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DETERMINATION OF CYSTEINESULFINATE, HYPOTAURINE AND TAURINE IN PHYSIOLOGICAL SAMPLES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cysteinesulfinate, hypotaurine and taurine, which are key metabolites of cysteine, can be separated from each other and other closely eluting amino acids in biological samples by reversed-phase high-performance liquid chromatography on a Waters Nova-Pak C_{18} column. Samples were derivatized with *o*-phthalaldehyde—2-mercaptoethanol prior to injection. The elution system consisted of 100 m*M* potassium phosphate buffer, pH 7.0, with 3% (v/v) tetrahydrofuran with an initial isocratic phase at 1.2% acetonitrile and a gradient from 1.2 to 12.8% acetonitrile. This method is suitable for measurement of the production of metabolites from cysteine by isolated cells and for analysis of plasma and tissue extracts. Low levels of hypotaurine in rat tissues were easily measured with this method and are reported here for the first time.

INTRODUCTION

A major pathway of cysteine catabolism in animals is thought to involve the oxidation of cysteine to cysteinesulfinate, the decarboxylation of cysteinesulfinate to hypotaurine, and finally the oxidation of hypotaurine to taurine [1, 2]. Radiotracer studies in intact male rats have demonstrated that 70—85% of the oxidative flux of intraperitoneally administered cysteine occurred via this taurine pathway [3, 4]. Both studies in intact animals [5] and in hepatocytes [6] have demonstrated a species difference between the rat and the cat in their capacity for taurine synthesis from cysteine. The cat's lower capacity for taurine synthesis is consistent with its requirement for dietary taurine [7], whereas the rat has a higher capacity for taurine synthesis and does not need taurine in its diet [5, 8]. Compared to most amino acids, the catabolism of

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cysteine, particularly in extrahepatic tissues, is poorly understood and needs further study.

Although a number of reversed-phase chromatographic methods for the determination of taurine and other metabolites of cysteine have been reported [9—14], none of these methods is appropriate for the simultaneous determination of cysteinesulfinate, hypotaurine and taurine in physiological samples. In particular, separation of these metabolites from alanine, glutamate, cysteate and α-, β- or γ-aminobutyrate has been a problem.

In this paper, we describe a reversed-phase high-performance liquid chromatographic (HPLC) method that allows quantitative analysis of cysteinesulfinate, taurine and hypotaurine as well as alanine, glutamate and α- and β- or γ-aminobutyrate in physiological samples. We also demonstrate the use of this method for measurement of the production of metabolites by rat hepatocytes incubated with cysteine and for analysis of metabolites of cysteine in rat tissues and human plasma.

EXPERIMENTAL

Chemicals

Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.); tetrahydrofuran from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); and phosphoric acid and monobasic potassium phosphate from Fisher Scientific (Pittsburgh, PA, U.S.A.). These were all of HPLC grade. All other chemicals were analytical grade. Amino acids and *o*-phthalaldehyde were obtained from Sigma (St. Louis, MO, U.S.A.); boric acid from Mallinckrodt (St. Louis, MO, U.S.A.); and 2-mercaptoethanol from J.T. Baker. Sep-Pak C_{18} sample clean-up cartridges were purchased from Millipore (Waters Chromatography Division, Milford, MA, U.S.A.).

Apparatus

The chromatographic system consisted of two Waters Model 510 pumps, a Waters Model 680 gradient controller, a WISP Model 710B automatic sample injector, a column temperature control block, a Nova-Pak C_{18} column (15 cm × 3.9 mm, 4-μm spherical particles) and a guard column dry-packed with Bondapak C_{18}/Corasil 37—50 μm bulk packing (all from Millipore, Waters Chromatography Division). Fluorescence was measured with a Spectra-Glo fluorometer (Gilson Medical Electronics, Middleton, WI, U.S.A.) equipped with a 5-μl flow cell and filters for excitation and emission peaks at 360 and 455 nm, respectively. The output signal from the fluorometer was set at 10 mV full scale, and the photomultiplier sensitivity switch was set at X1 and the range at 10 or 20. The fluorometer was connected to an HP 3392A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Chromatographic procedure

