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COMPARATIVE ANALYSIS OF EXTRACTION PROCEDURES AND CHROMATOGRAPHIC METHODS ON RAT BRAIN AMINO ACIDS

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SUMMARY

A statistical comparison of data on levels of amino acids obtained from analyses by an automatic analyzer and by thin-layer chromatography and paper chromatography showed good agreement between analyzer and thin-layer chromatographic data except for glutamine. Paper chromatography gave lower levels for glycine, alanine and glutamine. Comparison of analyzer data only obtained after extraction of the tissue with picric or perchloric acid revealed no significant differences in the levels of the amino acids studied.

INTRODUCTION

With the increasing use of thin-layer chromatography (TLC), automatic analysis and/or paper chromatography (PC) to study the concentrations of amino acids in the central nervous system (CNS), the question arises as to whether or not valid comparisons between these data can be made. Furthermore, even when the same technique has been employed to determine the levels of the amino acids, often the tissue has been prepared for analysis by different extraction procedures. Although it seemed worthwhile in general to ascertain whether such data were comparable, we had a particular interest in this problem. At one time or another each of these three methods had been used in our laboratory and we wanted to be able to compare data from our earlier studies with those from our later ones. Therefore, it was necessary to evaluate statistically the data we had obtained with each method. The results of a comparative analysis of our data on amino acid levels in the CNS obtained with these three techniques and with two different extraction procedures are the subject of this report.

TABLE I

R_F VALUES FOR THIRTY-SIX NINHYDRIN POSITIVE COMPOUNDS DETERMINED BY TWO-DIMENSIONAL TLC

Ninhydrin positive compound	*1st Phase*	*2nd Phase*	*Ninhydrin positive compound*	*1st Phase*	*2nd Phase*
DL-α-Amino butyric acid	0.82 ± 0.007	0.69 ± 0.005	DL-+ *allo*-Δ-Hydroxylysine·HCl	0.53 ± 0.000	0.28 ± 0.000
α-Amino isobutyric acid	0.89 ± 0.005	0.71 ± 0.013	L-Histidine	0.47 ± 0.008	0.33 ± 0.012
DL-β-Amino isobutyric acid	0.81 ± 0.007	0.59 ± 0.010	Hydroxy-L-proline	0.70 ± 0.005	0.41 ± 0.026
L-Alanine	0.69 ± 0.007	0.59 ± 0.070	DL-Homocystine	0.53 ± 0.002	0.15 ± 0.000
β-Alanine	0.71 ± 0.004	0.39 ± 0.005	L-Isoleucine	0.93 ± 0.006	0.86 ± 0.019
L-Arginine·HCl	0.57 ± 0.010	0.23 ± 0.020	DL-Lanthionine	0.69 ± 0.000	0.34 ± 0.000
L-Asparagine	0.46 ± 0.008	0.21 ± 0.005	L-Leucine	0.92 ± 0.010	0.88 ± 0.019
Aspartic acid	0.58 ± 0.001	0.07 ± 0.003	L-Lysine	0.56 ± 0.060	0.50 ± 0.040
L-Canavanine sulfate	0.71 ± 0.220	0.16 ± 0.000	L-Methionine	0.84 ± 0.014	0.75 ± 0.020
DL+*allo*-Cystathionine	0.11 ± 0.007	0.08 ± 0.005	DL-Pipecolic acid	0.91 ± 0.000	0.84 ± 0.000
L-Cysteic acid	0.60 ± 0.003	0.08 ± 0.004	L-Phenylalanine	0.86 ± 0.008	0.85 ± 0.017
L-Cysteine (free base)	0.69 ± 0.006	0.09 ± 0.010	L-Proline	0.80 ± 0.010	0.67 ± 0.009
L-Cystine	0.34 ± 0.010	0.08 ± 0.005	L-Serine	0.58 ± 0.007	0.36 ± 0.040
DL-Dopa	0.62 ± 0.005	0.28 ± 0.014	L-2-Thiol histidine	0.53 ± 0.000	0.29 ± 0.000
GABA	0.76 ± 0.005	0.58 ± 0.020	L-Threonine	0.67 ± 0.009	0.58 ± 0.050
L-Glutamine	0.54 ± 0.009	0.28 ± 0.020	L-Tryptophan	0.68 ± 0.009	0.64 ± 0.010
L-Glutamic acid	0.65 ± 0.010	0.10 ± 0.003	L-Tyrosine	0.85 ± 0.015	0.55 ± 0.038
L-Glycine	0.55 ± 0.009	0.38 ± 0.030	L-Valine	0.90 ± 0.015	0.79 ± 0.010

