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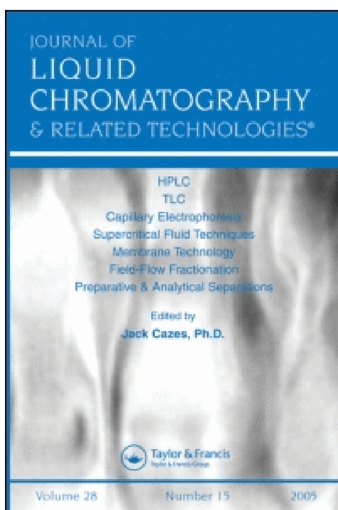

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Pekka Lähdesmäki[a]; Maritta Timonen[a]
[a] Department of Biochemistry, University of Oulu, Oulu, Finland

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FRACTIONATION INTO COMPONENTS OF A MIXTURE OF ACIDIC NINHYDRIN-POSITIVE COMPOUNDS OF MOUSE BRAIN EXTRACTS WITH THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

Pekka Lähdesmäki and Maritta Timonen
Department of Biochemistry, University of Oulu, SF-90570, Oulu 57, Finland

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ABSTRACT

A mixture of more than ten acidic or highly ionized ninhydrin-positive compounds of mouse brain extracts, occurring in ion-exchange chromatography from the beginning up to aspartic acid, was analyzed using ion-exchange and thin-layer chromatography standardized with known substances. These formed four peak groups in the chromatogram of an automatic amino acid analyzer. The first group contained cysteic acid, cysteinesulfinic acid and phosphoserine, which could be separated from each others only by thin-layer chromatography. Only phosphoserine could be identified in the brain extract (about 0.14 mmol/kg brain wet weight), however. Taurine (4.1 mmol/kg) and phosphoethanolamine (1.05 mmol/kg) in the second group could be satisfactorily separated from each others after the hydrolysis of glycerylphosphoethanolamine (about 0.6 mmol/kg) and certain acidic peptides with 6 mol/l HCl. Hypotaurine (0.03 mmol/kg) and urea (6.6 mmol/kg) were completely overlapped in the third peak, but urea was decomposed in the hydrolysis with 6 mol/l HCl. The fourth group consisted of aspartic acid (2.1 mmol/kg). A number of low-molecular weight peptides also appeared in the chromatograms, above all in the phosphoserine and taurine peaks, but they were eliminated by the hydrolysis. They contained, however, some of the above-mentioned critical amino acids (phosphoserine, taurine and aspartic acid).

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INTRODUCTION

Tissue extracts, and brain extracts in particular, contain a large number of acidic amino acids and certain other ninhydrin-positive compounds, which in the ion-exchange chromatography (e.g. in the automatic amino acid analyzers) can not be separated satisfactorily well from each others, but appear in two or three peaks in the chromatograms (1-6). This fraction (often called "taurine fraction") is a complex mixture of more than ten (see Ref. 1) acidic or highly ionized compounds of physiological importance, including taurine, phosphoserine, phosphoethanolamine, glycerylphosphoethanolamine, hypotaurine, cysteic and cysteinesulfinic acids, carbamylphosphate, urea and glucosaminephosphate. Moreover, the presence of a number of low-molecular weight peptides, including taurine and phosphoserine peptides, which are soluble in sulfosalicylic, trichloroacetic and perchloric acids used in the preparation of the tissue samples, has been reported (7,8).

Taurine (2-aminoethanesulfonic acid) is often the main constituent in the free amino acid pool, but how real are its concentration values calculated from the mixture of the most acidic amino compounds eluting in the ion-exchange chromatography just before aspartic and glutamic acids (5,7,9,10) ? For their strong ionization or having phosphoric acid groups or other extra acidic moieties, the amino acids of the "taurine fraction" as well as the peptides containing taurine and phosphoserine (7,8,11-15) behave similarly in the chromatography, in the ion-exchange chromatography in particular, and overlap each others. The first eluting acidic ninhydrin-positive compounds may thus distort seriously each others' quantitative determination. A good attempt

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to clarify the chromatographic determination of the components of the taurine peak has recently been made by Kontro *et al.* (5) and Marnela (10).

