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AZETIDINE-2-CARBOXYLIC ACID CONTAMINATED DIETARY PROLINE AS A CAUSE OF URINARY EXCRETION OF 4-AMINO-2-(S-CYSTEINYL)BUTYRIC ACID IN PATIENTS ON ORAL TREATMENT WITH A SYNTHETIC DIET

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SUMMARY

Three children with branched-chain ketoaciduria (maple syrup urine disease) were found to excrete an abnormal amino acid when they were on an artifical diet. This substance was identified as 4-amino-2-(S-cysteinyl)butyric acid with the use of column liquid chromatography, gas chromatography—mass spectrometry of various derivatives, and 360 MHz

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^{1}H-NMR spectroscopy. The same compound was detected in urine samples from subjects undergoing an oral loading test with L-proline. The chromatographic analysis of commercial proline from two sources indicated that one of the batches was contaminated (< 1%) with L-azetidine-2-carboxylic acid (the homologue of proline with a four-membered ring). The latter compound is probably metabolized by the human via ring-opening and addition of a cysteine moiety. It is highly probable that the artificial diet given to the patients contained the impure proline and that the L-azetidine-2-carboxylic acid in the proline gave rise to the excretion of the 4-amino-2-(S-cysteinyl)butyric acid.

INTRODUCTION

Patients with an inherited defect of amino acid metabolism such as phenylketonuria, maple syrup urine disease (MSUD), homocystinuria, tyrosinemia and others are treated by replacement of most of the natural dietary protein by an amino acid mixture, containing the minimal amount of the index essential amino acid necessary for normal growth.

When analyzing the urine of three patients with MSUD on dietary treatment, an abnormal ninhydrin-positive compound was observed. The phenomenon of the abnormal excretion was transient and had disappeared three years after its first observation.

In this paper we describe the identification of the abnormal ninhydrin-positive compound as 4-amino-2-(S-cysteinyl)butyric acid. We could also obtain evidence that azetidine-2-carboxylic acid was its precursor. The latter compound appeared to be a contaminant of commercially available proline. The use of this contaminated proline in the artificial diet most probably caused the biochemical anomaly in the patients' urine.

MATERIALS AND METHODS

General

L-Proline was obtained from two sources: D and J. L-Azetidine-2-carboxylic acid was purchased from Aldrich Europe (Beerse, Belgium).

Micro-scale two-dimensional thin-layer chromatography and column chromatography for routine analysis of ninhydrin-positive compounds in physiological fluids were performed as described previously [1] (see also ref. 2).

Gas—liquid chromatography (GLC) and combined gas—liquid chromatography—mass spectrometry (GLC—MS) of derivatized amino acids were carried out essentially as reported earlier [3]; oven temperatures were dependent on the type of compounds. High-resolution mass measurements and metastable measurements using a defocusing technique according to Barber and Elliott [4] were performed using an AEI MS-902 mass spectrometer (direct inlet system; electron-impact MS).

90-MHz and 360-MHz ^{1}H-NMR spectra were recorded with a Varian EM-390 and a Bruker HX-360 (Fourier Transform mode) spectrometer, respectively. Before analysis, amino acids were exchanged three times in ^2H_2O with intermediate lyophilization. Chemical shifts (δ) at a probe temperature of 25°C are given relative to sodium 5,5-dimethyl-5-silapentane-2-sulphonate in ^2H_2O as solvent (indirectly to acetone: δ = 2.225 ppm).

Isolation of 4-amino-2-(S-cysteinyl)butyric acid (ACBA)

The urine (10 ml) of a patient with MSUD on treatment was filtered and then applied to a small column (5 ml) of Dowex 50W-X8, 50—100 mesh, H^+. The resin was washed with 100 ml of water. Then, the urinary amino acids were eluted with 40 ml of 2 *M* ammonia. After evaporation of the solvent the residue was dissolved in 0.5 ml of 0.1 *M* pyridine—acetic acid, pH 3.50, and fractionated on a column of Aminex-MS fraction B, H^+ (150 × 0.6 cm). The elution was performed with the same buffer as mentioned above, at a flow-rate of 1.0 ml/min and a column temperature of 48.2° C. The unknown amino acid was present in the fractions between 350 and 400 ml of the eluate (ninhydrin detection) and proved to be pure by thin-layer chromatography. The yield was 4 mg.