MATERIALS AND METHODS

Since the procedures used in our laboratory to determine amino acid levels in the CNS by PC and by automated analysis have been fully described in previous papers[1–6], only the methodology for TLC will be given in detail here.

Preparation of tissue

Twelve Wistar rats bred in our animal colony were killed by decapitation. The calvarium was quickly removed; the brain was sectioned at the level of the calamus scriptorius, immediately frozen between sheets of dry ice and stored at −45° until extracted. The brain from each of six animals was homogenized with 15 volumes of 1.0% picric acid. The homogenate was centrifuged at 5000 r.p.m. for 20 min and the supernatant was decanted[2]. The protein pellet was washed twice with 4.0 ml of distilled water and centrifuged and the supernatant and the washings were pooled. To remove the picric acid, the solution was mixed with Dowex-2 (2 × 8–400) ion-exchange resin, 200–400 mesh, and filtered (Beckman Instruction Manual A-IM-3, 1965). The sample was lyophilized, dissolved in 4.0 ml of glass-distilled water and run through a 10 × 9 cm Dowex-50W (50 × 8–400), 200–400 mesh ion-exchange resin column which was then washed with 50.0 ml of glass-distilled water to remove biological contaminants[7,8]. This fraction also contains taurine, glycerophosphoethanolamine, phosphoethanolamine and urea (personal observations). The amino acids were then eluted with 30.0 ml of 8.0% ammonium hydroxide and lyophilized. The samples were dissolved in 1.5–2.0 ml of 10% isopropanol, centrifuged and stored at −45° until used. Aliquots of the samples were used for the determination of the levels of the amino acids by TLC, PC and the amino acid analyzer.

The remaining six brains were extracted by a method using 6.0% perchloric acid as described by AGRAWAL *et al.*[4], and amino acid levels in these six brains were determined by the amino acid analyzer only.

Thin-layer chromatography

Sonified (1.0 min) slurries of 16 g of Cellulosepulver MN 300 (Macherey, Nagel and Co.) washed according to the method of HAWORTH AND HEATHCOTE[9] to remove impurities and mixed with 75.0 ml of glass-distilled water and 5.0 ml of 100% ethanol were spread on the chromatoplate in a layer 0.4 mm thick by means of a Shandon Unoplan Leveller (Shandon Scientific Company, Inc., Sweickley, Pa.). The plates were developed in a Colab glass chromatank (Colab Laboratories, Inc., Chicago Heights, Ill.). By using a Teflon plate holder designed in our laboratory, as many as twenty plates could be developed at one time in one chromatank (Fig. 1).

The plates were spotted in duplicate with aliquots equivalent to 10.0 mg of tissue and run through two solvent systems. The developing solvents were 95% ethanol–double distilled water–acetic acid (35:15:1) and chloroform–methanol–ammonia (4:4:1). The first phase required 2.5 h running time and the second 1.5 h; the plates were dried after each phase. Filter paper placed on the four sides of the chromatank provided the saturated atmosphere necessary for good separation.

