A METHOD FOR THE GAS-CHROMATOGRAPHIC ANALYSIS OF N-ACETYL-ASPARTIC ACID IN BRAIN

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In the last few years the gas-chromatographic determination of amino acids has been the subject of an extensive study in this laboratory[1,2]. The esterification of the carboxylic group of the amino acids by treatment with diazopropane improves and shortens the preparation of these derivatives[3] for gas chromatography. Furthermore, this reagent allowed us to develop a rapid method for the quantitative determination of amino acid N-acyl derivatives (acetyl, formyl, etc.). A new method for the determination of the N-acetyl-aspartic acid (NAA) present in brain is now reported.

EXPERIMENTAL

Preparation of brain extracts

The rats and mice were decapitated and the brain rapidly removed (about 2 min) and homogenized in 75 % ice cold ethanol 1:10 (w/v). (Ethanol was chosen for the precipitation of the proteins because other precipitants, *e.g.* sulphosalicylic, trichloroacetic, perchloric or picric acids were found to lower the recovery and to interfere with the esterification of NAA.) After being kept for 1 h at 4°, the homogenates were centrifuged at 15,000 r.p.m. for 30 min. An aliquot of the supernatant (2.5 ml) was evaporated to dryness under vacuum (15 mm Hg).

The residue was redissolved in the same volume of water (2.5 ml) and centrifuged clear at 5,000 r.p.m. for 10 min. An aliquot of the supernatant (2 ml) was transferred to a 0.9 × 12 cm glass column packed with Zerolit 225, H^+ form (30–45 μ). The resin was then washed thrice with 3 ml portions of water. Amino acids and inorganic cations are retained on the resin column. NAA, anions and neutral molecules wash through.

The filtrate and washings were evaporated to dryness.

Preparation of N-acetyl-aspartic acid propyl ester

The residue was dissolved in 2 ml of methanol and then stirred with 5 ml of an ethereal solution of diazopropane. The diazopropane solution was prepared by adding 50 % aqueous KOH to N-propyl-N'-nitroso-nitroguanidine dissolved in ethyl ether. After 5 min, the sample was evaporated to dryness, redissolved in 0.25 ml of ethylene chloride and 2 μl, corresponding to 1.6 mg of brain, were injected into the chromatographic column.

Gas chromatography

The gas chromatograph used was Model C analytical unit P.AID (Carlo Erba, Milan), equipped with a hydrogen flame ionization detector.

The stationary phase was Carbowax 20 M, 1 %, on Gas chrom P (100 mesh) packed into a two-meter glass column (internal diameter 2 mm, external diameter 4 mm).

The flow rate of the carrier gas (nitrogen) was 9.2 ml/min. The column temperature was programmed from 150° to 200° with a linear increase of 18.3°/min.

Quantitative analysis

For calibration, calculations and identification the internal standard technique was used. Stearic acid methyl ester was chosen as the internal standard because of its suitable retention time. The area of the peaks was determined by measuring, in convenient units, the height and the width of the peak at half height.

A linear calibration curve was obtained for NAA by using different amounts of NAA (kindly supplied by Laboratorio Bioterapico Selvi, Milan) with a constant amount of the internal standard.

