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Hypotaurine disproportionation reaction: identification of products

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Summary. Hypotaurine, concentrated under reduced pressure in HCl solution, partially (30–40%) degrades into taurine (about 30%), 2-aminoethyl-2-aminoethanethiolsulfonate (about 10%) and ethanolamine. The degradation products were identified using LC/APCI-MS, NMR, amino acid analysis and various chromatographies. The identities were confirmed by comparing the HPLS, MS and NMR characteristics of authentic compounds. One of the degradation processes during concentration of HCl solution of hypotaurine is therefore a disproportionation reaction which can interfere with the experimental results, when studying hypotaurine in biological systems.

Keywords: Amino acids – Hypotaurine – LC/APCI-MS chromatography – Disproportionation reaction – 2-Aminoethyl-2-aminoethanethiolsulfonate

Abbreviations: LC/APCI-MS, liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry; TLC, thin layer chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Introduction

Hypotaurine is normally present in living beings in low amounts, of the order of μmol/g tissue (Huxtable, 1986), and the quantitative determination of hypotaurine in tissues requires a concentration step. *In vitro* experiments also often require concentration steps at reduced pressure. In order to identify the compounds obtained after concentration at reduced pressure of hypotaurine in diluted HCl, reaction products were analyzed by LC/APCI-MS. Quantitative data were obtained. We recently presented results dealing with chemical transformations shown by hypotaurine and cysteinesulfinic acid, which were observed after performing a concentration step under reduced pressure of a diluted solution of sulfinate in HCl of variable concentration (Duprè et al., 1996). The presence of other compounds at the end of

the concentration procedure, detected and in part identified on TLC and by amino acid analysis (Duprè et al., 1996), indicates that hypotaurine undergoes some degradation processes under these conditions: one of these is probably a disproportionation reaction, whose extent is not negligible. Disproportionation reactions were reported for some organic sulfinates to occur in anhydrous acid solvents at 70°C (Kice and Bowers, 1962), by thermal decomposition (Wellish et al., 1961) or in acid or alkaline solutions at high temperature (von Braun and Weissbach, 1930; Marvel and Johnson, 1948; Schöberl and Wagner, 1955a). We suggest that the reaction observed with hypotaurine, which occurs under apparently mild conditions, could be responsible for incorrect experimental results when studying hypotaurine in biological systems.

Material and methods

Materials

Hypotaurine, taurine, cystamine dihydrochloride and H_2O_2 were purchased from Sigma Chemicals (USA) (see Fig. 1 for structures of compounds). $NaBH_4$, dithionitrobenzoic acid and silica gel plates were purchased from Merck (Germany). Other reagents were of the best commercial source. Dansyl chloride was obtained from Fluka (Suisse); polyamide layer sheets were obtained from BDH Laboratory Supplies (England).

Preparation of hypotaurine samples

Hypotaurine (4 mg, 37 μmol), dissolved in 25 ml HCl (from 0.2 N to 6 N), was dried in a rotary evaporator under reduced pressure at water bath temperature of 40°. The residue was dissolved in 25 ml water and dried again. The residue, dissolved in 1.0 ml water and centrifuged, was analyzed as reported.

Synthesis of 2-aminoethyl-2-aminoethanethiolsulfonate (cystamine thiol-sulfonate)

Synthesis of 2-aminoethyl-2-aminoethanethiolsulfonate dihydrochloride was performed following reported methods (Cavallini et al., 1951; Maehly, 1966) with minor modifications. Cystamine dihydrochloride (1 g) was reacted with 1.02 ml 30% H_2O_2 in 4 ml water with efficient stirring. After 24 h at room temperature ethanol was added to the solution until precipitation began and the solution was left overnight in the cold. White crystals obtained were dried over P_2O_5 in a desiccator under *vacuo*. Yield: 85%, mp 166°C (uncorrected) (see Fig. 1 for structure of cystamine thiolsulfonate).

Instrumentation

The apparatus used for LC/MS analyses was a Hitachi L-6200 high-performance liquid chromatography (HPLC) instrument, equipped with a 5 μm Inertsil ODS-2 column (150 mm × 4.6 mm i.d.) from Gasukuro Kogyo (Tokyo, Japan) connected to a Hitachi M80B mass spectrometer/computer system through the APCI interface (Kodama et al., 1990). The nebulizer and vaporizer temperatures were 255°C and 380°C respectively. Analyses were performed with a mobile phase of 100 mM CH_3COONH_4:CH_3CN (80:20 v/v) at a flow rate of 0.9 ml/min.