Desulfurization with Raney nickel

The isolated urinary substance (1 mg) was dissolved in 2 ml of water and heated at 100° C for 2 h with 0.5 ml of a suspension of Raney nickel [5]. After decantation, the catalyst was washed three times with water at 100° C. The combined water phases were evaporated to dryness under reduced pressure and analyzed by two-dimensional thin-layer chromatography, column chromatography and GLC—MS (after trimethylsilylation).

Synthesis of 4-amino-2-(S-cysteinyl)butyric acid (ACBA)

For the synthesis of the title compound a similar strategy was followed as described earlier for isovalthine [6]. 4-Acetamidobutyric acid (1 g; 6.9 mmol) [7] was dissolved in 5 ml of freshly distilled thionyl chloride. The solution was kept at room temperature for 2 h and then warmed to 80° C. After the addition of 1.8 g of bromine (22.5 mmol), the mixture was refluxed for 3 h at 100° C. The excess thionyl chloride and bromine were removed by evaporation under reduced pressure. To the residue a mixture of 15 ml of water and 4 ml of triethylamine was added, followed by 1 g of L-cysteine (8.3 mmol). The suspension was stirred magnetically at room temperature for two days, whereby the pH was kept at 8.5. Subsequently, the precipitate was removed by filtration and the clear filtrate evaporated to dryness. The residue was suspended in 50 ml of 2 *M* HCl and refluxed for 3 h. The dark-brown syrup, obtained after evaporation of the acidic solution, was dissolved in 20 ml of water. The amino acid mixture was applied to a column (30 × 2 cm) of Dowex 50W-X8, 100—200 mesh, H^+. The resin was washed with 200 ml of water and the amino acids eluted with 250 ml of 3 *M* ammonia. The fraction containing the amino acids was evaporated to dryness. After esterification with methanolic HCl and trifluoroacetylation, GLC—MS showed the presence of ACBA, and in addition some contaminants, i.e. 4-aminobutyric acid, cystine, and S-carboxymethylcysteine.

The formed mixture of stereoisomers of ACBA gave rise to two peaks in the column chromatogram of the amino acid analyzer. However, in view of the reaction conditions used, it is tempting to assume that all four possibilities are present. The two peaks were fractionated on a preparative scale with a column (107 × 2.5 cm) of Aminex Q 150S, H^+, using 0.1 *M* pyridine—acetic acid, pH 3.50, at a flow-rate of 7.3 ml/min and a column temperature of 48.2° C. The

pooled fractions between 4700 and 5800 ml of eluate correlated mainly with the first peak of ACBA on the amino acid analyzer (composition: first peak, 82.2%; second peak, 17.8%). The pooled fractions between 6600 and 7100 ml correlated mainly with the second peak on the amino acid analyzer (composition: first peak, 24.6%; second peak, 75.4%). The yields of the lyophilized fractions were 35 and 25 mg, respectively.

Isolation of L-azetidine-2-carboxylic acid (ACA)

ACA-contaminated L-proline source D (100 mg) was fractionated on a column (100 × 2.5 cm) of Aminex-MS fraction B (H^+) with 0.1 *M* pyridine—acetic acid, pH 3.50, at a flow-rate of 5.0 ml/min and a column temperature of 50.0°C. ACA eluted just before proline as a broad peak. To obtain a total yield of 5 mg, the isolation procedure was repeated two times.

For the gas chromatographic determination of the absolute configuration of ACA on SP-1000 as non-chiral stationary phase [8], part of the sample was treated with (—)-2-butanolic HCl and trifluoroacetic anhydride.

Derivatization procedures

Esterification: amino acids (0.5 mg) were treated with methanolic, trideuteromethanolic, 1-butanolic, (±)-2-butanolic or (—)-2-butanolic 3 *M* HCl (1 ml) for 2 h at 100°C [3]. Acylation: alkyl esters of amino acids were acylated with trifluoroacetic anhydride (1 ml) for 4 h at 20°C [3]. Trimethylsilylation: amino acids (0.5 mg) were treated with N,O-bis-(trimethylsilyl)-acetamide—pyridine (1:1, v/v) for 30 min at 70°C.

