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GAS CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF *meta*- AND *para*-HYDROXYPHENYLACETIC ACIDS

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SUMMARY

m- and *p*-Hydroxyphenylacetic acids have been identified and quantitated in rat brain by gas chromatography of their pentafluoropropionyl hexafluoroisopropyl diesters. The procedure differs from those employed in earlier studies on the catecholic acids in that a support-coated open-tubular column was used, a preliminary purification of the acids by thin-layer chromatography was carried out, and excess derivatizing reagent was removed by extraction with a phosphate buffer.

INTRODUCTION

The quantitation of a number of pharmacologically active, arylalkylamines (that exist in minute quantities in the mammalian brain and that have been labelled "trace" amines) and their respective acid metabolites is important if a fuller understanding of their role in the central nervous system is to be obtained. Quantitation of urinary phenolic and catecholic acids has been achieved by gas chromatography[1–5] and liquid chromatography[6], the quantitation of the much lower levels of these acids in the brain however has generally required the use of gas chromatography–mass spectrometry systems[7–10]. Since a mass spectrometer is not readily available in all laboratories, however, a more sensitive gas chromatographic method, especially one that permits resolution of the *meta* and *para* isomers of hydroxyphenylacetic acids (*m*HPA and *p*HPA, respectively) is desirable.

In this paper we report such a procedure for the analysis of the acid metabolites of tyramine as their pentafluoropropionyl hexafluoroisopropyl (PFP-HFIP) diesters and its application to an analysis of rat brain. The major changes from previously published procedures include the use of a support-coated open tubular (SCOT) column, a preliminary purification of the acids in a brain extract by thin-layer chromatography (TLC), and removal of excess derivatizing agent by extraction with an aqueous buffer.

EXPERIMENTAL

Equipment

A Hewlett-Packard 5840A gas chromatograph equipped with a ^{63}Ni electron

capture detector and a 35 m × 0.5 mm I.D. × 1.2 mm O.D. SCOT column coated with OV-17 (Scientific Glass Engineering, Melbourne, Australia) was used. The flow-rate of the carrier gas (5% methane in argon) was 2.5 ml/min through the column and 40 ml/min through the detector. An initial oven temperature of 80° was programmed to increase at 4°/min. The injector port and detector temperatures were 200° and 300°, respectively.

Materials

Pentafluoropropionic anhydride and 1,1,1,3,3,3-hexafluoroisopropanol were purchased from Pierce (Rockford, Ill., U.S.A.). Pre-coated silica gel 60 TLC plates (E. Merck, Darmstadt, G.F.R., distributed by Brinkmann, Rexdale, Canada) were pre-treated by development in 1-butanol–pyridine–acetic acid–water (60:8:12:20) followed by heating at 100° for 1 h. All solvents were distilled before use.

Procedure

Male Wistar rats, weighing 150–200 g, were stunned and then killed by decapitation, the whole brain was immediately excised and cooled in an ice-cold saline solution. After blotting and weighing, the brain was homogenized in 6 ml of 0.4 *N* perchloric acid, to which was added a known amount (usually 75 ng) of *p*-hydroxyphenylpropionic acid (*p*HPP) as internal standard and *p*-hydroxybenzoic acid (3 μg) as carrier. After centrifugation at 25,000 *g* during 20 min at 0–4° (Sorvall RC5), the supernatant was transferred to a 15-ml glass centrifuge tube and adjusted to between pH 7–8 with 2 *N* potassium hydroxide in order to precipitate the perchlorate. The precipitate was removed by centrifugation. The resultant supernatant was then acidified to pH 1 with 6 *N* hydrochloric acid, saturated with sodium chloride and extracted three times with ethyl acetate (3 ml). The combined organic solvent extracts were then evaporated to dryness in a stream of nitrogen at 50° in a water-bath and the residue redissolved in ethyl acetate (200 μl). This extract was applied to the origin of a washed and oven-dried silica gel plate (20 × 20 cm) and developed (4–6 h) in isopropanol–1-butanol–*tert.*-butanol–ammonia (sp.gr. 0.8)–water (40:20:20:10:10). A mixture of authentic acids was separated on the same plate as the tissue extracts, and these were visualized (after the developed chromatogram had been air-dried) by spraying with aqueous sodium carbonate followed by diazotized *p*-nitroaniline. The appropriate zones from the tissue extract, which had been protected by being covered with a glass plate during spraying, were outlined and removed from the chromatogram and extracted with hot ethyl acetate (thrice 3 ml). The combined extracts were then evaporated to dryness in a stream of nitrogen, and the residue transferred to a 1-ml PTFE-lined screw-top capped "Reacti-Vial" (Pierce) using ethyl acetate (twice 500 μl). This solution was then also evaporated to dryness in a stream of nitrogen. The acids were derivatized by adding to the residue pentafluoropropionic anhydride (250 μl) and hexafluoroisopropanol (150 μl) followed by heating at 65° for 90 min. The reaction mixture was then concentrated to about 100 μl by evaporation in a stream of nitrogen. Benzene (400 μl) and 1 *M* phosphate buffer (pH 6.0) (400 μl) were added and the mixture shaken vigorously for 30 sec. The aqueous layer after being withdrawn with the aid of a Pasteur (disposable) pipette was discarded and fresh buffer added. The process was then repeated. The remaining benzene layer was then concentrated to about 50 μl by evaporation in a stream of nitrogen and an aliquot (0.1–