Amino acid analysis was performed with a Hitachi L-8500 instrument, using citrate buffer 0.068 M, pH 2.8. ^1H- and ^{13}C-NMR spectra (with dioxane as internal standard) were recorded by a Varian XL-300 instrument. TLC on 0.2 mm thick silica gel plates on aluminium sheets were developed with the solvent, *n*-butanol:methanol:acetic

Hypotaurine	NH_2-CH_2-CH_2-SO_2H
Taurine	NH_2-CH_2-CH_2-SO_3H
Cystamine	NH_2-CH_2-CH_2-S-S-CH_2-CH_2-NH_2
Cystamine thiolsulfonate	NH_2-CH_2-CH_2-SO_2-S-CH_2-CH_2-NH_2

Fig. 1. Structures of hypotaurine, taurine, cystamine and cystamine thiolsulfonate (2-aminoethyl-2-aminoethanethiolsulfonate)

acid:water (25:40:10:25) and the compounds were detected with ninhydrin. Derivatization with dansyl chloride was performed according to the following procedure. The compound (2 mg/ml, 15 μl) was mixed with 15 μl 0.2 M $NaHCO_3$ and 15 μl 18.5 mM dansyl chloride in acetone. The reaction mixture was left for 30 min at 45°C, and analyzed on bidimensional polyamine sheets [first solvent: water:formic acid (220:3); second solvent: *n*-butanol:methanol:acetic acid:water (25:40:10:25)]. Spots were detected by a UV lamp.

Thiol groups were determined by the Ellman method (Ellman, 1959).

Results and discussion

The mass chromatogram and spectrum of a hypotaurine solution in 2 N HCl are shown in Fig. 2A and 2B, respectively. Molecular ion $[M + H]^+$ (m/z 110) is observed as the base peak, in addition to the hydrated $[M + H + H_2O]^+$ (m/z 128) and $[M + H + 2H_2O]^+$ (m/z 146) and the dehydrated $[M + H - H_2O]^+$ (m/z 92) ions. Fragment ions with m/z 78, 65 and 62 are also present. The mass chromatogram of a solution of hypotaurine after the compound has been taken to dryness under reduced pressure at 40°C, as described in the experimental section, is shown in Fig. 3A. Spectra of some of the eluted fractions, recorded over a wide mass range in order to monitor the presence of compounds of low molecular weight, are also shown (Fig. 3B). Hypotaurine represents the main compound present in the solution after the concentration procedure (Fig. 3B) and accounts for more than 60% of the total compounds. Comparison with the mass spectrum of a pure sample of hypotaurine (Fig. 2B) shows, after the concentration step, an increment of the signal at m/z 62. Apparently a new compound is formed, whose signal adds to the signal of a fragment ion due to hypotaurine. This new compound at m/z 62 has been tentatively identified as ethanolamine. Identification was confirmed by coelution with a pure sample, which has been submitted to separation and mass analysis under the same conditions (data not shown). Other ions appear at m/z 76 and 185 and they will be discussed later. Under identical conditions LC/APCI-MS detected authentic taurine sample at lower sensitivity than the other compounds. Moreover there is an overlapping at m/z 126 of the signal of taurine with one of the signals of the solvent used in the chromatographic separation. Therefore mass data alone are not suitable for the quantitative evaluation of the amount of taurine present.

Amino acid analysis (Fig. 4) shows the presence of hypotaurine (about 60–65%), taurine (about 30%) and ethanolamine. One peak eluted soon after the β-alanine position has not been identified. A small amount of glycine was also found.

