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Note

Assay of urinary phenylacetic acid by high-performance liquid chromatography

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Phenylacetic acid (PAA) is a normal constituent of human urine, where more than 90% of it occurs as the glutamine conjugate [1—4], and ca. 150 mg is excreted by a healthy adult every 24 h. Urinary PAA is derived from L-phenylalanine either by decarboxylation to 2-phenylethylamine (PEA) and further deamination by monoamine oxidase to PAA, or by transamination to phenylpyruvic acid followed by decarboxylation to PAA.

PEA is a trace amine occurring in mammalian brain [5], which according to the PEA hypothesis of affective behaviour [6] acts as an endogenous amphetamine and may be involved in alertness, excitement and mood. Consequently a change in PEA concentration or in PEA turnover in the brain may influence affective state, or is even considered to be an aetiological factor in the pathogenesis of certain forms of schizophrenia [7]. In a recent report, Sabelli et al. [8] suggest that low urinary excretion of PAA, the major metabolite of PEA, may be a reliable marker for diagnosis of some forms of unipolar major depressive disorders. Other workers [4] observed a decreased PAA excretion in a group of chronic schizophrenic patients, particularly of the non-paranoid subtype. In the cerebrospinal fluid of unmedicated schizophrenics free PAA is significantly reduced [7].

In order to evaluate the importance of urinary PAA as a diagnostic aid in psychiatric disorders, we developed a high-performance liquid chromatographic (HPLC) assay of total PAA in urine. We believe that there is a place for this assay alongside gas chromatographic methods [2, 3, 9].

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EXPERIMENTAL

Materials

PAA (98.5% pure) was obtained from Janssen Chimica (Beerse, Belgium) and 3-phenyl-1-propanol (98% pure) was from Fluka (Buchs, Switzerland). Disposable extraction columns (1-ml bed volume) packed with octadecylsilane (C_{18}) bonded silica gel were from Baker Chemicals (Deventer, The Netherlands). Sephadex G-10 was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade.

High-performance liquid chromatography

The liquid chromatograph consisted of an M6000A pump, a Model 440 absorbance detector (254 nm) and a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.). Peak areas were measured with a CDP1 digital integrator from Pye Unicam (Cambridge, U.K.). The solvent system consisted of 0.01 mol/l acetic acid in methanol—water (2:3), pH 3.6. The flow-rate was 1.5 ml/min. Analyses were carried out at room temperature on a LiChrosorb 10 RP-18 column (25 cm × 4.6 mm I.D.; 10 μm particle size) obtained from Chrompack (Middelburg, The Netherlands).

Sample collection

Random urine samples from unselected hospitalized psychiatric patients were frozen at −20°C immediately after collection. None of the patients had serious somatic illness.

Method

PAA in urine was quantitated by HPLC after consecutive purification on a C_{18} extraction column and a Sephadex G-10 column. Urine was centrifuged after thawing and 2.0 ml of the supernatant fluid was added to 2.0 ml of 6 mol/l hydrochloric acid in a screw-cap test tube. Conjugated PAA was hydrolysed by placing the tube in a water bath at 90°C for 4 h. After cooling and centrifugation for 5 min, 3.5 ml of the supernatant were applied to a C_{18} extraction column, which had been conditioned by aspirating 1 ml of methanol and 1 ml of water through the column prior to use. The column was subsequently washed with 1.0 ml of water and 0.1 ml of methanol. The organic fraction containing PAA was eluted with 0.25 ml of methanol. This fraction was diluted with 0.75 ml of water, and 0.9 ml of the resulting mixture was applied to a Sephadex G-10 column (8 × 0.4 cm I.D.) equilibrated with 0.01 mol/l formic acid prior to use. The column was washed with 1.0 ml of 0.01 mol/l formic acid, and PAA was eluted with 1.0 ml of the same eluent. As we were unsuccessful in finding a standard that behaved identically with PAA on the Sephadex G-10 column, the standard 3-phenyl-1-propanol was added to the eluate after Sephadex G-10 chromatography. PAA was measured with the HPLC system as described above, typically by a 100-μl injection. PAA excretion was calculated from calibration curves of known amounts of PAA added to the external standard. The amount of PAA in urine was expressed per millimole of creatinine. Urine samples containing more than 10.0 mmol/l creatinine were diluted (1:1) with water before processing. Creatinine in urine was determined by the Jaffé reaction with alkaline picrate reagent [10].