Patients

D.G., a three-week-old girl, first child of Turkish parents, was admitted after a positive outcome of the Guthrie population screening test for MSUD. She was placed on the special diet, low in branched-chain amino acids. When 8 months old, the intake of contaminated proline was 2.9 g/day*. Urinary ACBA was 2.6 and 3.1 mmol/g creatinine on two different days. The disease of the patient was classified as the intermediate form of MSUD. In spite of metabolic dysregulations the development of the patient was satisfactory.

A.E., male, second child of healthy German parents, was treated for MSUD starting 5 days after birth. The outcome was favourable. At the age of 6 months the intake of contaminated proline was 3.3 g/day*. Urinary ACBA was 3.6 and 4.1 mmol/g creatinine.

G.T., female, third child of Turkish parents, was admitted at the age of 13 days with apathy, muscular hypotonia and feeding difficulties. Acute MSUD was diagnosed and treatment with the same diet was started. In spite of the dietary regimen, mental and motor development were severely retarded. The intake of contaminated proline at the age of 14 months was 3.9 g/day*. Urinary ACBA was 2.5 and 3.2 mmol/g creatinine.

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*As calculated retrospectively.

RESULTS

Identification of urinary 4-amino-2-(S-cysteinyl)butyric acid

Two-dimensional thin-layer chromatography of the urinary amino acids in the three patients on dietary treatment gave rise to an unknown heterogeneous greyish-blue spot, approximately in the position of oxidized cystathionine. Upon oxidation of the sample with H_2O_2 a small shift to the direction of the application point was observed (Fig. 1). On column chromatography the corresponding unknown compound eluted at the position of leucine.

For structural analysis, the compound was isolated by ion-exchange column chromatography. The influence of H_2O_2 on the position of the substance on a thin-layer chromatogram and a low 570/440 nm absorbance ratio of the column chromatographic fraction, suggested the presence of sulphur in the amino acid. Treatment with an iodoplatinate reagent [9] yielded within a few minutes a white spot on a pink background, indicative of sulphur, most probably in a thioether. Desulfurization with hydrogenated Raney nickel gave rise to the formation of alanine and 4-aminobutyric acid. Their identity was verified by GLC—MS of the corresponding trimethylsilyl derivatives [10]. Apparently, alanine and 4-aminobutyric acid were linked to each other via a sulphur bridge.

The sulphur content and the position of the bridge was further investigated by GLC—MS after three types of derivatization of the unkown substance: (1) pertrimethylsilylation (derivative I); (2) treatment with methanolic HCl followed by trifluoroacetylation (derivative II); (3) treatment with trideuteromethanolic HCl followed by trifluoroacetylation (derivative III). The mass

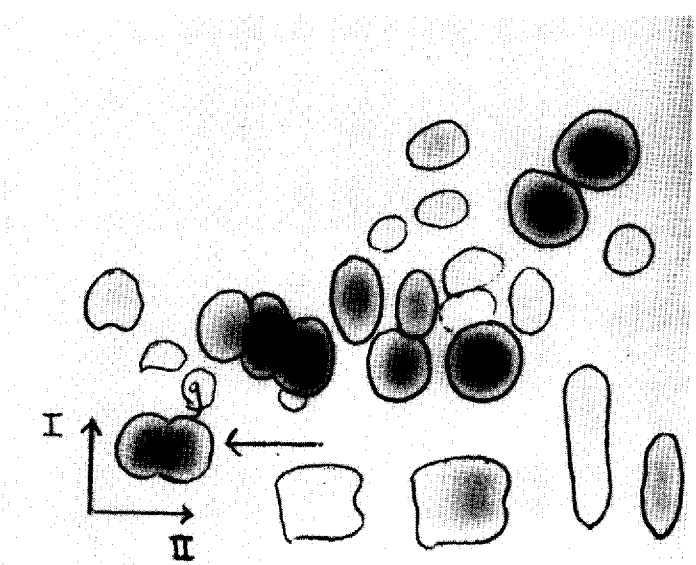


Fig. 1. Two-dimensional thin-layer chromatography on cellulose of urinary amino acids in a patient with MSUD on treatment with a dietary amino acid mixture; the applied material was oxidized with H_2O_2. The sulphur-containing amino acid is indicated with an arrow. Solvent I = *n*-butanol—pyridine—water (1:1:1, v/v), and solvent II = 88% phenol—25% ammonia—water (10:0.05:1, v/v). For further details, see refs. 1 and 2.

spectra of the pertrimethylsilylated amino acid (derivative I) and the N-trifluoroacetyl amino acid methyl ester (derivative II) are presented in Figs. 2 and 3, respectively.