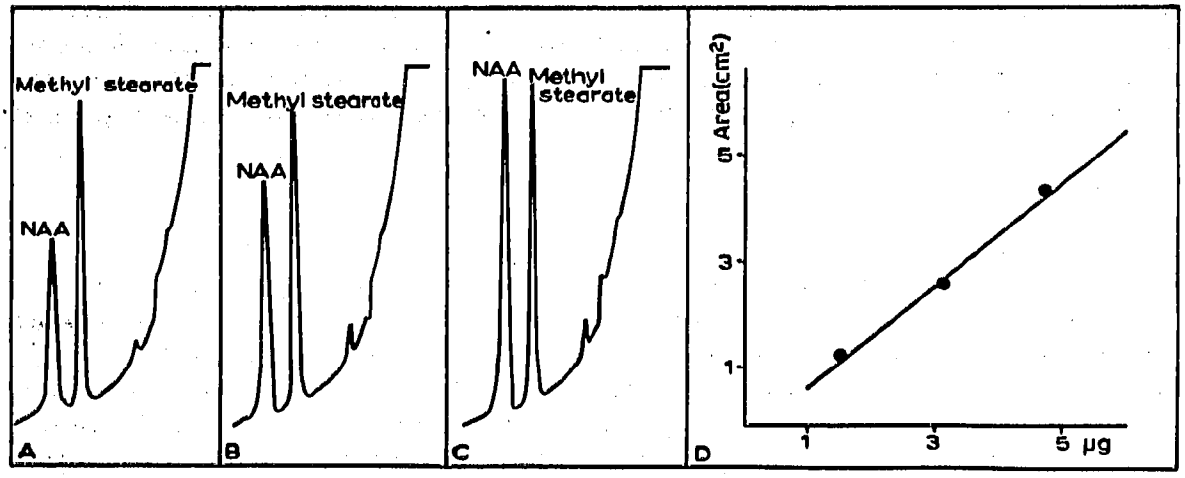

Fig. 1. The chromatograms A, B and C were obtained with increasing amounts of N-acetyl-aspartic acid (NAA) (1.6, 3.2 and 4.8 µg, respectively) and a constant amount of the internal standard. In D the relation between peak area and amount of NAA is shown.

In our experimental conditions the correction factor for the NAA propyl ester was 2.141. The results were linear over a range of 1 to 5 µg of NAA, with a recovery of the NAA added to brain around 110 % (see Fig. 1). A summary of the concentrations of NAA found in the brain of rats and mice is given in Table I.

TABLE I

N-ACETYL-ASPARTIC ACID (NAA) FOUND IN BRAINS OF RATS AND MICE
The number of determinations is given in brackets.

Animal species	*NAA content of brain mg/g ± S.E.*	
Male rats	1.53 ± 0.10	(4)
Female rats	1.45 ± 0.08	(4)
Male mice	1.28 ± 0.08	(4)
Female mice	1.22 ± 0.02	(4)

The method described can also be used for other N-acyl derivatives of amino acids. It should be noted that N-formyl-aspartic acid and N-acetyl-glutamic acid have a retention time different from the one characteristic for NAA.

Other methods used for the identification of NAA

The identity of NAA extracted from the brain was also confirmed by paper and thin layer chromatography, by comparison with synthetic NAA. The solvent system used was a mixture of butanol, methanol, and NH_4OH (4:1:5). After development, the chromatogram was dried and the NAA showed as a yellow spot on a blue background after dipping the paper into a solution of bromocresol green dissolved in acetone (0.01 %) with a drop of morpholine added[4].

The chromatogram, after location of the NAA, was subjected to acidic hydrolysis by spraying with a solution of 6 *N* HCl. The paper was heated for 20 min in an oven at 100° and after total evaporation of the HCl, the chromatogram was sprayed with ninhydrin (0.2 % in *n*-butanol). A violet spot appeared at the same point as that where the NAA was previously located. Before hydrolysis the ninhydrin reaction was negative.

DISCUSSION

In the last ten years several investigations[5–10] have shown that N-acetyl-L-aspartic acid (NAA) is present in the central nervous system of vertebrates.

In the literature two methods have been used for the estimation of NAA. The first one, devised by FLEMING (quoted in ref. 8) consists of the enzymatic hydrolysis of NAA to acetate and aspartate, transamination of aspartate to oxaloacetate, and reduction of the latter to malate. Since the reduction step involves the concomitant oxidation of NADH to NAD^+, the NAA is determined by measuring the difference in the optical density at 340 mμ.

The second method involves the chromatographic separation of the protein-free extract on a resin column: alternate effluent fractions are analyzed by the ninhydrin method, without or with previous mild acid hydrolysis.

The method described in this paper, suitable for the analysis of 1–2 μg of NAA, has the advantage over the previous methods of being simple and rapid. The reproducibility and the high sensitivity of the present method permits the investigation of the levels of NAA in various parts of the brain of small experimental animals.

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SUMMARY

A gas chromatographic method for the determination of N-acetyl-aspartic acid (NAA) in biological materials is presented. This technique permits the estimation of the NAA and possibly other acetyl amino acids in amounts as low as 1–2 μg. The recovery, the accuracy and the sensitivity of this method are discussed.

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