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PROCEDURAL DIFFICULTIES IN THE GAS–LIQUID CHROMATOGRAPHIC ASSAY OF THE ARYLALKYLAMINES

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SUMMARY

A gas–liquid chromatographic method for the estimation of 2-phenylethylamine in rat brain tissue is described. The procedure involves initial separation of the amine from homogenates with ion-exchange chromatography, subsequent acetylation of the eluate and extraction of the N-acetylated amine with ethyl acetate. The N-acetyl-2-phenylethylamine derivative is then converted to the pentafluoropropionyl derivative and quantitated with electron capture detection. Difficulties involved with alternative separative procedures and derivative formation are discussed.

INTRODUCTION

There has been considerable interest in recent years in a number of non-catecholic arylalkylamines which have been identified in the mammalian central nervous system (CNS). The pharmacological properties of these substances and their possible involvement in CNS function have stimulated attempts to develop methods for the quantitative determination of these amines in nervous tissue.

Methods currently available with the required sensitivity and specificity are the radioenzymatic and mass spectrometric (MS) assays. The enzymatic methods depend on the enzyme-catalysed transfer of a [^{3}H]- or [^{14}C]methyl group of S-adenosylmethionine to either the phenolic or the aliphatic amino function of the amine of interest (for review see ref. 1). The methylated product is then purified by solvent–solvent partition or paper chromatography, and quantified by liquid scintillation counting. MS methods involve the conversion of the amines to suitably volatile derivatives, gas–liquid chromatography (GLC) and subsequent quantification with a low-resolution mass spectrometer using multiple specific ion detection[2]. Alternatively, purification is carried out by classical chromatographic procedures and the derivative then evaporated into the ionisation source of a high-resolution mass spectrometer and quantified using the integrated ion current technique[3]. Each of the methods has its own advantages and disadvantages which space does not permit us to discuss here.

The use of GLC with electron capture (EC) detection offers a suitable alternative to the more expensive MS methods. However, GLC-EC requires care in its application as specificity is endowed in this case only by the preliminary purification of the tissue extract and the resolution of the gas chromatograph, the EC detector being

innately far less specific than the mass spectrometer. Nevertheless, we have found it feasible to use GLC-EC for the estimation of a number of these amines in tissue. The discussion will concern itself with some of the difficulties we have encountered in the development of a method for the determination of 2-phenylethylamine (PE) in rat brain tissue.

PE is stable under acidic conditions, and homogenisation of the brain tissue initially in 0.4 *N* perchloric acid at 0° was found to yield consistent recoveries of the amine. After centrifugation, the supernatant was adjusted to pH 8.0 with 4 *N* potassium hydroxide, and the potassium perchlorate removed by centrifugation before further analysis.

EXTRACTION BY LIQUID ION-EXCHANGE

In a previous publication[4] di(2-ethylhexyl) phosphate (DEHPA), a liquid cation exchanger, was used to recover noradrenaline and dopamine from aqueous solution (pH 8.0). The extraction of PE from pH 8.0 aqueous solution with an equal volume of 2.5% v/v solution of DEHPA in chloroform does give excellent recoveries of PE when the amine is then back-extracted into an equal volume of 0.5 *N* formic acid. However, sodium ions are removed from the aqueous phase by DEHPA and are subsequently found as sodium formate on evaporation of the formic acid solution. Sodium formate decomposes on addition of pentafluoropropionic anhydride (PFPA) with the presumed formation of sodium pentafluoropropionate. Following evaporation of the reagent and reconstitution of the sample in ethyl acetate, this salt decomposes at the injection port temperature of 250° required for the satisfactory chromatography of the PE-pentafluoropropionate (PE-PFP) derivative (the derivative of choice in this study). This problem can be overcome by removing the sodium ions from the ethyl acetate solution by percolating the sample through a column of Amberlyst 15, prior to GLC analysis. Amberlyst 15 is a macroreticular ion-exchange resin from which all water can be removed without causing a marked decrease in its ion-exchange capability. The PE-PFP is not bound to the resin and the effluent from the Amberlyst 15 column has satisfactory GLC properties. However, while columns of Amberlyst 15 are quite adequate for use with amounts of PE-PFP above 10 ng, below this level there is an apparent adsorption of the PE-PFP to the resin with a resultant loss of derivative. This loss increases proportionately as the amine concentration decreases and this procedure was therefore considered unsuitable for this analysis.