Standard and sample solutions were mixed 1:1 (v/v) with *o*-phthalaldehyde—2-mercaptoethanol derivatizing reagent, which was prepared each day by dissolving 35 mg of *o*-phthalaldehyde in 0.5 ml of 95% ethanol and mixing this with 50 ml of 100 m*M* borate buffer, pH 10.4, plus 0.10 ml of 2-mercapto-

ethanol. Within 1 min of mixing, 20 μl of the final solution were injected onto the column. The derivatizing reagent in an opaque vial at room temperature was stable for at least 24 h.

Two solutions were used to create the gradient for the mobile phase. Both contained a final concentration of 100 m*M* potassium phosphate buffer and 3% (v/v) tetrahydrofuran and were adjusted to a final pH of 7.0. One of the solutions (buffer B) also contained 40% (v/v) acetonitrile. Before they were used, these solutions were filtered through a 0.5-μm filter (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum.

A flow-rate of 1.0 ml/min was used. Column temperature was maintained at 41°C. The mobile phase was run isocratically for the first 1.5 min at 3% buffer B. The amount of buffer B was then increased to 32% over 17 min using curve profile 3 of the Waters Model 680 gradient controller. Buffer B was then linearly increased from 32% to 100% over 2 min and held at 100% for 4.5 min. At 25 min the percentage of buffer B was returned to 3% by a linear decrease over 2 min, and the column was allowed to equilibrate for at least 8 min before the next sample was injected. Thus, the total run time was about 35 min per sample.

As an alternative to the manual method of derivatization described above, the WISP Model 710B automatic sample injector was also used to perform the derivatization. Under conditions of no flow and no pressure in the HPLC system, 10 μl of the derivatizing reagent and 10 μl of the sample solution were sequentially injected into the tubing between the injector and the guard column (200 cm × 0.23 mm I.D. including that in the temperature-control block). After a 2-min delay period, the flow-rate was increased from 0 to 1.0 ml/min over 0.5 min and chromatography was carried out as described above except the isocratic phase was increased to 4.0 min. A 5-min delay period was added between runs to allow the system to return to conditions of no flow and no pressure prior to injection of the next sample. Total run time was about 47 min per sample.

Sample collection and preparation

Hepatocytes were prepared from male Sprague–Dawley rats (Blue Spruce Farms, Altamont, NY, U.S.A.) by the method of Berry and Friend [15] as modified by Krebs et al. [16]. Approximately 50 mg (wet weight) of cells were incubated with Krebs–Henseleit bicarbonate buffer, 2.5% (w/v) dialyzed bovine serum albumin and 25 m*M* L-cysteine in a final volume of 2.5 ml at pH 7.4. The contents of the flask were equilibrated with a gas mixture of oxygen–carbon dioxide (95:5), and then the flask was sealed and incubated for 40 min at 37°C in a shaking water bath. The incubation was stopped by addition of 0.5 ml of 6 *M* perchloric acid, and the contents of the flask were centrifuged at 2000 *g* for 10 min to remove the protein. The pH of the supernatant was then adjusted to 6–7 with 10 *M* potassium hydroxide, and the sample was centrifuged at 2000 *g* for 10 min to remove potassium perchlorate. The supernatant was frozen and stored at −20°C until it was chromatographed.

Tissues were obtained from male Sprague–Dawley rats that weighed 200–250 g. Rats were killed by decapitation, and liver, kidney, heart and brain were removed immediately. Tissues were frozen on dry ice and stored at −20°C

until they were prepared for analysis. Tissues were homogenized in 5% (w/v) 5-sulfosalicylic acid (routine procedure), 5% (w/v) perchloric acid or 50 m*M* potassium phosphate (pH 6.8) to prepare 20% (w/v) homogenates. When recoveries were done, standards were added to the above solutions prior to tissue homogenization. Tissue homogenates prepared with potassium phosphate were heated for 4 min in a boiling water bath to denature the protein. All homogenates were centrifuged at 5000 *g* for 10 min to remove the precipitated protein, and the supernatants were neutralized with 10 *M* potassium hydroxide. The supernatant fractions were chromatographed immediately or frozen for later analysis.