Simple hydrolytic and chromatographic means, which for their part bring facilitation to the exact quantitative determination of the individual components of the "taurine fraction" are given here. The methods were standardized with pure amino acids and some peptides as well as with certain taurine and phosphoserine peptides prepared from calf brain synaptosomes (7). The results, although not yet definitive, will show that serious attention must be paid to the interpretation of the chromatograms regarding compounds eluting before aspartic acid.

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MATERIAL AND METHODS

Whole brains of NMRI mice were used, but the subcellular fractions were prepared from calf brains. Newly removed brains were rinsed free of external blood clots, and the cortical layer from calf brains separated with a knife. The synaptosomes were prepared from the homogenates in cold 0.32 mol/l sucrose by the gradient centrifugation method of Whittaker *et al.* (16).

Tissue samples were homogenized in 5 % trichloroacetic acid (TCA), the precipitated proteins removed by centrifugation, and the TCA then removed by shaking with diethyl ether. The residual solution was lyophilized and the amino acids and peptides then dissolved in water. When 15 % perchloric acid (PCA) was used, the precipitable proteins were first removed by centrifugation and the supernatant then neutralized with KOH and the potassium perchlorate centrifuged. The neutral, protein-free extracts were lyophilized and the residue taken up in acidified water and used for chromatogra-

phic analysis. The extracts from the synaptosomes were prepared in a similar manner.

The amino acid samples were quantitatively analyzed in an automatic amino acid analyzer (Kontron Liquimat III), equipped with a two-channel peak integrator. Ion-exchange chromatography in separate short columns according to Garvin (17) also was used. Thin-layer chromatography was performed on silica gel (Kieselgel G) plates of thickness 0.25 mm. Two-dimensional chromatograms were developed with 70 % ethanol in water and 75 % phenol in water. The dried plates were sprayed with ninhydrin (0.5 % solution in butanol:acetic acid, 20:1, pH 3.3). The spots were developed for 10 min at 105 ^{o}C.

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Two unknown hydrolyzable spots on the TLC plates from calf brain synaptosomes (7,9) were projected to the corresponding unsprayed plates, the silica gel scraped up and the peptides extracted with water and lyophilized. They were analyzed for the amino acid composition after the hydrolysis with 6 mol/l HCl, and used as such for studying the chromatographic mobility in an amino acid analyzer.

Commecial pure amino acids (E. Merck, Darmstadt and Fluka AG, Buchs, Basel, Switzerland) and γ-glutamyltaurine (Chinoin Pharmaceuticals, Budapest) were used as standards.

RESULTS

As shown in Figure 1, the initial part of a chromatogram of the PCA extract of mouse brain, obtained with a modern "high resolution" automatic amino acid analyzer, contained more than ten different ninhydrin-positive compounds, which could not be separated satisfactorily well from each others. Using known amino acid

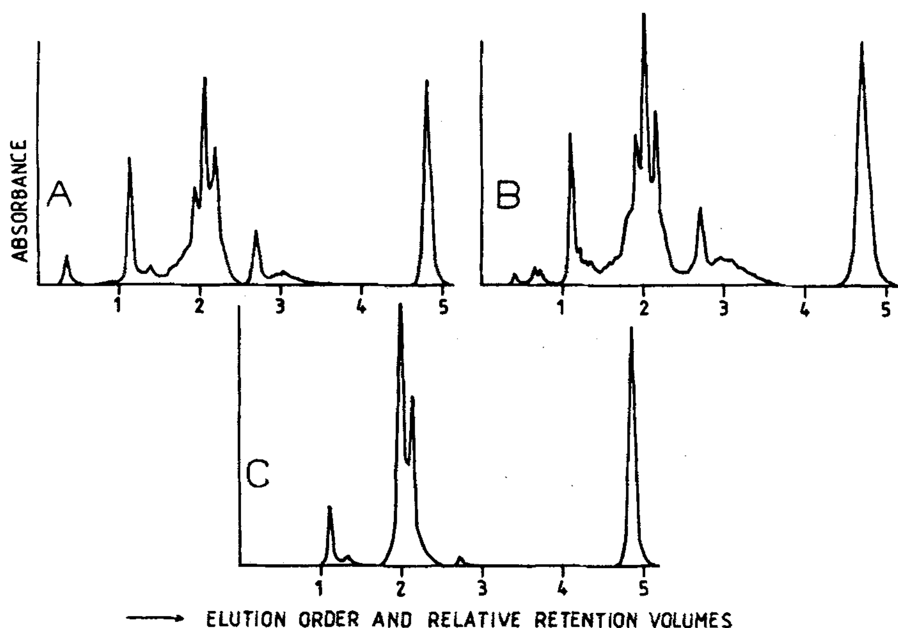

Figure 1. Initial parts of the ion-exchange chromatograms of the amino compounds of mouse brain extracts before (A) and after the hydrolysis with 2 mol/l (B) or 6 mol/l (C) HCl from a "high resolution" automatic amino acid analyzer (Kontron Liquimat III). For identification, see Figure 2.