ACETYLATION IN AQUEOUS SOLUTION

To overcome the problem of salts being carried through the procedure and causing subsequent interference in the GLC analysis, an aqueous acetylation step was included. Primary amino and phenolic functional groups can be acetylated in slightly alkaline aqueous solution (pH 7.5–8.5) very efficiently by the addition of acetic anhydride[5]. The resultant acetylated amines are then efficiently removed from the aqueous phase (N-acetyl-PE 90%) by a single extraction with ethyl acetate. Evaporation of the organic phase indicates that no salts are partitioned from the aqueous phase and reaction of the N-acetyl-PE with 100 μl PFPA in 20 μl ethyl acetate for 30 min at

60° results in the formation of a derivative suitable for electron capture detection. While the structure of the N-acetyl-PE has been confirmed by MS, the structure of its pentafluoropropionyl derivative has not yet been elucidated, though it may result from a cyclisation and rearrangement similar though not identical to that suggested for N-acetyltryptamine and N-acetyl-5-hydroxytryptamine by Cattabeni *et al.*[6].

REMOVAL OF EXCESS REAGENT

The standard procedure for removal of the excess reagent and its byproducts before GLC is to evaporate to dryness and reconstitute in a small volume of organic solvent such as ethyl acetate. However, the pentafluoropropionyl derivatives of these amines are extremely volatile and considerable losses are incurred by this procedure. This problem can be circumvented in methods in which a mass spectrometer is used as the detector by using deuterated internal standards whose volatility will presumably differ only slightly from that of the non-deuterated analogue. Alternatively, a close chemical analogue of the compound of interest can be introduced as an internal standard and a calibration curve constructed against the compound of interest. However, in our hands the use of analogues led to an unacceptable degree of variability and an alternative was sought. The N-acetylpentafluoropropionyl PE derivative undergoes aqueous hydrolysis with less facility than the PE-PFP derivative. Consequently, rather than evaporating the reaction mixture under nitrogen it is partitioned between cyclohexane and a saturated solution of sodium tetraborate; the N-acetylpentafluoropropionyl PE derivative preferentially partitions into the organic layer, which contains essentially no contaminants from the PFPA. An aliquot of the organic layer has excellent GLC characteristics. This approach, *i.e.*, acetylation, extraction, reaction, and partition, has therefore allowed us to circumvent several problems, namely salt contamination in the reaction mixture, disadvantageous extraction conditions of the amines from aqueous solution and volatility of the pentafluoropropionyl derivatives.

ADDITIONAL PURIFICATION PROCEDURES

It was found that the acetylation procedure by itself was not an adequate purification procedure prior to GLC, and two alternative preliminary purification procedures were investigated.

Thin-layer chromatography

The extract, subsequent to acetylation, was subjected to thin-layer chromatography (TLC) on silica gel with ethyl acetate or ethyl acetate–methanol (9:1) as the solvent. The R_F values of a number of acetylated amines are shown in Table I. The appropriate area of the plate was then removed and eluted with ethyl acetate containing 0.01 *N* HCl. This procedure was perfectly adequate using 25 ng standard of PE derivative but as the amount of PE decreased below 5 ng recovery of the derivative from the plate was decreased.

Ion-exchange chromatography

The second approach was to use ion-exchange columns for the absorption of

TABLE I

R_F VALUES OF ACETYL DERIVATIVES OF SOME AMINES ON SILICA GEL

Solvent systems: (A) ethyl acetate; (B) ethyl acetate–methanol (9:1).

Acetylated derivative of	*Solvent system*	
	A	*B*
2-Phenylethylamine	0.27	0.72
Benzylamine	0.35	0.69
p-Tyramine	0.21	0.62
Octopamine	0.12	0.52
Noradrenaline	0.13	0.55
Dopamine	0.19	0.65
Adrenaline	0.14	0.56
p-Chlorophenylethylamine	0.23	0.60
Amphetamine	0.36	0.69
5-Hydroxytryptamine	0.15	0.58
Tryptamine	0.20	0.62