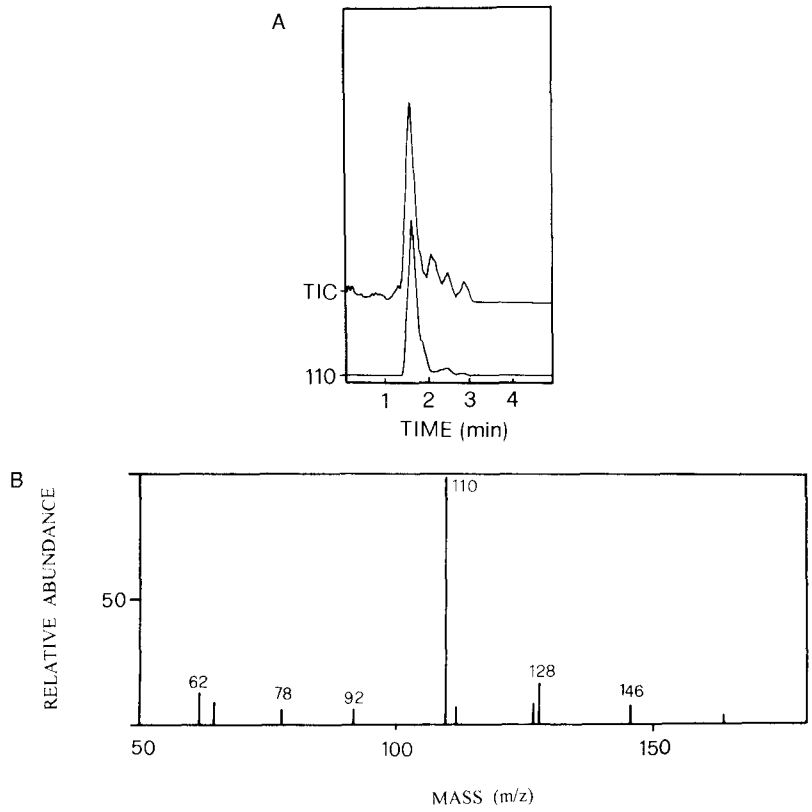

Fig. 2. Mass chromatogram (**A**) and mass spectrum (**B**) of an authentic sample of hypotaurine dissolved in 2N HCl. Chromatographic conditions are described in the experimental section. Mass spectrometer was scanned from 50 to 180 at a rate of 4s per scan

Of particular interest is the compound at m/z 185, accounting for at least 10% of the total amount. Thiolsulfonates are reported as products of the disproportionation reaction of sulfinates (Schöberl and Wagner, 1955a; Quaedvlieg, 1955; Kice and Bowers, 1962; Field et al., 1964). In order to verify whether this reaction has taken place, we synthesized 2-aminoethyl-2-aminoethanethiolsulfonate (cystamine thiolsulfonate) following the published method (Maehly, 1966) with minor modifications of the crystallization procedure. The compound was purified by precipitation and crystallization with ethanol from a water solution in the cold, thus avoiding precipitation from hot acetic acid.

The spectral data of the synthesized compound are in agreement with the structure of 2-aminoethyl-2-aminoethanethiolsulfonate dihydrochloride. In particular, in the ^{1}H-NMR spectrum (D_2O) the methylene CH_2-SO_2 protons are found shifted at low field (δ = 4.05) and coupled with the adjacent CH_2-NH_2 group resonating at δ = 3.6. The two additional methylene signals appear at δ = 3.55 and 3.45. Furthermore, the ^{13}C-NMR spectrum (D_2O) reveals four distinct signals centered at δ = 60.3 (C-SO_2), 41.9 (C-S), 36.9 and 35.5 (2 × C-N) in accordance with the different chemical environments of