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or peptide samples, the retention volumes of 14 different compounds were determined (Figure 2). These formed four peak groups, which could be separated quite well from each others, but inside the groups the peaks of different compounds had almost the same retention volumes. The first group contained carbamylphosphate, cysteic acid, cysteinesulfinic acid, phosphoserine, γ-glutamyltaurine and certain other taurine peptides (see Ref. 8). The second group contained taurine, phosphoethanolamine, glycerylphosphoethanolamine and certain phosphoserine and other acidic peptides (see Ref. 8), and the third

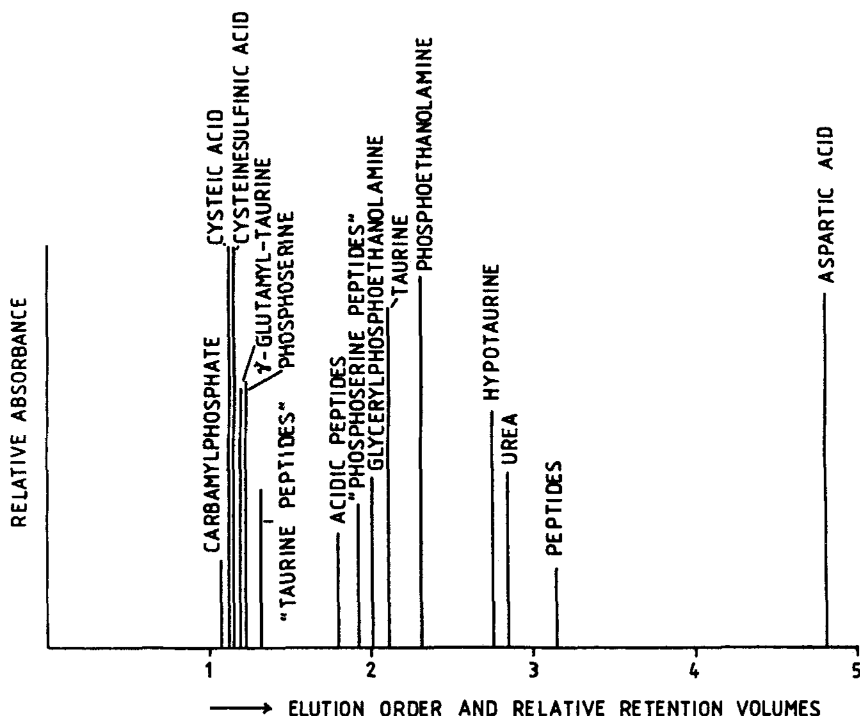

Figure 2. Retention volumes of 14 different acidic ninhydrin positive compounds analyzed with an automatic amino acid analyzer (Kontron Liquimat III) from standard amino acids, γ -glutamyl-taurine and certain other peptides prepared from calf brain synaptosomes. The hight of the absorbance lines depicts approximate molar sensitivity of the ninhydrin reaction.

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group hypotaurine, urea and again some synaptosomal peptides. The fourth group consisted of aspartic acid.

The concentrations of the amino compounds of the PCA extract of mouse brain, calculated from the chromatograms of Figure 1, are given in Table 1 before and after the hydrolysis with 2 and 6 mol/l HCl. Most of the peaks or peak groups of the brain extract could be identified with the standard samples (Figure 2), but very low concentrations of unknown compounds still ap-

TABLE I

Concentrations of the acidic ninhydrin-positive compounds of mouse brain after extraction with perchloric acid and after the hydrolysis with 2 and 6 mol/l HCl.