An 0.11% solution of ninhydrin in 100% ethanol–acetic acid (66:23) was used as the color reagent. The plates were dipped in the ninhydrin solution and allowed to stand overnight at room temperature or, for quick analysis, were heated at 60°

TABLE II

STATISTICAL ANALYSIS OF TWO EXTRACTION PROCEDURES AND THREE METHODS OF DETERMINING BRAIN AMINO ACIDS

Perchloric acid samples were drawn from a separate set of extracts; picric acid samples were aliquots drawn from the same extracts; ( ) = number of determinations; values expressed as µmoles/g wet weight rat brain tissue.

	Glutamic acid	*GABA*	*Glutamine*	*Aspartic acid*	*Glycine*	*Alanine*	*Threonine*	*Serine*
Analyzer[a] (6)	8.35 ± 0.47	3.06 ± 0.47	5.30 ± 0.63	2.20 ± 0.14	1.04 ± 0.10	0.75 ± 0.06	0.29 ± 0.02	1.09 ± 0.03
Analyzer[b] (6)	8.18 ± 0.21	2.87 ± 0.20	5.03 ± 0.21	1.99 ± 0.08	0.87 ± 0.04	0.73 ± 0.01	0.31 ± 0.03	1.10 ± 0.05
"P"[c]	N.S.[d]	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
TLC[b] (6)	7.76 ± 0.50	2.94 ± 0.27	4.02 ± 0.44	2.12 ± 0.39	—	0.79 ± 0.17	0.46 ± 0.07	—
"P"	N.S.	N.S.	<0.02	N.S.		N.S.	N.S.	
PC[b] (6)	8.63 ± 0.52	2.61 ± 0.18	4.27 ± 0.37	2.09 ± 0.40	0.61 ± 0.05	0.58 ± 0.01	0.40 ± 0.03	0.89 ± 0.06
"P"	N.S.	N.S.	<0.02	N.S.	<0.05	<0.05	N.S.	N.S.

[a] = 6% Perchloric acid.
[b] = 1% Picric acid.
[c] "P" = level of probability.
[d] N.S. = not significant.

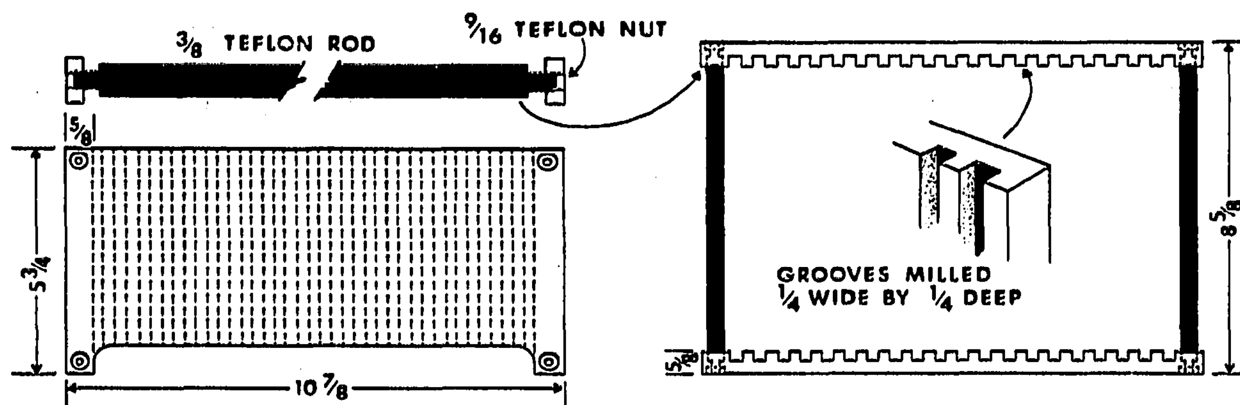

Fig. 1. Specification drawing of Teflon plate holder used for thin-layer chromatography. All measurements are in inches.

for 8 min. The spots were removed from the plates and the color was eluted first with 1.0 ml of a 0.5% solution of ninhydrin in 80% ethanol for 15 min and then by adding 4.0 ml of 70% acetone to the 0.5% ninhydrin for 30 min. The samples were centrifuged to remove the cellulosepulver.