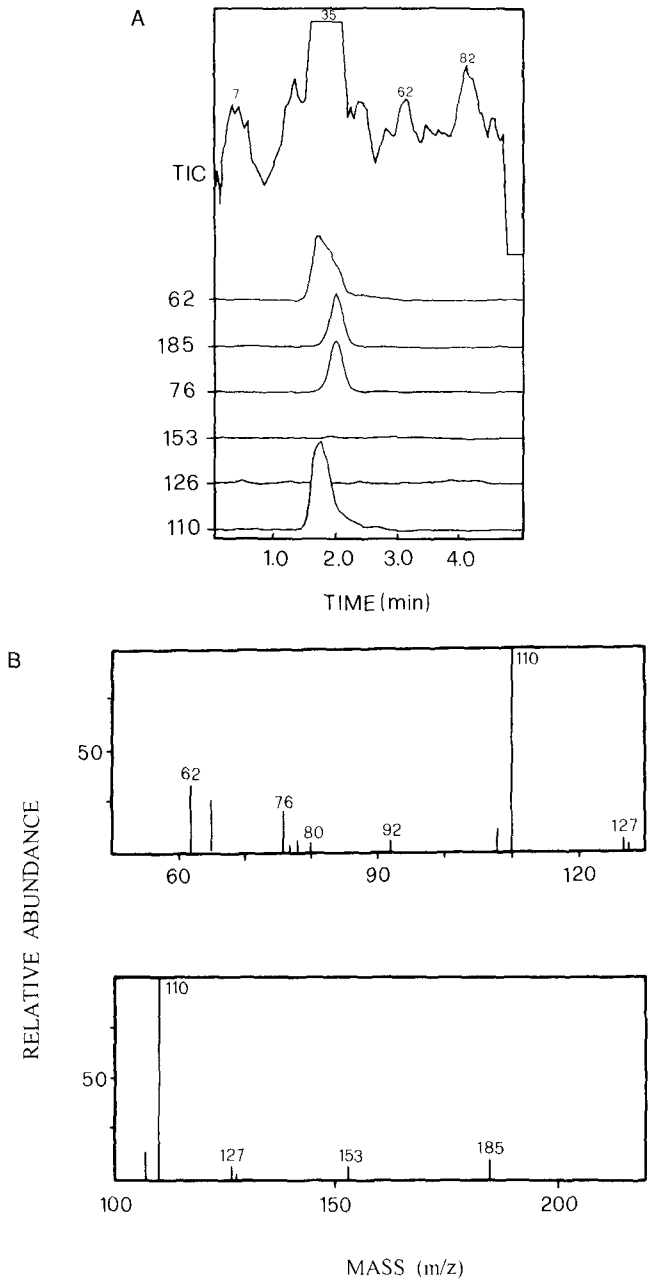

Fig. 3. Mass chromatogram (**A**) and mass spectra (**B**) of an hypotaurine solution in 2N HCl, after the concentration procedure under reduced pressure, as described in the experimental section. **A** Total ion chromatography and selected ion monitoring are reported. (M + H) values of identified compounds are: hypotaurine (110), taurine (126), 2-aminoethyl-2-aminoethanethiolsulfonate (185), ethanolamine (62). Compound with m/z value of 76 is discussed in the text. **B** Mass spectra of samples [(33*43) − 20], scanned from 50 to 130 and from 100 to 220

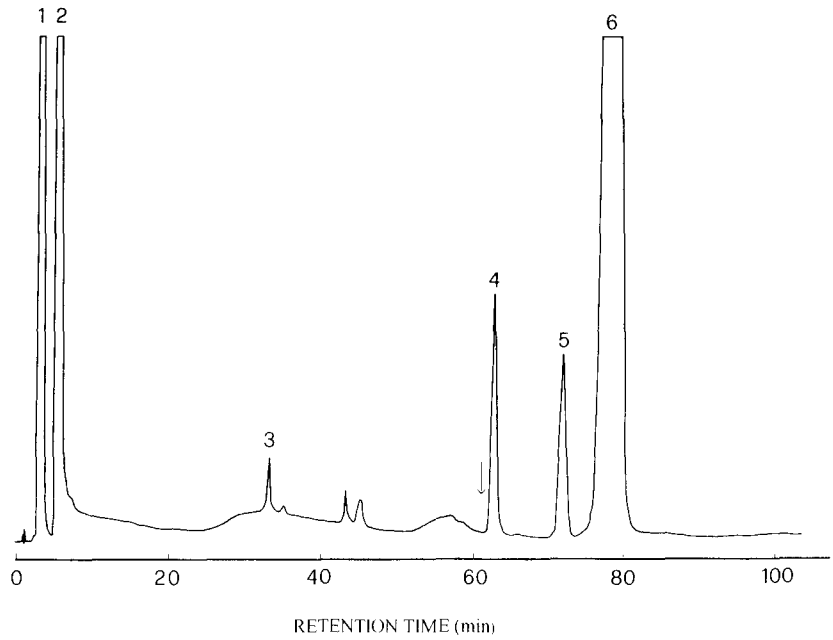

Fig. 4. Amino acid analysis of an hypotaurine solution in 2 N HCl, after the concentration procedure under reduced pressure, as described in the experimental section. Analyses conditions are given in the experimental section. Peaks are identified as follows: taurine (*1*), hypotaurine (*2*), glycine (*3*), unknown compound (*4*), ethanolamine (*5*), ammonia (*6*). The arrow indicates the elution position of β-alanine

each methylene group. In addition, we observed a similar difference in the chemical shifts of the two methylene carbon atoms of hypotaurine (58.2 and 36.2 δ for C-SO_2 and C-N, respectively). These data are in accordance with those reported for other thiolsulfonates (Bass and Evans, 1980).

Figures 5A and 5B show the mass chromatogram and spectrum of the synthesized 2-aminoethyl-2-aminoethanethiolsulfonate. Together with the main peak at m/z = 185, peaks at m/z 108 (fragment ion $[H_2N\text{-}CH_2\text{-}CH_2\text{-}SO_2]^+$), and m/z = 153 $[(H_2N\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}NH_2 + H)^+]$ are observed. The presence of peaks at m/z = 30 and 44 indicates that fragmentation also occurs at the primary amine end. Fragment ion $[H_2N\text{-}CH_2\text{-}CH_2\text{-}S^+]$ at m/z = 76, which is reported as one of the major fragment ions of thiolsulfonates (Block et al., 1975), was also present.