Blood from adult women was collected into tubes that contained heparin and centrifuged at 2000 *g* for 10 min to obtain plasma. Care was taken not to disturb the buffy coat when plasma was removed. Plasma protein was precipitated by addition of 1 vol. of 35% (w/v) 5-sulfosalicylic acid to 9 vols. of plasma. After centrifugation of each sample at 2000 *g* for 10 min, the supernatant was removed, neutralized with 10 *M* potassium hydroxide, diluted with 2 vols. of water to 1 vol. of supernatant, frozen and stored at −20°C until it was analyzed.

Although it was generally not necessary, some of the deproteinized tissue supernatants, particularly those prepared from brain, were passed through Sep-Pak C_{18} clean-up cartridges prior to chromatography. Before use, these cartridges were prepared by sequentially washing them with 20 ml of methanol, 20 ml of 0.1% (v/v) trifluoroacetic acid (TFA) in water and 10 ml of 1.0% TFA in methanol—water (20:80, v/v). A 1-ml aliquot of the sample was then mixed with 2 ml of 0.1% TFA in methanol—water (30:70) and pushed through the cartridge with a syringe. This was followed with 2 ml of 0.1% TFA in methanol—water (30:70) to completely elute the amino acids. All 5 ml of the eluate were collected and adjusted to a final volume of 10 ml. Recovery of cysteinesulfinate, hypotaurine and taurine standards carried through this procedure alone or in the presence of tissue extracts ranged from 91 to 101%, and tissue extracts chromatographed with or without this clean-up procedure gave similar values.

RESULTS AND DISCUSSION

The effect of the concentration of *o*-phthalaldehyde in the derivatizing reagent on the formation of fluorescent derivatives of cysteinesulfinate, hypotaurine and taurine is shown in Fig. 1. As little as 0.004% (w/v) *o*-phthalaldehyde was sufficient to yield maximal fluorescence with taurine or hypotaurine, but 0.06% *o*-phthalaldehyde was required to obtain maximal derivatization of cysteinesulfinate. Higher levels of *o*-phthaladehyde did not affect the fluorescence yield. A concentration of 0.07% *o*-phthalaldehyde was used routinely.

A progressive decrease in fluorescence with increasing time after the derivatizing reagent was mixed with solutions of each compound in liver extract is shown in Fig. 2. Fluorescence yield from the *o*-phthalaldehyde derivatives of both taurine and hypotaurine was decreased markedly when solutions were injected 4 min after mixing compared to fluorescence obtained

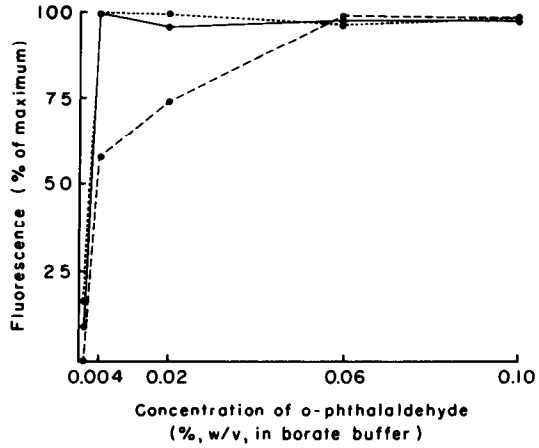

Fig. 1. Effect of *o*-phthalaldehyde concentration of the formation of fluorescent derivatives of cysteinesulfinate (— — —), hypotaurine (—), and taurine (- - -). Each amino acid (100 pmol per 10 μl of liver extract) was derivatized and chromatographed by the standard method described in the text. Each point represents the average of duplicate determinations.

