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Note

Artifacts in the gas chromatographic analysis of some sulfur-containing amino acids

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A screening program for the detection of inborn errors of metabolism is carried out routinely in this laboratory. To supplement this program, a method to quantify urinary and serum amino acids by gas chromatography (GC) utilizing N-trifluoroacetyl-*n*-butyl (TAB) esters is being developed. Of particular interest is the quantification of sulfur-containing amino acids commonly excreted in excess amounts in disorders such as cystinuria and homocystinuria. In the course of this investigation it has been determined that derivatization of the disulfide amino acids (cystine and homocystine) results in the artifactual formation of a new species, a mixed disulfide, which elutes between the two parent compounds on the chromatogram. This observation has initiated this study to determine the mode of formation of the mixed disulfide and any effect on quantitative analysis.

EXPERIMENTAL

All amino acids used in this study were obtained from Sigma (St. Louis, Mo., U.S.A.) and were chromatographically pure. 3.0 *N* HCl in *n*-butanol and TAB derivatives of cysteine (0.05 *M*) and cystine (0.05 *M*) were obtained from Regis (Morton Grove, Ill., U.S.A.) and trifluoroacetic anhydride from Pierce (Rockford, Ill., U.S.A.).

Preparation of derivatives

The "direct esterification–acylation" procedure (micro-method) as described by Zumwalt *et al.*[1,2] was used for conversion of the amino acids to their TAB esters. Tranexamic acid (*trans*-4-aminomethylcyclohexanecarboxylic acid) was added as an internal standard and was well resolved from the surrounding amino acid peaks. Mixing during derivatization was enhanced by sonication.

Gas chromatography

The analytical separations were carried out with a Tracor Model 550 dual-column gas chromatograph. The column was a 1.8 m (6 ft.) glass coil (4 mm I.D.) packed with 1% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). Operating conditions included: injector zone, 250°; detector bath, 300°; nitrogen flow-rate, 30 ml/min; inlet pressures: nitrogen, 40 p.s.i.;

hydrogen, 25 p.s.i. and air, 40 p.s.i. The column oven temperature was programmed from 80° at 2°/min for the amino acid mixtures and at 4°/min for the standards. Quantitative data were obtained with an Infotronics Model CRS-204 automatic digital integrator.

Mass spectrometry

Mass spectra were recorded on an LKB-9000-S combination GC–mass spectrometry (MS) instrument with a 3.7 m (12 ft.) coiled glass column (4 mm I.D.) packed with 1% OV-17 on 80–100 mesh Gas-Chrom Q (Applied Science Labs.) as the inlet. The ionizing and accelerating potentials were 70 eV and 3.5 kV, respectively, the trap current was 60 μA, the source temperature was 270°, and the column oven temperature was programmed at either 2° or 4°/min from 80°. All identified components were confirmed by GC–MS.

RESULTS

The chromatogram in Fig. 1 shows a mixture of the TAB derivatives of seven sulfur-containing amino acids with tranexamic acid included as the internal standard. The cysteine–homocysteine mixed disulfide elutes between the corresponding symmetrical disulfides, as would be expected. To determine the relative amounts of the mixed disulfide formed, a mixture containing equal amounts of cystine and homocystine was derivatized and chromatographed. The resulting data revealed the

