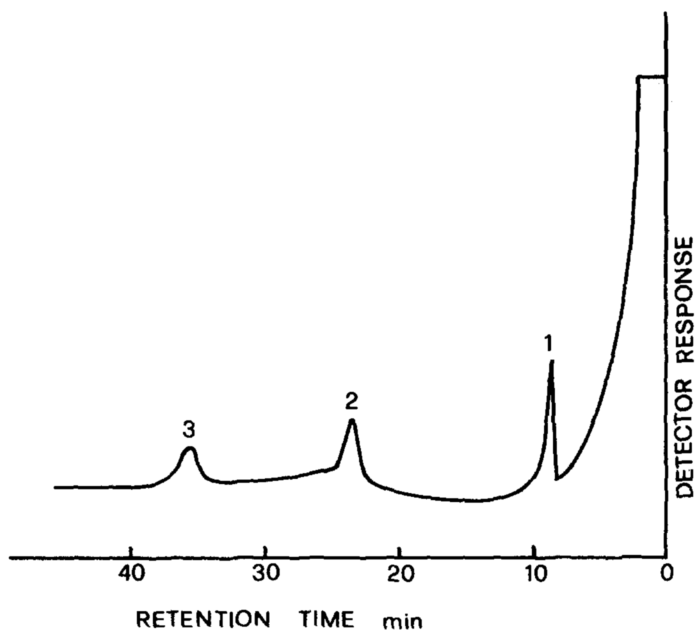

Fig. 5. Gas chromatographic separation of the methyl derivatives of the ketimine oxidation products. For analysis conditions see text. Peaks: 1 = oxo-TM; 2 = oxo-TMC; 3 = oxo-HTZC.

fraction of the compound is monomethylated, while the remainder is dimethylated (Fig. 2). Similarly, TMDC and HTZDC have three functions that can be methylated. The mass spectra of the two peaks of each compound revealed di- and trimethylated derivatives (Figs. 3, 4). GC analyses performed at different reaction times always gave the same results.

Fig. 5 shows the chromatographic separation of a mixture of the oxidation products of the three ketimines. Unlike the reduction products, these compounds show a single peak.

The sensitivity of the method was not the same for all the compounds: 10 ng for ketimines and 5 and 20 ng respectively for reduced and oxidized derivatives. The results are highly reproducible. The calibration graphs were linear in the experimental range (0.01–10 μg of each compound). For the reduced compounds which form two methyl derivatives, quantitation was performed by summing the areas of the two peaks.

Work is in progress to apply this procedure to the detection of cystathionine cyclic derivatives in the brain where the high content of cystathionine[11,12] suggests also the possible occurrence of its metabolic derivatives.

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