Comparison of the mass spectra of the three derivatives I—III showed that only one sulphur atom is involved in the linkage between alanine and 4-aminobutyric acid (I: *m/z* 567, M_I-CH_3; II: *m/z* 410, measured formula $C_{12}H_{12}N_2O_5SF_6$, $M_{II}-CH_3OH$; III: *m/z* 413, $M_{III}-C^2H_3OH$). The three mass spectra suggested this sulphur atom to be attached at C-3 of alanine, thus making it a cysteinyl residue (I: *m/z* 218; II: *m/z* 184, measured formula $C_5H_5NO_3F_3$; III: *m/z* 187). The determination of the linkage between cysteine and 4-aminobutyric acid was difficult. The presence of *m/z* 174 and 188 in the mass spectrum of I could be explained by the attachment of the sulphur atom of the cysteinyl group at C-2 of 4-aminobutyric acid. The presence of the peaks at *m/z* 126 and 140 in the mass spectra of both II and III supported this con-

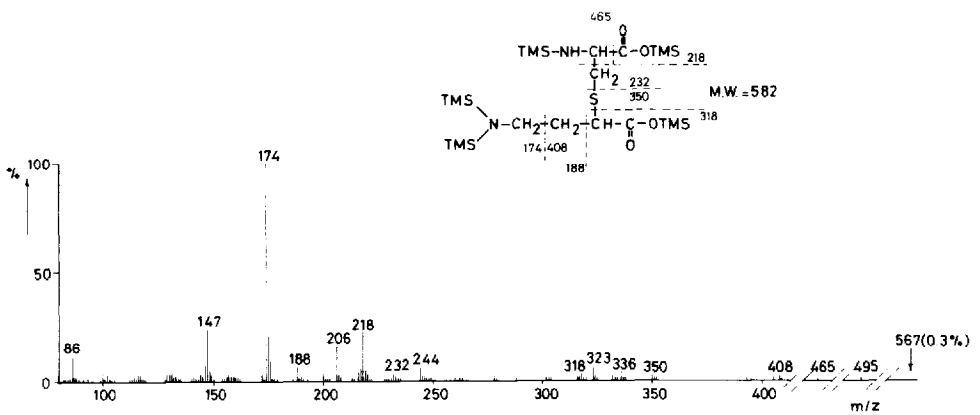


Fig. 2. Electron-impact (70 eV) mass spectrum of pertrimethylsilylated 4-amino-2-(S-cysteinyl)butyric acid.

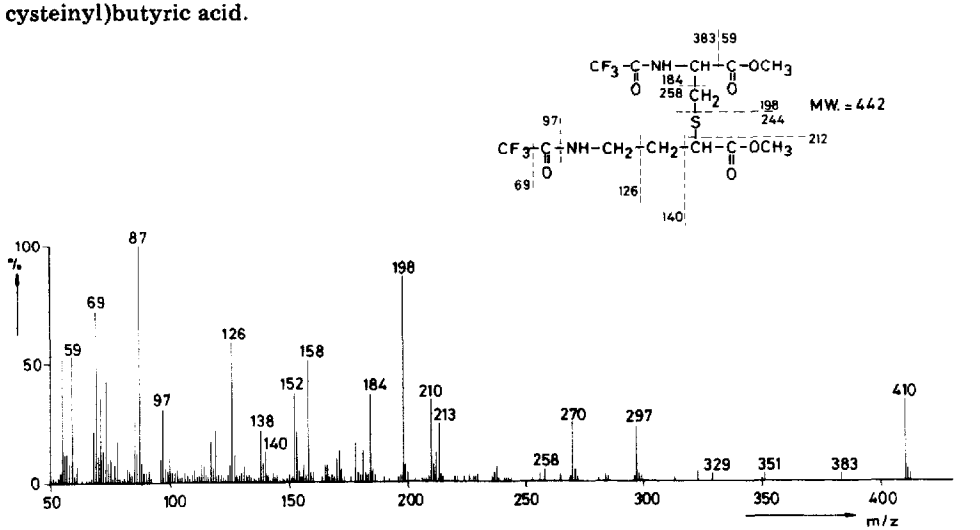


Fig. 3. Electron-impact (70 eV) mass spectrum of N-trifluoroacetyl 4-amino-2-(S-cysteinyl)-butyric acid methyl ester.

