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Note

Rapid and simple procedure for the determination of urinary phenylacetic acid using derivatization in aqueous medium followed by electron-capture gas chromatography

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2-Phenylethylamine (PEA) is a trace amine which has been implicated in the etiology and pharmacotherapy of a number of psychiatric and neurological disorders, including depression [1–6], schizophrenia [7,8], aggression [9–13] and dietary migraine [14]. Phenylacetic acid (PAA), the major metabolite of this amine, has been the subject of interest in psychiatric research for many years. Plasma and urinary levels of PAA have been suggested as state markers for depression [15] since it has been determined that these levels were considerably and consistently lower in patients with major depression relative to those in normal subjects.

Most of the reported gas chromatographic (GC) methods for analyzing the acidic metabolites of biogenic amines involve organic extraction of the metabolites from an acidic medium and evaporation of the organic layer to complete dryness before derivatization is performed under anhydrous conditions. We have used such a procedure [16] for simultaneous analysis of urinary *m*- and *p*-hydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindole-3-acetic acid, acid metabolites of the biogenic amines *m*- and *p*-tyramine, dopamine and 5-hydroxytryptamine, respectively. In our hands this procedure produced variable recoveries of PAA. Because of this we have developed a separate assay procedure for quantifying PAA using aqueous extractive derivatization and electron-capture GC.

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EXPERIMENTAL

Chemicals and reagents

Chemicals and reagents were purchased from the following companies: Fisher Scientific (Fair Lawn, NJ, U.S.A.) for sodium hydroxide, hydrochloric acid, ammonium hydroxide, disodium ethylenediaminetetraacetate (EDTA), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$); Aldrich Chemicals (Milwaukee, WI, U.S.A.) for dicyclohexylcarbodiimide (DCC), pentafluorophenol (PFPh-OH), 4-chlorophenylacetic acid (*p*-Cl-PAA); Sigma (St. Louis, MO, U.S.A.) for sodium phosphate dibasic (Na_2HPO_4); Caledon (Georgetown, Canada) for glass-distilled toluene and acetonitrile.

Preparation of pH 6 0 phosphate buffer solution

NaH_2PO_4 solution (2 *M*) was mixed with Na_2HPO_4 (2 *M*) in the ratio of 88.9:11.1 [17]. The mixed solution was then adjusted to pH 6.0 using sodium hydroxide.

Urine samples

Urine samples (24 h) from normal subjects were collected in specimen containers to which 20 ml EDTA solution (2%) had been added. Aliquots of the samples were stored at −30 °C until analyzed. For the 48-h period prior to urine collection and during urine collection, the subjects were on a low-amine diet, with restrictions similar to those used in patients taking monoamine oxidase inhibitors [18].

Extraction and derivatization

A portion (25 μl) of a 24-h urine sample was added to test-tubes to which a fixed amount of internal standard compound, *p*-Cl-PAA, had been added. An equal volume of 6 *M* hydrochloric acid was then added to each mixture. The tubes were capped and left in a boiling water bath for 1 h [19]. After cooling, the volume in the tubes was made up to 200 μl with water before the addition of 400 μl sodium phosphate buffer (2 ml, pH 6.0). After brief mixing, the following solutions were added sequentially to each tube: 200 μl toluene, 200 μl DCC solution [5 μl/ml toluene–acetonitrile (9:1, v/v)], 200 μl PFPh-OH solution [5 μl/ml toluene–acetonitrile (9:1, v/v)]. The two phases were shaken on a mechanical shaker for 15 min. After a brief centrifugation, the organic layers were transferred to 1.5-ml conical polypropylene microcentrifuge tubes with snap-on caps (Bio Plas, San Francisco, CA, U.S.A.) which contained 400 μl of 0.5 *M* ammonium hydroxide. The microcentrifuge tubes were vortexed for 5 s and centrifuged to separate phases. The organic phases were retained for GC analysis. The concentrations of the resultant derivatives were measured using the ratio of peak heights of the compounds of interest to that of the internal standard. For measurement, a standard (calibration) curve, constructed by plotting peak-height ratios against concentrations, was prepared for each batch of samples subjected to analysis. The calibration curve was constructed by adding known, varying amounts of authentic

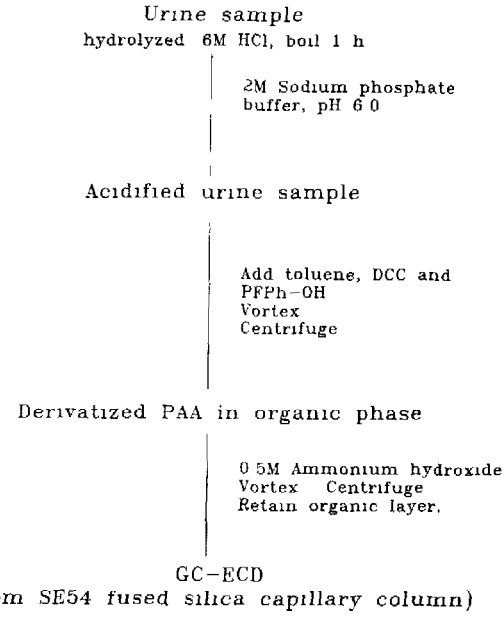

Fig. 1. Assay procedure for analysis of PAA

PAA standard and a fixed amount of internal standard to a series of tubes and carrying these tubes through the assay procedure in parallel with the sample tubes.