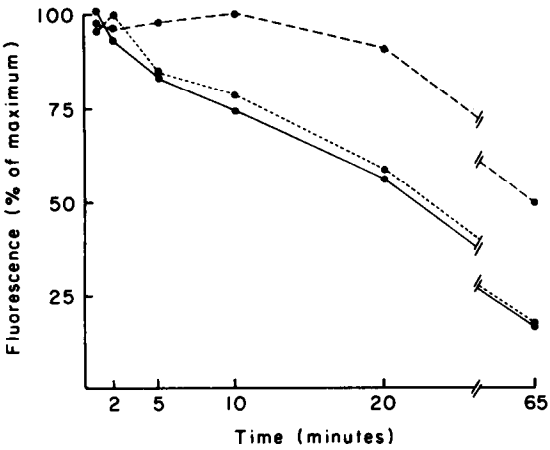

Fig. 2. Time course of the stability of *o*-phthalaldehyde—2-mercaptoethanol derivatives of cysteinesulfinate (— — —), hypotaurine (—), and taurine (- - -). Each amino acid (75 pmol per 10 μl of liver extract) was mixed with derivatizing reagent and injected at the indicated time. Other chromatographic conditions were as described in the text. Each point represents a single determination.

when samples were injected 1 or 2 min after they were derivatized. Fluorescence yield from the derivative of cysteinesulfinate was substantially more stable and did not decrease markedly until after 20 min. All samples were routinely injected within 1 min after mixing with the derivatizing reagent.

Standard curves for cysteinesulfinate, hypotaurine and taurine are shown in Fig. 3. The fluorescence intensity was proportionate to the amino acid content of the standard solution between 0 and 1000 pmol per 10-μl aliquot for all three compounds. The slopes of the standard curves for taurine and hypotaurine were similar and steeper than the slope of the curve for cysteinesulfinate.

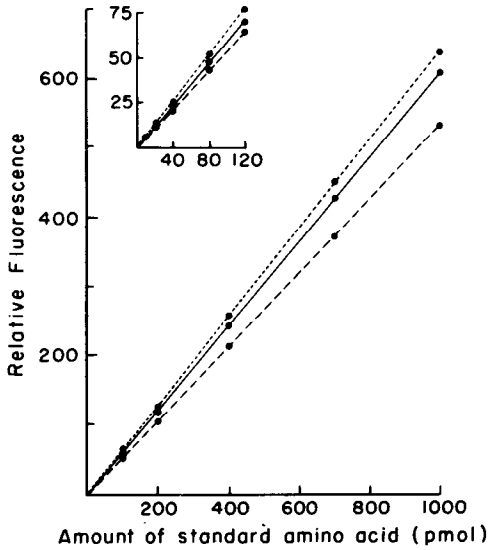

Fig. 3. Effect of the concentration of cysteinesulfinate (— — —), hypotaurine (—), or taurine (- - -) on fluorescence yield. Each amino acid (10—1000 pmol per 10 μl in water) was derivatized and chromatographed by the standard method described in the text. Each point represents the average of duplicate determinations.

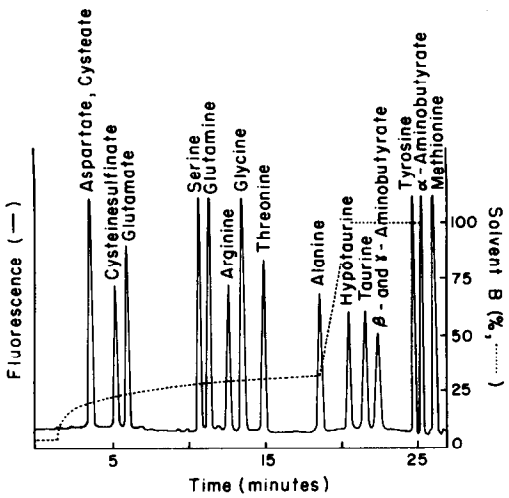

Fig. 4. Representative chromatogram for the analysis of a mixture of amino acids. The amino acids were derivatized and chromatographed by the standard method described in the text. The isocratic and gradient phases of the run are indicated as the percentage of buffer B.