clusion. However, the occurrence of *m/z* 87 in the mass spectrum of II and *m/z* 90 in that of III, being in both cases the most abundant ions, suggested a connection with C-4. Because of this contradiction the mass spectrometric origin of *m/z* 87 was studied in more detail. Metastable measurements for II, using the defocusing technique of Barber and Elliott [4], indicated that the most prominent precursor-ion of *m/z* 87 appeared to be *m/z* 213. No evidence was obtained for precursor-ions > *m/z* 213. It is possible to presume that the fragmentation pattern includes formation of *m/z* 213 and the subsequent transition to *m/z* 87 via a simple cleavage. It is reasonable to suppose that the fragment ion *m/z* 87 in the mass spectrum of II (and *m/z* 90 in that of III) does not yield direct structural information. Taking into account the various mass spectrometric data, the most likely position of the sulphur atom of the cysteinyl residue is at C-2 of 4-aminobutyric acid.

To obtain conclusive evidence for the suggested structure, the underivatized amino acid was investigated by 360-MHz ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectrum recorded in ^2H_2O at pH 7 is depicted in Fig. 4. The various signals were assigned by comparison with the ^{1}H-NMR spectra of L-cysteine, 4-aminobutyric acid and 4-amino-2-hydroxybutyric acid [11], recorded under the same conditions (not shown here).

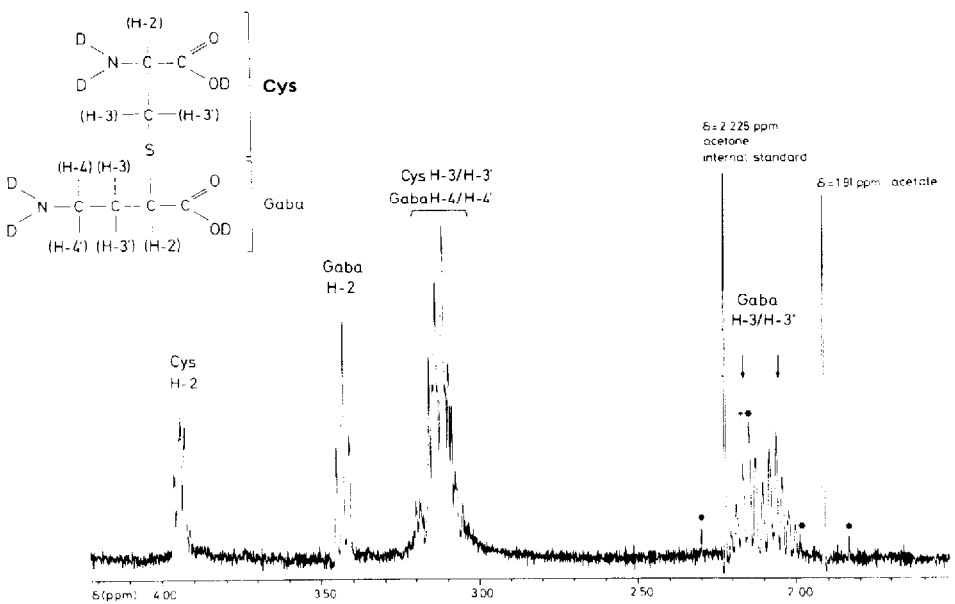


Fig. 4. Resolution-enhanced, 360-MHz ^{1}NMR spectrum of 4-amino-2-(S-cysteinyl)butyric acid, recorded in ^2H_2O at pH 7. Asterisks (*) denote spinning side bands. Gaba = 4-aminobutyric acid.

The cysteinyl part of the molecule was demonstrated by the presence of a double doublet at δ = 3.95 (Cys H-2), and two double doublets in the δ-region 3.05–3.20 (Cys H-3/H-3'). Compared with the spectrum of L-cysteine, no large differences in chemical shift were observed. The 4-aminobutyric acid part of the molecule gave rise to a triplet at δ = 3.45 (GABA* H-2), multiplets at δ =

*4-Aminobutyric acid.