Fig. 1 depicts a flow chart that summarizes the procedure.

Gas chromatography–mass spectrometry (GC–MS)

Analysis of the derivative of PAA was performed on a Hewlett-Packard (HP) 5880A gas chromatograph equipped with a 7672A automatic sampler (splitless injection mode), 5880A terminal and a 15-mCi ^{63}Ni source electron-capture detector. Separation of the derivatives was carried out on a fused-silica capillary column (SE-54, 15 m×0.25 mm I.D., 0.25 μm film thickness, J. & W. Scientific, Rancho Cordova, CA, U.S.A.). Helium at a flow-rate of 2 ml/min was used as the carrier gas and 5% methane in argon at a flow-rate of 35 ml/min was used as make-up gas to the detector. The oven programme employed was as follows: an initial temperature of 100 °C was maintained for 0.5 min before being raised at a rate of 20 °C/min to 140 °C, which was maintained for 5 min before being raised again at 5 °C/min to 280 °C. The injection port temperature was set at 200 °C and that of the detector at 300 °C. Retention times were 5.8 and 10.8 min for derivatives of PAA and *p*-Cl-PAA, respectively.

In preliminary studies, GC–MS was used to confirm the structure of the derivative. The mass spectrometer was an HP5985A operated in electron-impact mode, with the inlet being an HP5840A gas chromatograph. The system also consisted of an HP2648A graphics terminal, an HP 9876A printer, an HP7920 disc drive and an HP 21MX Series E computer. Conditions of operation were as follows: ion source temperature, 200 °C; interface temperature, 275 °C; column pressure, 34.5 kPa; accelerating voltage, 2200 eV; ionization voltage, 70 eV; scan speed, 100

a.m.u./s; dwell time, 200 ms. The same capillary column and oven temperature programme as described above for GC analysis were used.

RESULTS AND DISCUSSION

Carbodiimides have been shown to be useful condensing agents in promoting esterification [20,21], and DCC is known to be useful in such reactions even in aqueous solutions [22]. The coupling of PAA and the fluorinated phenol to form an ester in the presence of DCC has been demonstrated in the present report to occur even under relatively mild aqueous conditions. The structure of the derivative obtained from this procedure was established by GC–MS, with *m/z* values of the molecular ion and those of significant fragment ions being supportive of the structure shown in Fig. 2.

Losses of PAA can be a major problem when procedures require evaporation of organic solvent which contains PAA and/or its derivatives [19,23,24]. This present procedure has the advantage of directly extracting and derivatizing PAA under aqueous conditions, and analyzing the derivative on a gas chromatograph

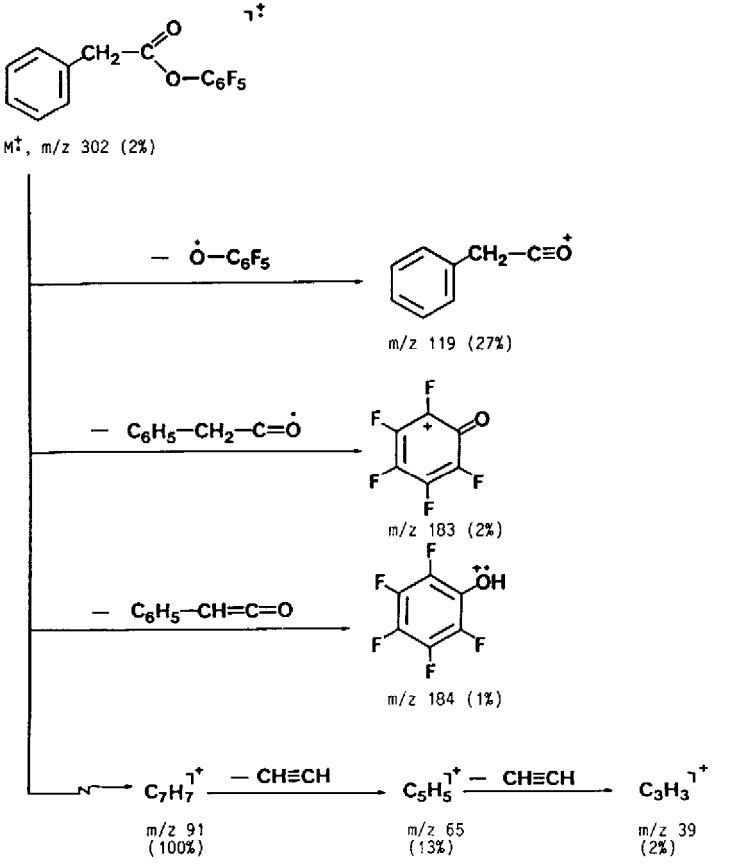

Fig. 2. Proposed mass fragmentation pattern of the derivative of PAA. Values in parentheses represent relative abundance