The separation of a mixture of standard amino acids is shown in Fig. 4. A plot of the gradient is superimposed on the chromatogram. Cysteinesulfinate, hypotaurine and taurine were clearly resolved from other closely eluting amino acids. The first 1.5 min of the run was isocratic at 1.2% acetonitrile in 100 m*M* potassium phosphate buffer with 3% tetrahydrofuran, pH 7.0 (3% buffer B); this allowed separation of cysteinesulfinate from glutamate. The acetonitrile

concentration was then increased from 1.2 to 12.8% (32% buffer B) over 17 min as shown in Fig. 4; this gave separation of alanine, hypotaurine, taurine, γ-aminobutyrate and β-aminobutyrate. γ-Aminobutyrate eluted slightly (ca. 0.2 min) earlier than β-aminobutyrate when standards were run separately, but they produced a single peak when both were present in the same mixture of standard amino acids. The concentration of acetonitrile was rapidly and linearly increased to 40% (100% buffer B) to elute any compounds remaining on the column that might interfere with subsequent runs. It should be noted that we have always obtained superior separation of these amino acids on the Nova-Pak C_{18} column compared to other C_{18} columns we have tried. If only taurine and hypotaurine are of interest, the sample run time may be decreased by using a higher initial concentration of acetonitrile (ca. 6%) and running a similar gradient without an isocratic phase at the beginning of the run.

The separation shown in Fig. 4 is very reproducible. The coefficients of variation for the retention times of hypotaurine and taurine were 0.23 and 0.25%, respectively, for a liver sample that was repeatedly chromatographed.

Both cysteine and cystine gave very low fluorescence yields with *o*-phthalaldehyde—2-mercaptoethanol that were below the limits of detection even when as much as 10 nmol of either compound were derivatized and injected. The elution positions of cysteine and cystine under standard run conditions were determined with L-[^{35}S]cysteine and L-[^{35}S]cystine; cysteine was eluted with the void volume at 2.5 min and cystine was eluted at 3.5 min. Thus, both eluted well before cysteinesulfinate, which eluted at 5.2 min (Fig. 4). Neither ethanolamine, *o*-phosphorylethanolamine nor glycerophosphorylethanolamine, which sometimes interfere with measurements of taurine in biological samples by ion-exchange chromatography [17—19], coeluted with any of the metabolites of cysteine in our system. Elution times were 29.0, 15.3 and 15.0 min for ethanolamine, *o*-phosphorylethanolamine and glycerophosphorylethanolamine, respectively.

Both tetrahydrofuran concentration, as reported by Eslami and Stuart [10], and column temperature had substantial effects on the separation of the compounds shown in Fig. 4. Low concentrations of tetrahydrofuran (ca. 3%) significantly improved the resolution between hypotaurine and alanine and slightly improved the resolution of taurine and γ- or β-aminobutyrate. Increasing the column temperature from ambient to 41°C substantially improved the resolution of taurine and γ- or β-aminobutyrate. For samples that did not contain γ- or β-aminobutyrate, satisfactory resolution of hypotaurine and taurine could be obtained at ambient temperature.

We developed this method primarily for analysis of the in vitro production of metabolites of cysteine. A chromatogram of the acid-soluble extract from an incubation of rat hepatocytes with 25 m*M* cysteine is shown in Fig. 5. As with standard solutions, excellent resolution of cysteinesulfinate, hypotaurine and taurine from closely eluting amino acids was obtained. No peak in the position of cysteinesulfinate or hypotaurine was observed in the extract of hepatocytes incubated without cysteine, suggesting that other compounds present in rat liver do not interfere with analysis of these two compounds. A small amount of taurine was present in the extract of hepatocytes incubated without substrate as would be expected because taurine is normally found in tissues in relatively high concentrations.