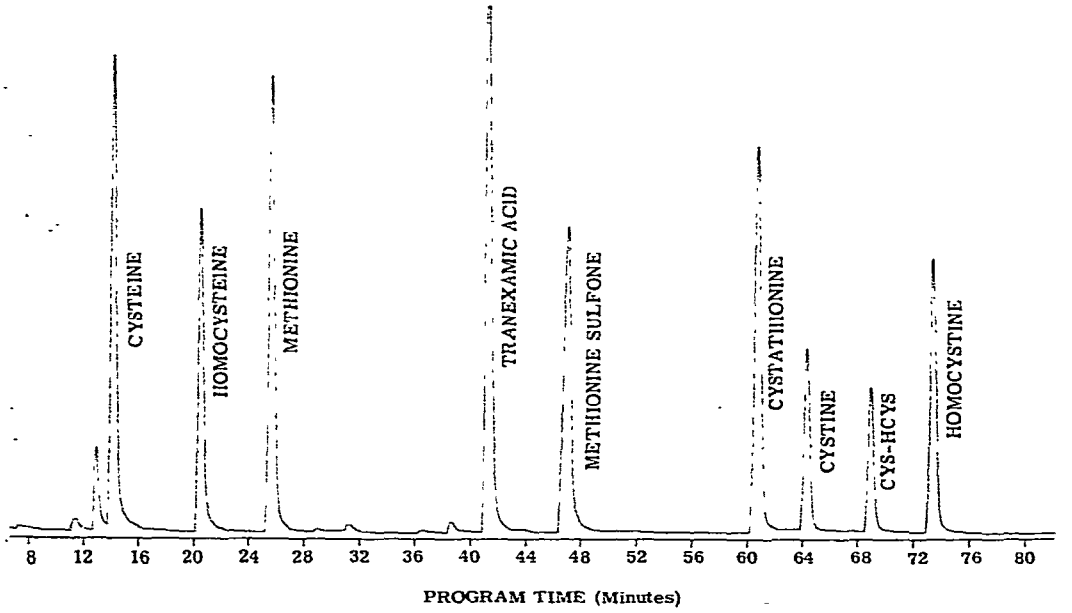

Fig. 1. GC separation of seven sulfur-containing amino acids as the TAB derivatives. Tranexamic acid is the internal standard. CYS-HCYS = mixed disulfide. Conditions: column, 1.8 m × 4 mm I.D., packed with 1% OV-17 on 100–120 mesh Gas-Chrom Q; injector temperature, 250°; detector temperature, 300°, nitrogen flow-rate, 30 ml/min; temperature programming, 2°/min from 80°.

relative proportion of each component: cystine, 36.6%; cysteine–homocysteine disulfide, 11.4%; homocystine, 52.0% (assuming no response factors). Derivatization with and without a nitrogen atmosphere revealed no significant differences in the values.

Empirically, formation of the artifact resulted from the process of derivatization or as a result of an "on-column" reaction. To determine its origin, several experiments with various combinations of standards were carried out. Initially, cystine and homocystine were derivatized independently yielding their respective TAB esters, which were co-injected into the chromatograph. The formation of less than 1% of the mixed disulfide (assuming no response factors) revealed that the conversion reaction did not occur to any appreciable extent on the column but occurred during derivatization.

It was noted that cystine derivatized along with tranexamic acid yielded 95.6% TAB-cystine plus the remaining 4.4% as TAB-cysteine. The possibility of on-column formation was again ruled out by chromatographing standard TAB-cystine (Regis) with and without the internal standard. The lack of cysteine formation supported our original conclusion.

Likewise, cysteine derivatized with the internal standard yielded 96.0% TAB-cysteine and 4% TAB-cystine. Standard TAB-cysteine (Regis) was chromatographed and revealed no cystine formation on the GC column.

Derivatization of equal amounts of cysteine and homocystine resulted in the formation of cysteine (23.0%), cystine (14.3%), cysteine–homocysteine disulfide (22.4%) and homocystine (40.3%). Also, preparation of derivatives by transesterification through the methyl esters as opposed to direct formation of butyl esters (mixing enhanced by sonication) revealed no appreciable change in the yields.

Similar studies with homocysteine were not carried out because only the thiolactone was available, although the TAB derivative of homocysteine was formed by derivatization of the thiolactone.

Figs. 2–5 show the mass spectra of the derivatized amino acids. All spectra contain significant molecular ions (*m/e* 369, 544, 558, 572) and are easily identifiable

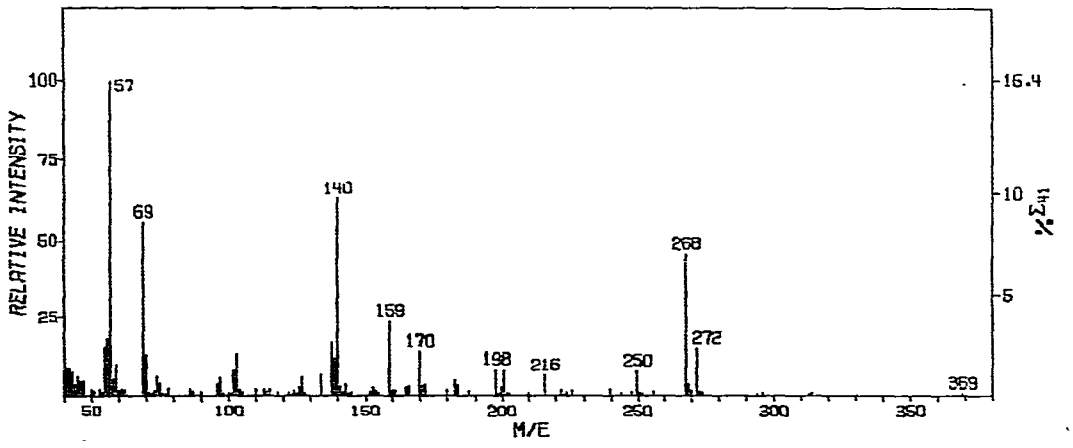

Fig. 2. Mass spectrum of TAB-cysteine.