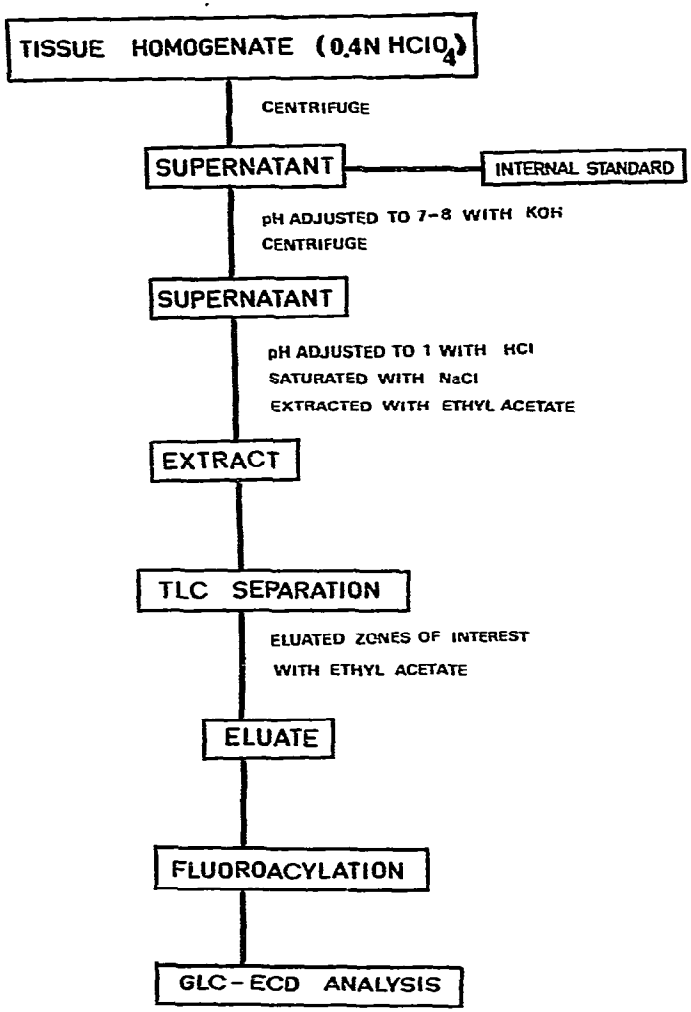

Fig. 1. Schematic diagram for the isolation, derivatization and chromatographic separation of *m*HPA and *p*HPA.

0.2 μl) injected onto the column. A schematic diagram of the above procedure is presented in Fig. 1.

Analysis

Calibration curves (see Fig. 2) for *m*HPA, *p*HPA and *p*HPP (which was used as an internal standard) were prepared by derivatizing standard solutions of known concentrations in the range 250 pg/μl to 10 ng/μl and measuring the response (peak area) of the detector.

RESULTS AND DISCUSSION

The essentials of the procedure described above have been employed in the analysis of urinary catecholic acids[3,4,11]. Pearson and Sharman[11] employed such a