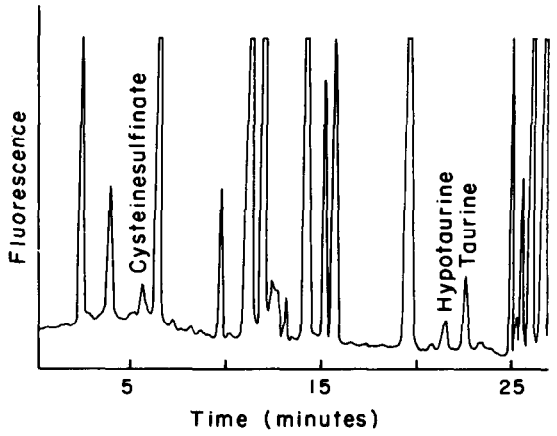

Fig. 5. Representative chromatogram of acid-soluble extract from an incubation of rat hepatocytes with cysteine. Approximately 50 mg of cells were incubated for 40 min with 25 m*M* cysteine; the acid-soluble extract was derivatized and chromatographed as described in the text.

Recovery of standard cysteinesulfinate, hypotaurine or taurine added to similar hepatocyte incubation mixtures prior to addition of perchloric acid ranged from 80 to 100%. Mean production of cysteinesulfinate, hypotaurine and taurine by hepatocytes incubated with 25 m*M* cysteine was 5.0, 4.2 and 0.5 pmol min^{-1} mg of $cells^{-1}$ (wet wt), respectively, for cells from three rats.

Sample chromatograms from the analysis of tissue and plasma samples are shown in Fig. 6. All runs were performed under the standard conditions described in Experimental. Taurine was present in all samples. Low levels of hypotaurine were consistently detected in liver, heart and kidney (not shown), but cysteinesulfinate was not detected in any of the samples. Taurine was resolved from the substantial amount of γ-aminobutyrate in brain samples; the resolution was improved by a further increase in the column temperature to 45°C (data not shown). The analysis of taurine and hypotaurine in tissues was reproducible. The coefficients of variation for determinations of hypotaurine and taurine in rat liver were 1.6% and 1.3%, respectively.

Values obtained for hypotaurine and taurine concentrations in various rat tissues and in human plasma analyzed by this method are summarized in Table I along with literature values for taurine [20—29]. The taurine concentrations obtained with this method agree closely with the literature values. Hypotaurine levels in normal rat tissues have not been reported previously.

Recoveries of cysteinesulfinate, hypotaurine and taurine from rat liver homogenized in sulfosalicylic acid (our standard procedure) were determined. These were also compared to recoveries for the same standards from samples homogenized in perchloric acid or homogenized in phosphate buffer and then heated at 100°C to precipitate the protein. The results are shown in Table II. Recovery of all three standards was greater than 90% when liver was homogenized in sulfosalicylic acid, and this appeared to be the best of the three methods for sample preparation. Recoveries were slightly lower when liver was homogenized in perchloric acid. Cysteinesulfinate was not recovered from liver prepared by heat precipitation of protein, but the hypotaurine peak increased