RESULTS AND DISCUSSION

Under standard HPLC conditions, PAA and 3-phenyl-1-propanol have retention times of 8.3 and 17.0 min, respectively. 3-Phenyl-1-propanol was selected from a number of compounds (including 2-phenylpropionic acid, 3-phenylpropionic acid and benzoic acid) as the most suitable standard. As shown in Fig. 1, PAA and the standard are well resolved from interfering peaks. The limit of detection of PAA, as defined by a signal-to-noise ratio of 3 at 0.005 a.u.f.s., is 16 ng. This corresponds with a limit of detection of 28 ng/ml PAA in urine. The linearity of the assay was determined from the HPLC response of various known amounts of PAA to the external standard. The assay is linear up to at least 3 mmol/l PAA in the original solution. This value is approximately equivalent to four times the mean expected value of the PAA concentration in a 24-h urine of a healthy adult [8]. The major part of PAA in urine is conjugated with glutamine. The optimal time of hydrolysis in acidified urine, studied by varying the time of hydrolysis at 90°C from 0.5 to 5 h, is 4 h. This is in full agreement with the results of Gusovski et al. [9].

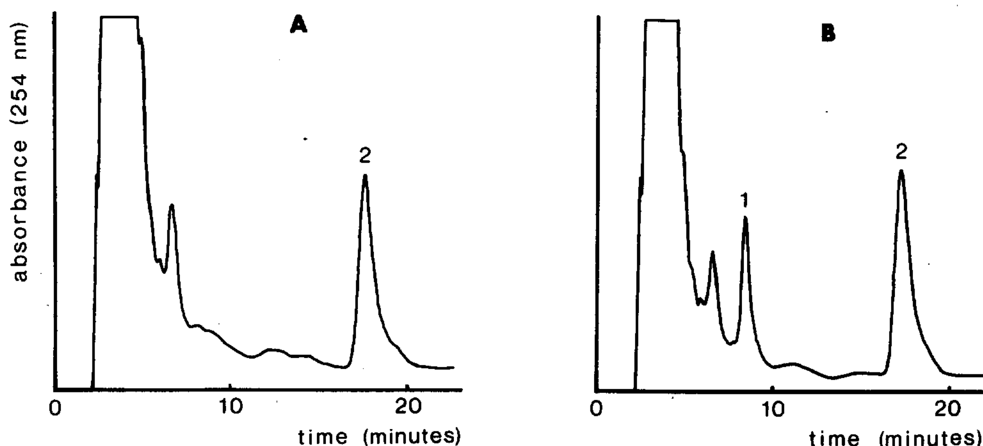

Fig. 1. HPLC profile of phenylacetic acid in urine before (A) and after (B) acid hydrolysis of conjugates. 3-Phenyl-1-propanol was used as the external standard. Conditions for HPLC as described in Experimental. Peaks: 1 = phenylacetic acid: 2 = 3-phenyl-1-propanol.

Recoveries

To C_{18} extraction columns and Sephadex G-10 columns, 2 ml of known solutions of PAA in water were applied. Before HPLC, 3-phenyl-1-propanol was added to the eluates as the standard and PAA was quantitated by the HPLC system as described above. Recovery (mean ± S.D.; n = 5) of PAA from the C_{18} extraction column is 93% ± 2% and from the Sephadex G-10 column it is 63% ± 3%, which is acceptable as this recovery is reproducible and because further elution of the Sephadex G-10 column gives rise to interfering peaks in the final chromatogram.

Reproducibility

For the determination of the intra-assay coefficient of variation (C.V.) five

samples were processed on the same day. The inter-assay C.V. was determined from five samples assayed on different days within one week. The concentration of PAA in the samples was 0.44 mmol/l of urine. Intra-assay and inter-assay C.V. values are 1% and 3%, respectively.

Interferences

Systematic interference studies were not carried out. From the investigation of urine samples from patients we conclude that the following drugs do not interfere with the assay: lithium carbonate, carbamazepine, flurazepam, flunitrazepam, lorazepam, temazepam, pimozide, mianserin, sulpiride, clomipramine, thioridazine, promethazine, *cis*-clopenthixol, glibenclamide, furosemide, triamterene, phenprocoumon and digoxin.

PAA excretion in urine

Differences depending on sex were not observed. Therefore, values of PAA excretion (mean ± S.E.M.) are given for the whole group. The urinary PAA excretion was 96.6 ± 10.8 μmol/mmol of creatinine (n = 35). Comparison with other published methods is complicated by the fact that other workers present their results as milligrams of PAA excreted per 24 h. Calculation of our results on the basis of a mean creatinine excretion of 12.8 mmol per 24 h [11] gives an approximate 24-h excretion of PAA, as measured by the method presented here, of 168.0 ± 18.8 mg per 24 h. This is in excellent agreement with the reported values (mg PAA per 24 h): 137.4 ± 15.8 [3], 162 ± 19 [4] and 141.1 ± 10.2 [8, 9] obtained by other methods.

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