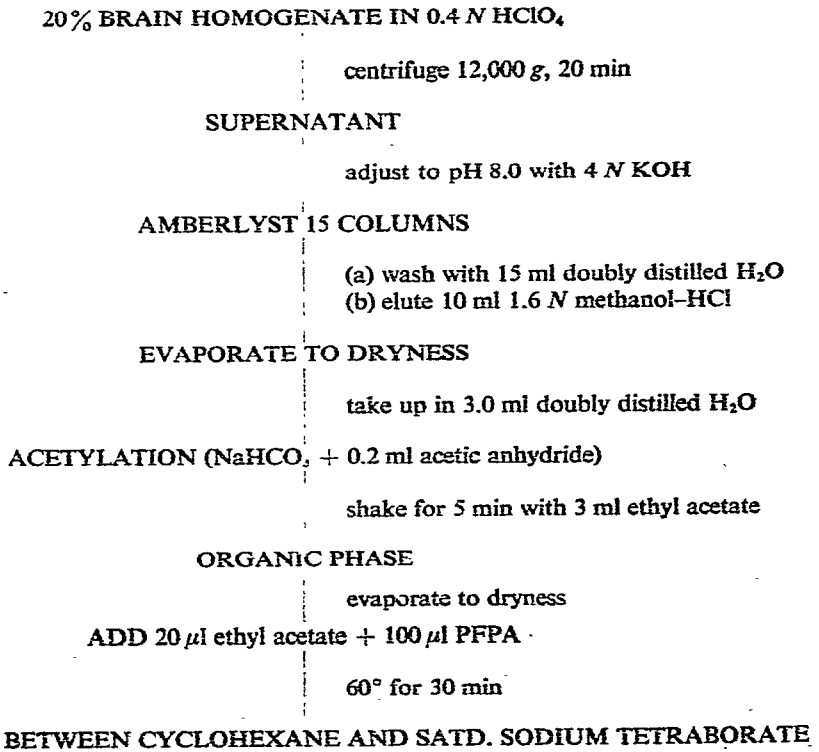

Fig. 1. Schematic representation of the method.

the amines prior to the acetylation stage. This procedure had the advantage that the supernatant from the original brain homogenate could be considerably purified before acetylation. The clear supernatant from the tissue homogenate after precipitation of the potassium perchlorate with KOH was percolated through Amberlyst 15 columns (4 cm × 0.6 cm) at a flow-rate of about 20 ml/h. The columns were then washed with distilled water and the amines eluted with 1.6 *N* methanolic HCl. Amberlyst 15 was

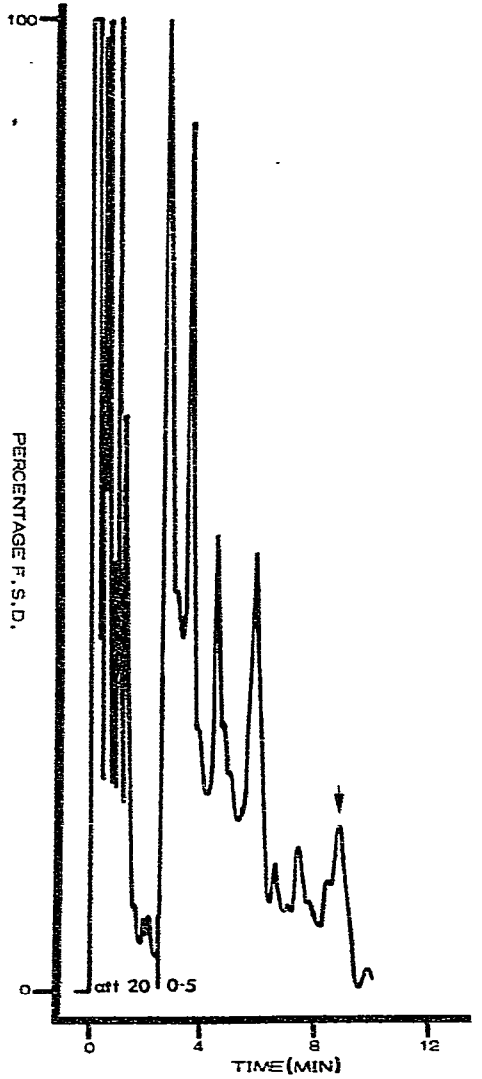

Fig. 2. A typical gas chromatographic record obtained from 2 g whole rat brain tissue. The peak marked with an arrow is that due to phenylethylamine. It was absent in the reagent blank samples which consisted of an equivalent volume of 0.4 *N* perchloric acid taken through the whole procedure. The gas chromatography was carried out on a Hewlett-Packard 5713 fitted with a ^{63}Ni electron capture detector; the injector port and detector temperature was 250°; the column was a 6 ft. × 4 mm I.D. glass column packed with 3% OV-3 on 100–120 mesh Gas-Chrom Q, at 130°.

used, because, with this eluent, the columns did not shrink and cause a reduction in flow-rate as occurred with the Dowex columns. The methanolic HCl eluate was then evaporated to dryness at 40° under vacuum and the residue taken up in distilled water and subjected to acetylation. These conditions resulted in 80–90% recovery of the PE from the Amberlyst columns.

The reaction of the N-acetyl-PE with PFPA was investigated in a number of solvents and under varying conditions, however, the reaction appeared to have gone to completion after 30 min at 60° using 20 μl ethyl acetate and 100 μl PFPA. Less than 5 pg of the resultant derivative could be detected on column with a signal-to-noise ratio of about 5:1.

A schematic representation of the method used is shown in Fig. 1 and a typical GC record in Fig. 2. With this method the levels of PE in whole rat brain are found to be 1.1 ± 0.2 ng/g (mean $\pm$ S.E.M.), which is in good agreement with other published figures[2,3,7].

CONCLUSION

In conclusion, the methodology outlined here is theoretically suitable for the GLC-EC determination of the series of compounds of the arylalkylamine type. The extension of this work to other compounds of this series is in progress.

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