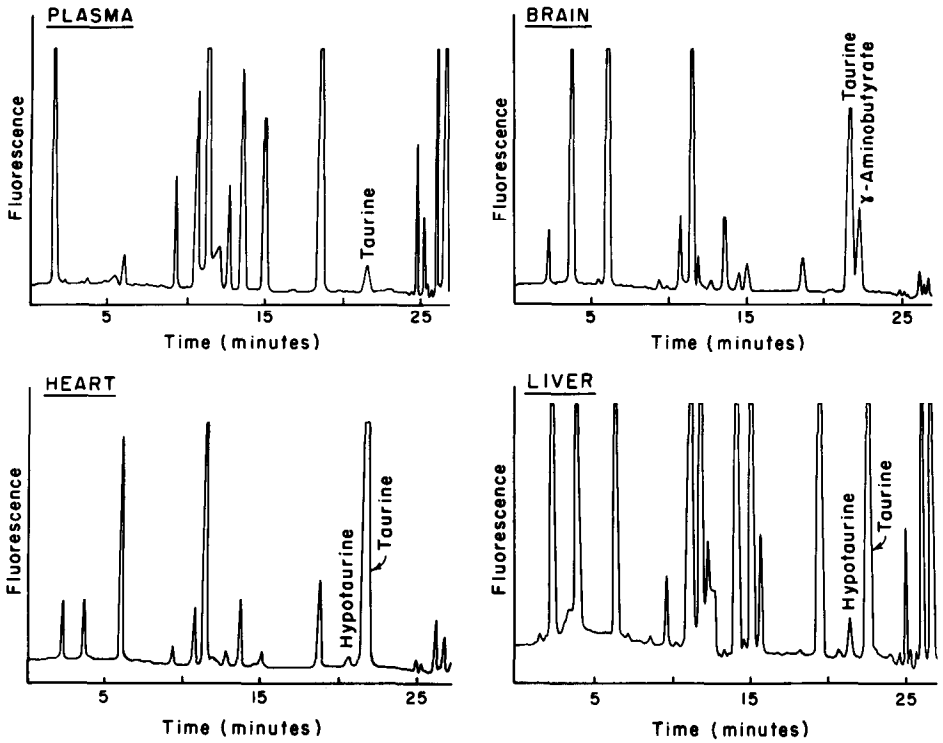

Fig. 6. Representative chromatograms for the analysis of taurine and hypotaurine in extracts of plasma, liver, brain and heart. Standard run conditions as described in the text were used for all samples.

TABLE I

DETERMINATION OF HYPOTAURINE AND TAURINE CONCENTRATIONS IN RAT TISSUES AND HUMAN PLASMA BY HPLC

Concentrations are expressed as μmol/g of tissue or μmol per 100 ml of plasma. Values are means ± S.D. for tissues from four rats or plasma from four women.

Tissue	Hypotaurine	Taurine	Mean literature value for taurine	References
Heart	0.27 ± 0.04	25.0 ± 0.6	24.3	20—23
Brain	ND*	6.5 ± 0.8	5.2	20—24
Liver	0.07 ± 0.02	1.4 ± 0.8	2.1	20—23, 25
Kidney	0.23 ± 0.05	8.9 ± 0.6	9.3	20—23
Plasma	ND	4.0 ± 0.7	4.4	26—29

*ND = Not detected.

markedly. When standard solutions were heat-treated, no loss of cysteinesulfinate was observed. Thus, cysteinesulfinate apparently was converted enzymatically to hypotaurine during heat treatment, and this method of sample preparation should not be used for cysteinesulfinate determinations.

Recoveries of cysteinesulfinate, hypotaurine and taurine from other tissues

TABLE II

RECOVERY OF CYSTEINESULFINATE, HYPOTAURINE AND TAURINE FROM RAT LIVER PREPARED FOR HPLC ANALYSIS BY THREE METHODS

Values are means ± S.D. for three to five determinations.

Method	Percentage of standard recovered*		
	Cysteinesulfinate	Hypotaurine	Taurine
Homogenization in 5% (w/v) sulfosalicylic acid	92 ± 8	95 ± 6	103 ± 7
Homogenization in 5% (w/v) perchloric acid	88 ± 12	83 ± 3	97 ± 10
Homogenization in 0.05 *M* phosphate buffer with heat precipitation of protein	0	82 ± 2	96 ± 3

*The concentrations of added standards were 20 μM for cysteinesulfinate, 20 μM for hypotaurine and 40 μM for taurine in the 20% (w/v) homogenate.

were similar to those reported for liver in Table II, except the recovery of cysteinesulfinate from heat-treated samples was better. Recovery of cysteinesulfinate from samples prepared by heat precipitation of protein was 63% for kidney and 91% for brain.