TLC of the synthesized 2-aminoethyl-2-aminoethanethiolsulfonate showed a major spot (R_f = 0.22) very close to cystamine (R_f = 0.25) together with low variable amounts of hypotaurine and taurine. Ninhydrin reaction gave only low color. Presence of hypotaurine and taurine on TLC plates indicates that 2-aminoethyl-2-aminoethanethiolsulfonate probably begins to decompose during the chromatographic procedure. This compounds has been reported in the past to be rather labile (Cavallini et al., 1951, 1953), and other thiolsulfonates are unstable in aqueous solutions as well (Jocelyn, 1972). This compound behaves as a strong basic compound, when submitted to ion-exchange chromatography. Under conditions allowing for the

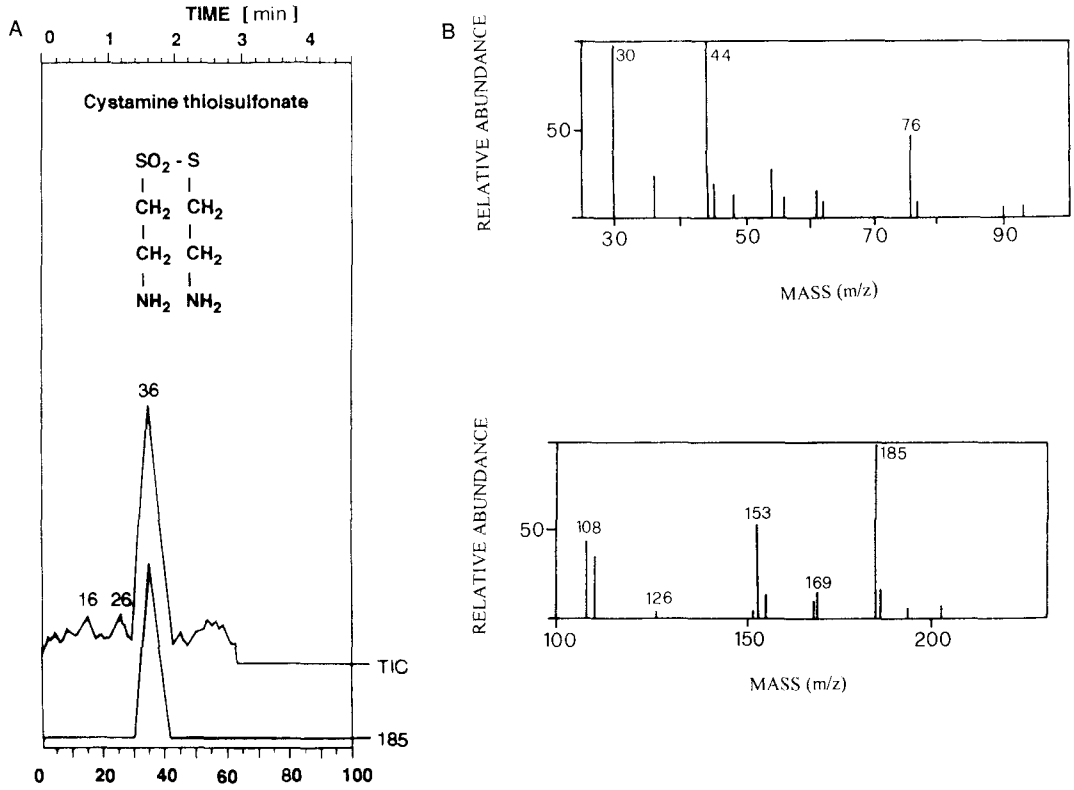

Fig. 5. Mass chromatogram (**A**) and mass spectrum (**B**) of authentic sample of 2-aminoethyl-2-aminoethanethiolsulfonate dihydrochloride. Chromatographic conditions are described in the experimental section. Mass spectrometer was scanned from 25 to 100 and from 100 to 230 at a rate of 4 s per scan