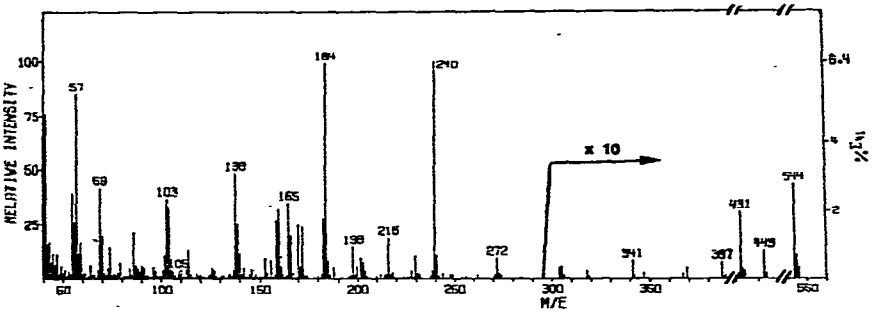

Fig. 3. Mass spectrum of TAB-cystine.

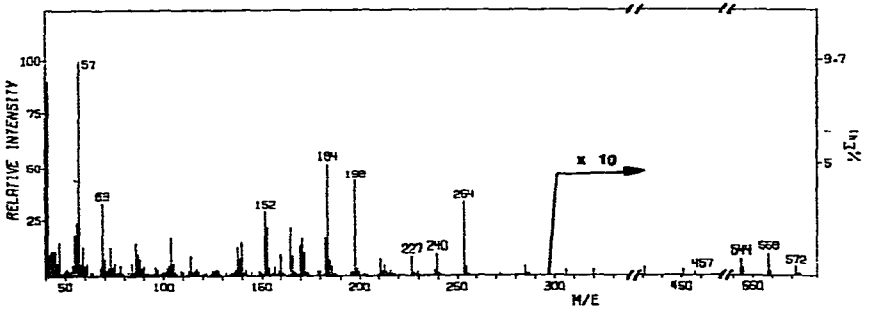

Fig. 4. Mass spectrum of TAB-cysteine–homocysteine mixed disulfide.

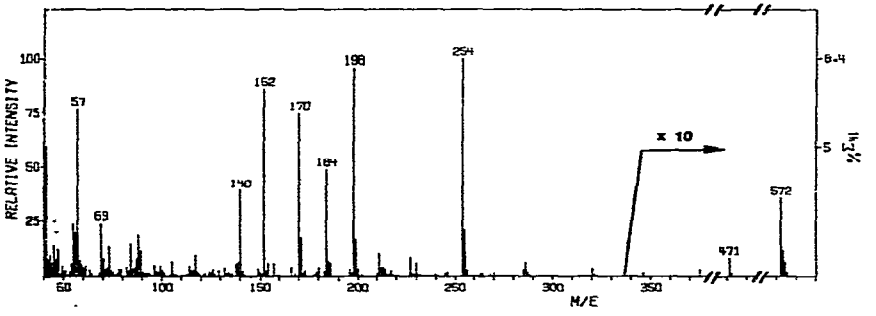

Fig. 5. Mass spectrum of TAB-homocystine.

from spectra of other normally encountered amino acids. Partial fragmentation pathways for the molecules are also indicated in Fig. 6.