2.05 and 2.16 (GABA H-3/H-3′) and two multiplets in the δ-region 3.05—3.20 (GABA H-4/H-4′). Compared with the ^{1}H-NMR spectrum of free 4-aminobutyric acid, a relatively high downfield shift was observed for GABA H-2 (δ = 2.29; 2H → δ = 3.43; 1H). A similar phenomenon for GABA H-2 was observed in the spectrum of 4-amino-2-hydroxybutyric acid. The other protons of the 4-aminobutyric acid part of the molecule resonate in the same δ-range as found for 4-aminobutyric acid and 4-amino-2-hydroxybutyric acid. These data clearly indicate that in the isolated substance the sulphur atom is attached to C-2 of 4-aminobutyric acid, leading to the structure of ACBA.

The identity of the urinary ACBA was verified by comparison with the synthetic stereoisomers. It turned out that the first eluting peak of the mixture of stereoisomers on the amino acid analyzer co-chromatographed with the urinary compound.

Identification of L-*azetidine-2-carboxylic acid in commercial* L-*proline*

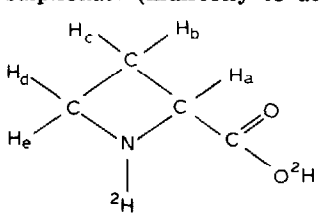
Because of the occurrence of ACBA in the urine of a patient and a healthy control after loading with L-proline source D, it was assumed that this proline sample should contain a precursor of the new sulphur-containing amino acid. Two-dimensional thin-layer chromatography of the commercial L-proline showed, just below the proline spot, an unknown spot that colored yellow with the ninhydrin reagent [2]. On the amino acid analyzer the compound eluted at the position of glutamic acid as a broad peak. The compound was separated from L-proline by preparative ion-exchange chromatography and identified by 360-MHz ^{1}H-NMR spectroscopy and GLC—MS.

The 360-MHz ^{1}H-NMR spectrum corresponded with that of commercial L-ACA. In Table I the various chemical shifts and coupling constants are summarized (these data could not be deduced earlier from the 60-MHz ^{1}H-NMR spectrum reported by Aldrich [12].

TABLE I

360-MHz ^{1}H-NMR DATA OF L-AZETIDINE-2-CARBOXYLIC ACID OBTAINED IN ^2H_2O AT A PROBE TEMPERATURE OF 25°C AND pH 7.95

Chemical shifts δ are given in ppm relative to internal sodium 5,5-dimethyl-5-silapentane-2-sulphonate (indirectly to acetone: δ = 2.225 ppm). Coupling constants J are given in Hz.



Proton	Chemical shift (ppm)	Coupling constants (Hz)
H_a	4.81	J_{ab}, 8.0; J_{ac}, 10.1
H_b	2.55	J_{bc}, −12.2; J_{ba}, 8.0; J_{be}, 9.8; J_{bd}, 8.3
H_c	2.79	J_{cb}, −12.2; J_{ca}, 10.1; J_{ce}, 6.1; J_{cd}, 9.5
H_d	4.10	J_{db}, 8.3; J_{dc}, 9.5; J_{de}, −10.6
H_e	3.93	J_{eb}, 9.8; J_{ec}, 6.1; J_{ed}, −10.6; J_{eN}, 0.8

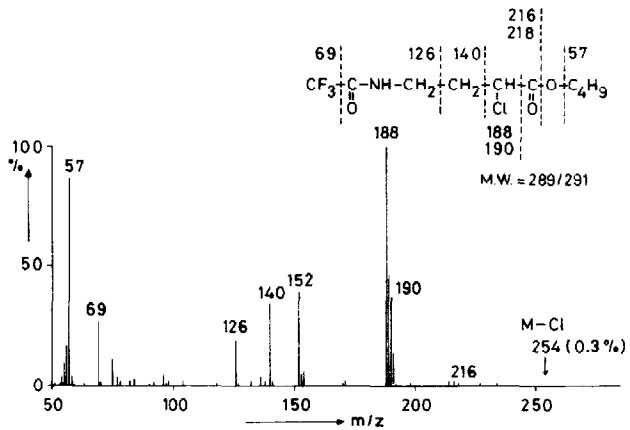


Fig. 5. Electron-impact (70 eV) mass spectrum of N-trifluoroacetyl 4-amino-2-chlorobutyric acid 1-butyl ester.