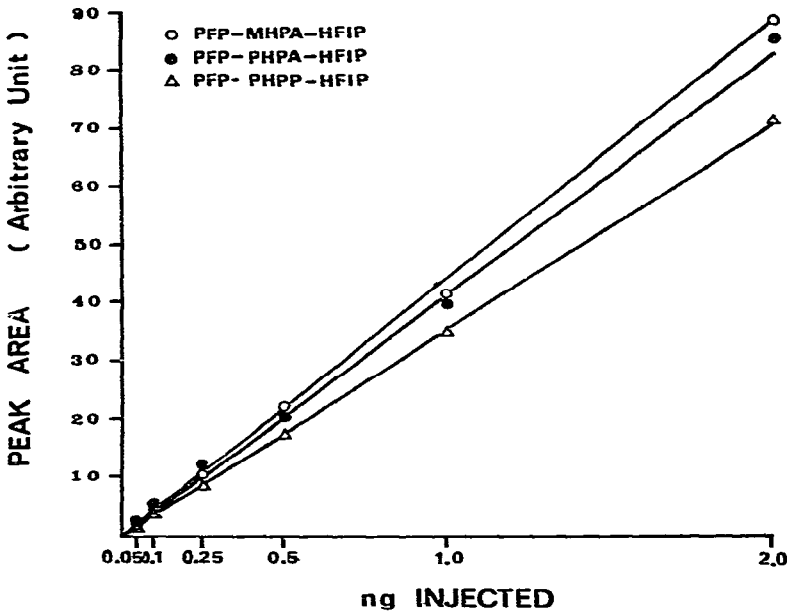

Fig. 2. Typical calibration curves for *m*HPA, *p*HPA and *p*HPP.

procedure for the analysis of dihydroxyphenylacetic acid, homovanillic and isohomovanillic acids in brain. In our hands, however, their procedure proved to be unsatisfactory for an analysis of the phenolic acids, probably because of the large (sometimes even total) losses of the derivatives that occurred during removal of the excess reagent.

As can be seen from Fig. 3 the derivatization of the hydroxy acids occurs in two steps, the first (without isolation of the intermediate) involves reaction of the phenolic hydroxyl group with pentafluoropropionic anhydride to produce an ester and of the carboxyl group to produce a mixed anhydride; this anhydride then subsequently reacts with the hexafluoroisopropanol to give the diester.

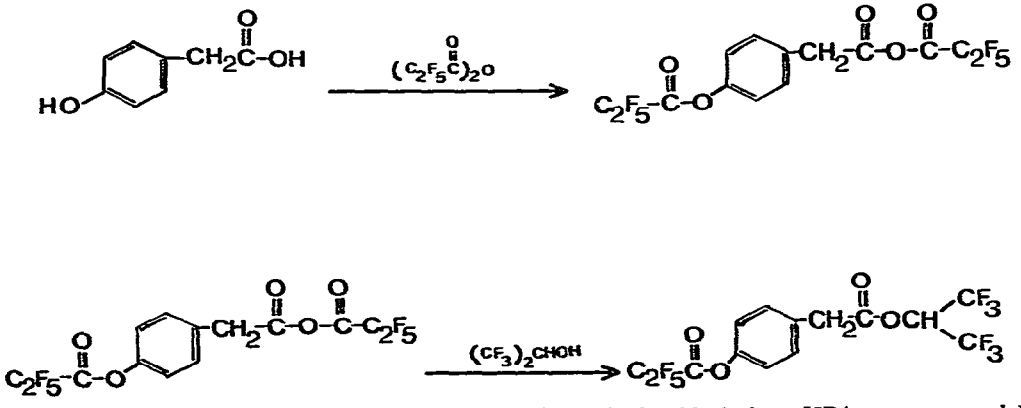

Fig. 3. Reaction sequence for the derivatization of arylalkyl acids (using *p*HPA as an example).

In order to permit quantitative determination, by this gas chromatographic procedure of the derivatized acids from brain tissue, it was necessary first to remove the excess of fluorinated derivatizing agent whose presence in an injected sample would cause saturation of the electron capture detector. A saturation could also occur by derivatization, on the column, of any underivatized acids that remained from an earlier separation. Such undesirable on-column derivatization has been shown to occur when pentafluoropropionic anhydride is injected onto a column containing dopamine[12], which, although possessing different properties to the acids, could give rise to ghost peaks and/or erroneous results.

Quantitation of the acid metabolites of the catecholamines by gas chromatography of their fluorinated derivatives[3,4] has proved to be fairly straightforward, really as a consequence of the relatively large amounts of acids being analysed and because of the relatively low volatility of their derivatives. Consequently, it proved possible to remove excess fluorinated reagents simply by evaporation, although some authors have reported losses of these derivatives if the evaporation is continued for too long a period[3,4,11]. The fluorinated derivatives of *m*HPA and *p*HPA, however, are more volatile and less abundant than those of the catecholic acids, and it was observed that the removal of excess reagents by evaporation to dryness in a similar manner resulted in substantial losses of the acid derivatives (as much as 100% if the amount of acid present was less than 100 ng).