This HPLC method for the determination of all three of the metabolites of cysteine in the taurine pathway should facilitate further work in the area of cysteine metabolism. It should also be useful for the measurement of taurine and hypotaurine levels in biological samples. Taurine, hypotaurine and cysteinesulfinate are resolved from compounds that presented difficulties with previously published methods, and the sensitivity is sufficient to allow measurement of as little as 10 pmol of these compounds. The method is applicable to all physiological samples we have tried.

ACKNOWLEDGEMENT

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REFERENCES

1 T.P. Singer, in D.M. Greenberg (Editor), Metabolism of Sulfur Compounds, Metabolic Pathways, Vol. 7, Academic Press, New York, 1975, p. 535.
2 A.J.L. Cooper, Ann. Rev. Biochem., 52 (1983) 187.
3 K. Yamaguchi, S. Sakakibara, J. Asamizu and I. Ueda, Biochem. Biophys Acta, 297 (1973) 48.
4 M.H. Stipanuk and M.A. Rotter, J. Nutr., 114 (1984) 1426.
5 J. De La Rosa and M.H. Stipanuk, Comp. Biochem. Physiol., 81B (1985) 565.
6 J. De La Rosa and M.H. Stipanuk, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 44 (1985) 1591.
7 K. Knopf, J.A. Sturman, M. Armstrong and K.C. Hayes, J. Nutr., 108 (1978) 773.
8 W.G.M. Hardison, C.A. Wood and J.H. Proffitt, Proc. Soc. Exp. Biol. Med., 155 (1977) 55.
9 D.C. Turnell and J.D.H. Cooper, Clin. Chem., 28 (1982) 527.

10 M. Eslami and J.D. Stuart, J. Liquid Chromatogr., 7 (1984) 1117.
11 D. Hill, L. Burnworth, W. Skea and R. Pfeifer, J. Liquid Chromatogr., 5 (1983) 2369.
12 G.H.T. Wheler and J.T. Russell, J. Liquid Chromatogr., 4 (1981) 1281.
13 J.D. Stuart, T.D. Wilson, D.W. Hill, F.H. Walters and S.Y. Feng, J. Liquid Chromatogr., 2 (1979) 809.
14 W.G. Martin, B.L. Robeson and L.S. Cadle, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 288.
15 J.M. Berry and D.S. Friend, J. Cell Biol., 43 (1969) 506.
16 H.A. Krebs, N.W. Cornell, P. Lund and R. Hems, in F. Lundquist and N. Tygstrup (Editors), Regulation of Hepatic Metabolism, Academic Press, New York, 1974, p. 726.
17 S. Gurusiddaiah and R.W. Brosemer, J. Chromatogr., 223 (1981) 179.
18 J. Garvin, Arch. Biochem. Biophys., 91 (1960) 219.
19 T.L. Perry, D. Stedman and S. Hansen, J. Chromatogr., 38 (1968) 460.
20 J. Awapara, J. Biol. Chem., 218 (1956) 571.
21 J.B. Lombardini and E.V. Medina, J. Nutr., 108 (1978) 428.
22 J.A. Sturman, J. Nutr., 103 (1973) 1566.
23 D.G. Spaeth and D.L. Schneider, in R. Huxtable and A. Barbeau (Editors), Taurine, Raven Press, New York, 1976, p. 35.
24 J.A. Sturman, D.K. Rassin and G.E. Gaull, Life Sci., 21 (1977) 1.
25 Y. Pierre and F. Chatagner, Reprod. Nutr. Develop., 21 (1981) 555.
26 K.C. Hayes and J.A. Sturman, Annu. Rev. Nutr., 1 (1981) 401.
27 M. Young and M.A. Preston, Obstet. Gynaec. Brit. Cwlth., 76 (1969) 333.
28 J.C. Dickinson, H. Rosenblum and P.B. Hamilton, Pediatrics, 45 (1970) 606.
29 M.D. Armstrong and K.N. Yates, Amer. J. Obstet. Gynecol., 88 (1964) 381.