without the need of an evaporation step. The procedure is highly reproducible, with coefficients of variation of 3.0, 3.4 and 3.7% for 500- and 1000-ng standards and aliquots of a urine sample, respectively, carried through the procedure in six different experiments in each case. It provides derivatives with good chromatographic properties (typical gas chromatograms are shown in Fig. 3). They are highly sensitive to electron-capture detection. The minimum detectable quantity, as determined by a signal-to-noise ratio of 2, is less than 3 ng (represents 5 pg on-column). If necessary, sensitivity can be enhanced by using smaller volumes of toluene. A calibration curve is constructed for each assay, and the range of concentrations used for routine analysis is from 0.125 to 2 μg. This range adequately covers the levels of PAA encountered in 25 μl of urine from patients and

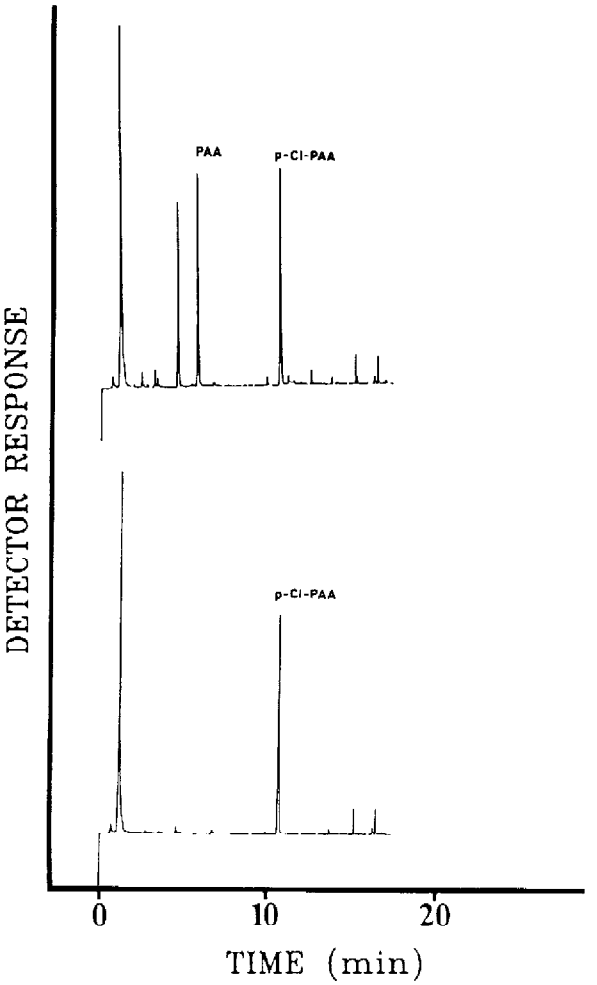

Fig. 3. Gas chromatograms of (top) derivatized urine sample and (bottom) water blank with internal standard added. PAA = derivatized phenylacetic acid; *p*-Cl-PAA = derivatized *p*-chlorophenylacetic acid (internal standard), the peak corresponding to derivatized *p*-Cl-PAA was not present in blanks unless the internal standard was added.

normal subjects, and the calibration curves have been shown to be linear (correlation coefficients > 0.99 obtained routinely). The wash with 0.5 *M* ammonium hydroxide helps eliminate a large solvent peak which is contributed by the excess PFPh-OH reagent. The total PAA value from 24-h urine samples of twenty normal subjects was found to be 86.5 ± 63.1 mg per 24 h (mean ± S.D.). In a study in which GC with flame ionization detection was used for analysis, Sabelli et al. [25] reported that in 70% of their healthy volunteers excretion of PAA ranged between 70 and 175 mg per 24 h. Davis and Boulton [26] used GC combined with high-resolution (integrated ion current) MS to study longitudinal urinary excretion of PAA in a human male and reported an average urinary excretion of total PAA of 97.3 mg per 24 h.

This novel procedure is simple, very rapid and convenient, and requires 25 μl or less of urine for analysis; it could be easily adopted by most laboratories doing similar research work. Attempts have been made to extend the assay to acid metabolites of other trace amines of psychiatric interest. Although in its present form, the assay has proved unsatisfactory for analysis of *m*- and *p*-hydroxyphenylacetic acid (metabolites of *m*- and *p*-tyramine, respectively), preliminary results from our laboratory indicate that it will be suitable for analysis of indole-3-acetic acid, the acid metabolite of tryptamine.

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