DISCUSSION

The general reaction for this phenomenon can be described as follows:

$$RS\text{–}SR + R'S\text{–}SR' \rightarrow TAB\text{–}RS\text{–}SR + TAB\text{–}RS\text{–}SR' + TAB\text{–}R'S\text{–}SR'$$

Partial cleavage of the amino acids probably occurs during the esterification step of the derivatization followed by random recombinations of the fragments producing

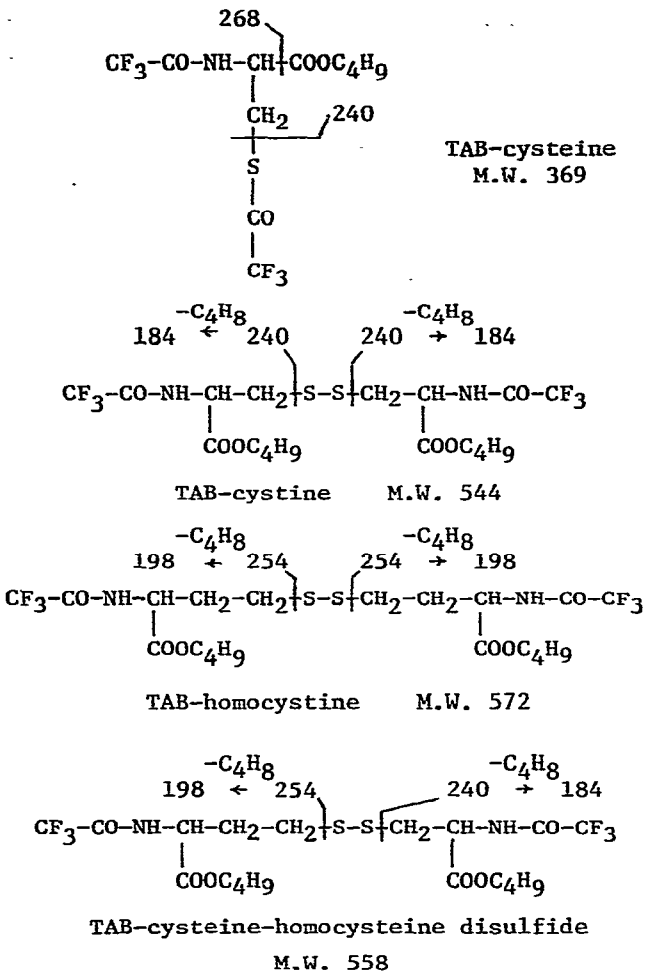

Fig. 6. Structures and partial fragmentation of various amino acid TAB derivatives.

the mixed disulfide. The likelihood of the cleavage occurring during trifluoroacetylation is minimal because the resulting free sulfhydryl groups would be rapidly acylated.

The mass spectrum of TAB-cysteine is shown in Fig. 2. The molecular ion is present at *m/e* 369 as well as ions at *m/e* 268 (loss of $-CO-O-C_4H_9$) and *m/e* 240 (loss of $-S-CO-CF_3$). Figs. 3 and 4 depict mass spectra of TAB-cystine (M^+ at *m/e* 544) and TAB-homocystine (M^+ at 572), respectively. Cleavage of the sulfur–carbon bonds lead to the characteristic fragments of *m/e* 240 from cystine and *m/e* 254 from homocystine, respectively, as shown in Fig. 6.

The mass spectrum of TAB-cysteine–homocysteine disulfide is given in Fig. 4. The parent ion is at *m/e* 558. The fragment ions at *m/e* 254 and 198 characterize the homocysteine branch and the fragment ions at *m/e* 240 and 184 originate from cysteine. The molecular ion at *m/e* 558 is surrounded by two peaks of lower intensity

at *m/e* 544 and 572, molecular ions for TAB-cystine and TAB-homocystine, respectively. These ions probably originate from scrambling during the ionization process.

TAB esters of amino acids have excellent GC and GC–MS properties. However, quantitative analysis of cystine and homocystine in the presence of each other, other disulfides, or free sulfhydryl groups can lead to erroneous results. If only trace amounts of the interfering substances are present, then the total error is probably insignificant. However, when nearly equal amounts of the hybridizing amino acids are present, as much as 11% error can be introduced into the analysis.

Cystinuria is sometimes treated with penicillamine to induce metabolically conversion of excess cystine excreted to a cysteine–penicillamine disulfide[3]. Homocystinuric patients are treated with cystine in the same manner[4]. The analyst monitoring amino acid excretion profiles of patients with these disorders should be aware of the induced (by drug therapy) or artifactual (by derivatization) formation of these mixed disulfides.

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REFERENCES

1 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 55 (1971) 267.
2 R. W. Zumwalt, K. Kuo and C. W. Gehrke, *J. Chromatogr.*, 57 (1971) 193.
3 J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson, *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1972, p. 1514.
4 J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson, *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York, 1972, p. 410.