separation of acidic compounds in the amino acid analyzer the presence of small amounts of hypotaurine was observed (data not shown). After derivatization with dansyl chloride, hypotaurine, cystamine and 2-aminoethyl-2-aminoethanethiolsulfonate were separated and identified by mono- or bi-dimensional chromatography on polyamine sheets (compounds located by UVA lamp). These compounds have R_f values of 0.35, 0.72 and 0 [water : formic acid (220 : 3) as solvent] or 0.34, 0.78 and 0.82 [*n*-butanol : methanol : acetic acid : water (25:40:10:25)] respectively. A more tedious separation procedure by paper chromatography has been published (Jayson et al., 1964). It has been reported (Schöberl and Wagner, 1955b; Field et al., 1961) that thiolsulfonates easily react with thiols with a stoichiometry of 1:2, giving first unsymmetrical disulfides and then, in the presence of excess thiol, symmetrical disulfides. On the contrary, the reaction rate of 2-aminoethyl-2-aminoethanethiolsulfonate with cysteine, mercaptoethanol or dithiothreitol was very low, as may be evidenced by following the decrease of thiols with DTNB.

A synthesized sample of 2-aminoethyl-2-aminoethanethiolsulfonate characterized as above has the same elution time of the unknown compound at

m/z = 185, thus confirming the identification. Linearity in the range 50–600 ng (Fig. 6) allows quantitative determination.

The disproportionation reaction of sulfinates is a well documented reaction (Kice and Bowers, 1962). However it was reported to occur under strong anhydrous acid conditions, and has been studied with aliphatic or aromatic sulfinates; data on the reaction of natural sulfinates, as hypotaurine or cysteinesulfinic acid, are not reported.

Two consecutive disproportionation reactions may be considered to run independently, in order to explain the presence of the observed compounds. Hypotaurine under the strong acidic conditions occurring at the end of the concentration process disproportionates according to equation 1:

$$3\ R\text{-}SO_2H \rightarrow R\text{-}SO_2\text{-}S\text{-}R + R\text{-}SO_3H + H_2O \qquad (1) \qquad R = NH_2\text{-}CH_2\text{-}CH_2\text{-}$$

The presence of the ion peak at m/z 185 and of taurine (amino acid analysis, Fig. 4) confirm that this reaction takes place. The second disproportionation reaction of the thiolsulfonate, according to equation 2

$$5\ R\text{-}SO_2\text{-}S\text{-}R + 2H_2O \rightarrow 3\ R\text{-}S\text{-}S\text{-}R + 4\ R\text{-}SO_3H \qquad (2)$$

should be evidenced by the presence of cystamine among the reaction products. A low intensity peak at m/z 153 was present in the mass spectrum of hypotaurine after the concentration process (Fig. 3B). A mass spectrum of cystamine shows the presence of signals at m/z 153 (about 50% of the base peak, molecular ion), 108 as base peak ($H_2N\text{-}CH_2\text{-}CH_2\text{-}SS^+$) and 76 ($H_2N\text{-}CH_2\text{-}CH_2\text{-}S^+$) almost as high as the base peak (data not shown). This fragmentation pattern is in accordance with the literature data (Gupta et al., 1981; Nibbering et al., 1993). Fragment ions with these m/z values are present also in the mass spectrum of hypotaurine after the concentration process (Fig. 3B) and in the mass spectrum of the thiolsulfonate (Fig. 5B). It is therefore not possible to discriminate, on the basis of the mass spectra, the extent of the two reported disproportionation reactions. The presence of small amounts of

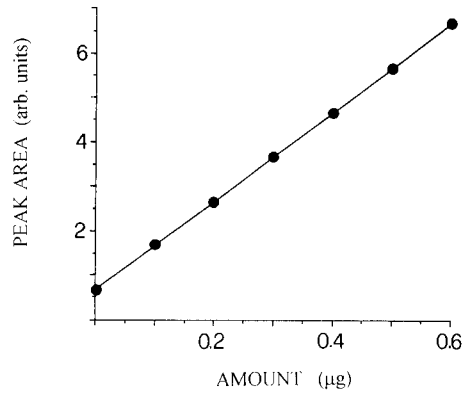

Fig. 6. Calibration curve for 2-aminoethyl-2-aminoethanethiolsulfonate dihydrochloride, showing concentrations plotted against peak area. Areas were obtained from single ion monitoring at m/z = 185

ethanolamine indicates that hypotaurine under these conditions also loses the sulfinic group.

We may conclude: i) that the first disproportionation reaction is quantitatively more important and that thiolsulfonate does not disproportionate further to a large extent; ii) that about 35% of the initial amount of hypotaurine disappears in these reactions; and iii) that the presence of taurine among the products does not necessarily indicate that a simple oxidation reaction of hypotaurine takes place.

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