The amino acid standards were obtained from Mann Research Laboratories, New York, N.Y. The standard stock solutions were prepared by dissolving the amino acid in 10% isopropanol to contain 5.0 µg/10.0 µl. In recovery experiments the amino acid standards were added directly to the tissue homogenate. Standard aliquots of 5 µg were spotted on plates and run simultaneously with the sample plates.

Color concentration was determined on a Beckman DU spectrophotometer and compared with that produced by 5.0 µg of the amino acid standard.

Since only glutamic acid, GABA, glutamine, aspartic acid, glycine, alanine, threonine and serine can be readily determined in this way, the RESULTS AND DISCUSSION relate only to these eight compounds.

Paper chromatography

Paper chromatography was performed according to the method of MOUREK *et al.*[6].

AUTOMATED ANALYSIS

Analyses of the amino acid levels were made on a Beckman automatic amino acid analyzer Model 120C by a method previously described[5].

An analysis of variance of unequal samples and Student's t test were used to determine significant differences.

RESULTS AND DISCUSSION

Each of the R_F values for the amino acid standards shown in Table I is the mean of six analyses ± SEM. In most instances distinct separation of the amino

acids was achieved by the technique described above. Glycine–serine and isoleucine–leucine tended to overlap slightly, but could be separated successfully when removed from the plate. Cystine trailed during the first solvent phase. Except for proline and hydroxyproline, which gave a yellow color, all of the commonly occurring amino acids eluted from the Dowex-50W column gave a light blue to dark purple color with ninhydrin.

In control experiments the recovery of glutamic and aspartic acids, glutamine, GABA and alanine ranged between 90 and 110% with a 95% average. By adding the standard amino acids directly to the tissue homogenates, not only the sensitivity of the TLC procedure but also the effectiveness of the method of extraction could be assessed. The procedures employed eliminate biological contaminants from the tissue extract and also remove from the cellulose powder contaminants that produce distortions on TLC[7–9].

The TLC procedure described here is a relatively rapid and accurate method for the qualitative and quantitative determination of the concentrations of amino acids in nervous tissue. Standard amino acids in quantities as low as 5 μg and amino acids in aliquots equivalent to 10 mg or less of nervous tissue can be accurately analyzed. For those amino acids present in lower concentrations a larger aliquot can be used to intensify the color of the spots. These solvent systems give compact spots and except for cystine, control trailing, so that the separate spots can be removed from the chromatoplate and the color eluted for colorimetric quantitation with relative ease. The systems also give highly reproducible R_F values as shown by the low SEM values in Table I.

A comparison of values obtained from material extracted with either a 1% picric acid or a 6% perchloric acid solution (Table II) and analyzed on an automatic analyzer showed no differences between these extraction media. There was a slight but significant difference between the levels of glutamine as determined by automated column analysis and by TLC (Table II): that determined by TLC being approximately 1.0 μmole/g wet tissue weight lower than that determined by automated analysis. On the other hand, PC gave significantly lower values in the levels of glutamine, glycine and alanine (Table II) than TLC or automated analysis.

It was concluded that the two extraction procedures were equally effective, and that there were relatively few differences between the analyses of samples for the amino acids whether performed on the automatic analyzer or on TLC. In contrast, the more significant differences observed with PC were probably due to the method itself, especially since there were no differences between the levels of brain amino acids determined in the two groups of rats by automatic analysis (Table II) when the methods of extraction were being compared.

There is considerable advantage in using TLC for biological application since 10 mg or less of tissue produces well separated spots on the chromatograms and a large number of samples can be analyzed at one time. TLC is a rapid, reproducible method for studying amino acid concentrations in the small areas and nuclei of the brain in animals of any age. Moreover, to obtain a quick, accurate indication of the amino acids present, smaller amounts of blood, cerebrospinal fluid and urine are required for TLC than for the automatic analyzer.

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