After treatment with 1-butanolic HCl and trifluoroacetic anhydride, identification of N-trifluoroacetyl 4-amino-2-chlorobutyric acid 1-butyl ester (Fig. 5) was made by GLC—MS. Derivatization of commercial L-ACA gave rise to the same product. Only a trace of intact N-trifluoroacetyl ACA 1-butyl ester was observed. As has been demonstrated previously, ACA is decomposed by hydrochloric acid to yield 4-amino-2-chlorobutyric acid among other degradation products [13].

The absolute configuration of the isolated ACA was determined indirectly by capillary GLC of the N-trifluoroacetyl 4-amino-2-chlorobutyric acid (—)-2-butyl ester on SP-1000 [8]. Commercial L-ACA was derivatized using either (—)- or (±)-2-butanol. The N-trifluoroacetyl 4-amino-2-chlorobutyric acid (—)-2-butyl ester obtained from L-ACA gave rise to one main GLC peak, but owing to the presence of a small amount of the (+)-enantiomer in the commercial (—)-2-butanol sample [(—)/(+) = 94:6], a small peak with a higher retention time was also observable. From this observation it can be concluded that the conversion of L-ACA into the chiral 4-amino-2-chlorobutyric acid occurs stereospecifically. GLC of the (±)-2-butyl ester showed two peaks. The peak with the lowest retention time corresponded with the main peak derived from authentic L-ACA treated with (—)-2-butanol. Therefore, the other peak must belong to L-ACA treated with (+)-2-butanol. As was discussed earlier [14], on non-chiral stationary phases L-enantiomers treated with (+)-2-butanol have the same retention time as D-enantiomers treated with (—)-2-butanol. The (—)-2-butyl ester derivative corresponding with the isolated ACA co-eluted with the (—)-2-butyl ester derivative of the L-enantiomer.

In conclusion, the commercial L-proline used contained L-azetidine-2-carboxylic acid. Quantitative analysis of the proline sample by column chromatography demonstrated the presence of 1.9% of the latter substance.

Recent investigations of new batches of L-proline (source D) showed that L-ACA was no longer detectable. Analysis of L-proline (source J) did not lead to the finding of ACA.

DISCUSSION

The abnormal amino acid ACBA was initially observed in the urine of three patients with MSUD, all on dietary treatment in the same university pediatric hospital. The phenomenon was transient. MSUD patients from elsewhere did not excrete this product. This course of events pointed rather to an exogenous origin of the abnormal compound or its precursor than to hitherto unknown errors of metabolism in MSUD. The accidental finding of ACBA in the urine of a patient who was loaded with commercially available proline led to the recognition that ACA, present as a contaminant by up to 1.9% in this imino acid sample, was the precursor. Also a healthy control subject, who was loaded with this contaminated proline, excreted ACBA.

The formation of ACBA from ACA may proceed via a direct attachment of L-cysteine to L-ACA. Another mode of formation might be via 4-amino-2-chlorobutyric acid, which could arise from ACA with gastric hydrochloric acid. This intermediate could react with L-cysteine in turn, to form ACBA. Now, it is highly probable that the proline used in the special MSUD diet also contained ACA. Later, when proline without ACA was used in the diet, patients no longer excreted ACBA.

Up to now L-ACA has not been found in human physiological fluids. However, this imino acid occurs in the free form in a variety of plants such as *Convallaria majalis* L. (lily-of-the-valley) and *Polygonatum officinale* [15—18]. The compound can act as a proline analogue, being incorporated into proteins [19].

Incorporation of ACA in proteins resulting in impaired biological activity [19] may be harmful to individuals daily exposed to oral loading with this compound. However, there is no evidence of damage in our patients. Two out of three had a good outcome; in one, G.T., with the severe form of MSUD, treatment was less successful, but the clinical picture did not point to causal factors other than can be expected in this disorder. A considerable part, 40% in our control, of the ACA intake was eliminated as ACBA in the first 8 h after administration. Nevertheless, we should be careful when giving a formula diet with free amino acids to patients. The amino acids used should be carefully analyzed for impurities. In general, the daily administration of small amounts of potentially toxic impurities to patients may cause damage that is difficult to recognize afterwards.

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