Compound	Concentrations mmol/kg wet weight		
	as such	2 M HCl	6 M HCl
unknown	0.01	-	-
unknown	-	0.02	-
Cysteic acid Cysteinesulfinic acid Phosphoserine	} 0.25	} 0.18	} 0.14
unknown	0.01	0.01	0.01
Peptides (ninhydrin-positive)	0.02	0.02	-
"Taurine peptides" (ninhydrin-negative)	-	0.27	-
Peptides (ninhydrin-positive)	0.55	0.50	-
"Phosphoserine peptides" (ninhydrin-negative)	-	0.21	-
Glycerylphosphoethanolamine	0.72	0.55	-
Taurine	4.10	4.90	4.88
Phosphoethanolamine	1.70	1.04	1.65
Hypotaurine Urea	} 6.86	} 6.59	0.03 -
Aspartic acid	2.06	6.87	7.50

Results are means of 5-6 experiments, S.D. being about 10 %. The order of substances refer to Figure 1. The concentrations of the peptides and unknown compounds were calculated using alanine as a standard.

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peared both before and after the hydrolysis. Certain peptides or peptide groups appeared between the single amino acids. A part of these became visible not until after the hydrolysis with 2 mol/l HCl (for 30 min in a boiling water bath), which is known to hydrolyze the acyl groups from the amino group. This procedure converts the originally N-acetylated peptides ninhydrin-positive. All the peptides disappeared in the hydrolysis with 6 mol/l HCl. The amount of aspartic acid increased 3 fold in the hydrolysis with 2 mol/l HCl, and was derived apparently from the hydrolysis of N-acetylaspar-

tate present in brain tissue (18), and still a little in the hydrolysis with 6 mol/l HCl, this aspartate being derived from the hydrolyzable peptides (4,9). The peaks of hypotaurine and urea were completely overlapped (Figure 2), but urea disappeared in the HCl hydrolysis, and a small amount of hypotaurine remained after the hydrolysis. As satisfied with the HCl treatments of the standards, hypotaurine, taurine, phosphoethanolamine, phosphoserine, cysteic acid and cysteinesulfinic acid did not be decomposed either in the 2 or 6 mol/l HCl solution.

The peaks of taurine, phosphoethanolamine and glycerylphosphoethanolamine appeared very close together (see also Ref. 5). A small decrease in the concentration of phosphoethanolamine due the hydrolysis with 2 mol/l HCl was observed, indicating that its original peak contained some hydrolyzable, ninhydrin-positive material. At the same time the amount of taurine increased slightly, but it is not known, what this increment in the taurine peak arises from, but probably from the large number of different acidic N-acetylaspartyl peptides (8), which are converted ninhydrin-positive by the hydrolysis with 2 mol/l HCl. Glycerylphosphoethanolamine was completely hydrolyzed to phosphoethanolamine, after which the determination of taurine and phosphoethanolamine was more reliable.

Cysteic and cysteinesulfinic acids and phosphoserine could not be separated from each others, but their common amount decreased significantly after the acid hydrolysis, indicating again some hydrolyzable material present in their original peak. In two-dimensional thin-layer chromatography, using 70 % ethanol and 75 % phenol in water, these critical amino acids could be separated very well from each others (Figure 3). Phosphoserine was

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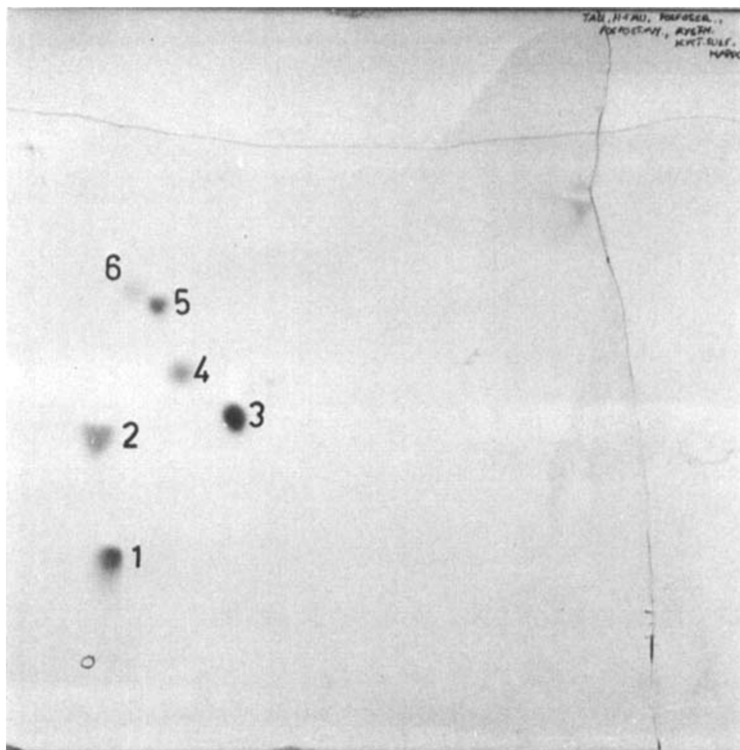