The use of PFP-methyl esters[3,9,10] as derivatives was also investigated. Although these are somewhat less volatile than the PFP-HFIP derivatives, some losses still occurred during evaporation of excess reagents. In addition, their sensitivity was lower, and they seemed unsuited to the analysis of phenylacetic and mandelic acids.

The procedure outlined in the methods therefore takes advantage of the stability of esters towards aqueous buffer at a pH 6.0 and the instability of PFP anhydride under the same conditions. The hydrolyzed anhydride (pentafluoropropionic acid) is soluble in the buffer, whereas the acid derivative (an ester) is insoluble and remains in the benzene layer. Excess hexafluoroisopropanol is rapidly and completely removed during concentration of the reaction mixture owing to its high volatility.

A further complication in the analysis of trace amounts of the hydroxyphenylacetic acids concerns the interference of the many other compounds co-extracted with the acids from the brain homogenate. Many of these may be neutral compounds. which ought therefore to be amenable to separation during the ether or ethyl acetate extractions. Although such extractions did produce some improvement, the very low brain levels of the acids necessitated operation of the gas chromatograph at attenuations so low that even trace amounts of contaminants gave rise to significantly large peaks. Most of these interfering compounds were removed during TLC separation of the extract of the brain homogenate.

The calibration curves of *m*HPA, *p*HPA and *p*HPP (see Fig. 2) are linear over the range 50 pg to 2 ng (acid injected) and it is apparent that the detector response is nearly identical for *m*HPA and *p*HPA. It was somewhat less so for *p*HPP.

A chromatogram of a standard mixture of the three acids (Fig. 4) indicates that retention times of 9.9 (*meta*), 10.35 (*para*) and 13.6 min (internal standard) are obtained, under the chromatograph conditions described earlier. These figures are reproducible to within $\pm 3\%$ even in the case of biological samples.

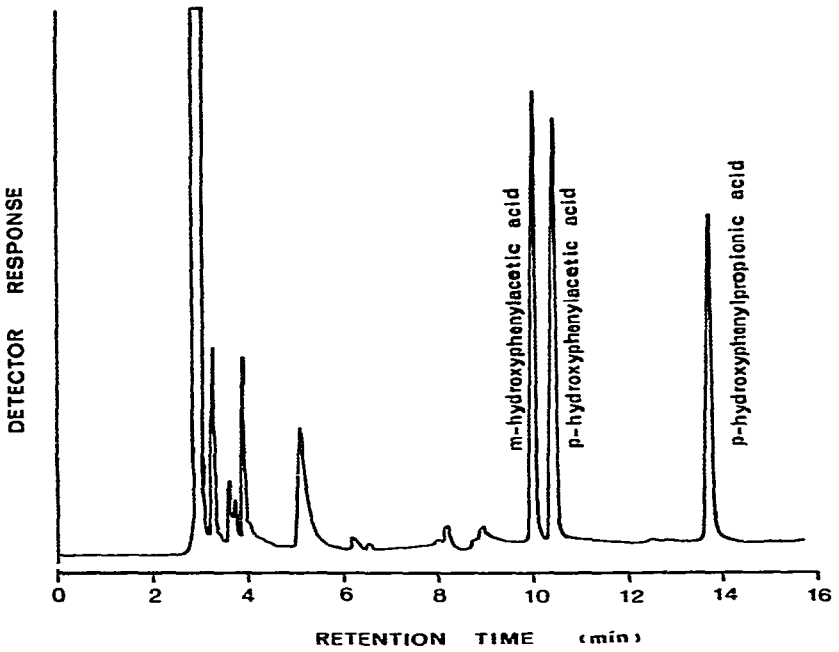

Fig. 4. Gas chromatogram of a standard mixture of the derivatives of *m*HPA, *p*HPA and *p*HPP.

The values obtained for *p*HPA and *m*HPA in preliminary analyses of the rat brain were, respectively, 17.1 $\pm$ 3.2 ($n = 3$) and 9.4 $\pm$ 1.3 ng/g ($n = 3$). This is the first time, so far as we are aware, that *m*HPA has been identified and quantitated in brain. If these two values are combined, the combined value is very close to the concentration claimed by Karoum *et al.*[8–10] for *p*HPA in brain. As the method of Karoum *et al.* did not distinguish between *meta* and *para* isomers, it is likely that this explains the discrepancy between the published values and those listed in this paper.

ACKNOWLEDGEMENT

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