Figure 3. A two-dimensional thin-layer chromatogram of certain acidic amino acids, analyzed from a standard sample on 0.25 mm thick silica gel plates. The R_f values in brackets are $R_{fI}:R_{fII}$ = 70 % ethanol: 75 % phenol in water. 1 = phosphoethanolamine (0.17:0.03), 2 = phosphoserine (0.40:0.01), 3 = hypotaurine (0.50: 0.27), 4 = taurine (0.57:0.17), 5 = cysteinesulfinic acid (0.71:0.12), 6 = cysteic acid (0.73:0.07).

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identified in the mouse brain extract (Figure 4), but no traces of cysteic or cysteinesulfinic acid were observed. The first peak group (phosphoserine-cysteic acid group, Figure 1-2) contained thus apparently only phosphoserine after the hydrolysis with 6 mol/l HCl.

The separation of taurine and phosphoethanolamine from each others was unsuccesful in the ion-exchange

Figure 4. A two-dimensional thin-layer chromatogram of mouse brain extract, treated with 2 mol/l HCl for 30 min and run under identical conditions to that of Figure 3. 1 = probably phosphoethanolamine, 2 = probably phosphoserine.

chromatography by the method of Garvin (17, Table 2). Several other acidic amino acids came partially in the same fraction with these, but phosphoserine not. This old method is thus unsatisfactory for the determination of taurine, since it is contaminated by other acidic amino acids. More useful might be the o-phthalaldehyde-

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TABLE II
Recovery percentages of taurine and certain other acidic compounds in ion-exchange chromatography performed according to the method of Garvin (17).

Compound	Recovery %
Taurine	100.0
Hypotaurine	26.2 ± 5.0
γ-Glutamyl-taurine	27.4 ± 15.2
Cysteic acid	22.6 ± 18.1
Phosphoethanolamine	115.2 ± 9.8
Phosphoserine	8.2 ± 2.8

Results (means ± S.D.) are from 3 determinations. The recovery value of taurine was taken as 100 and others expressed relative to this.

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urea reaction of Gaitonde and Short (19), since it is more specific for taurine, even though performed after the passage of the tissue extract through the ion exchange resins, when there again is a possibility of contamination.

The acidic low-molecular weight peptides occurring in the chromatograms between the amino acids have been studied elsewhere (4, 7-9). They contained 5-6 different amino acids among which acidic amino acids, aspartic and glutamic acids, phosphoserine and taurine have a main role.

DISCUSSION AND CONCLUSIONS

The complicated composition of the fraction of the acidic ninhydrin-positive compounds in tissue extracts has been known for several years; at least 10-15 different amino acids or their derivatives have been reported in the ion-exchange chromatograms in two or three peak grouos before aspartic and glutamic acids (see e.g. Ref. 1, p. 50-51). Numerous attempts have been made to divide

these mixtures of compounds into fractions of individual substances, but the problem is still largely the same: the most acidic compounds, being eluted in the volume of cysteic acid and taurine in partucular, are completely overlapped.

The strongly acidic functional groups in the compounds dealed here make their pI values low. For instance the pK_a values of taurine are 1.5 and 8.74, giving the pI value of 5.12 for it (20). The situation is the same with most of the above-mentioned amino acids and peptides, which contain extra acidic groups. The free acidic groups in them seem to be the main chromatographic determinant in the ion-exchange chromatography, and for this reason several different compounds fall into the same fraction.

The present study led us to the conclusion that the direct analysis of a brain extract does not give correct results about the concentrations of the acidic amino acids. Better quantitative results can be obtained by comparing the analysis results after the hydrolysis of the sample with 2 and 6 mol/l HCl. In some cases qualitative thin-layer chromatography is essential for the identification of the amino acids.

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ACKNOWLEDGEMENTS

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