Journal of Chromatography, 570 (1991) 376–381
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6013

Short Communication

High-performance liquid chromatographic determination of phenylacetic acid in human plasma extracted with ethyl acetate

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(First received April 11th, 1991; revised manuscript received May 21st, 1991)

ABSTRACT

This paper describes a high-performance liquid chromatographic method with ultraviolet detection for measuring plasma phenylacetic acid. This simple and reliable method consists of an acid hydrolysis of conjugated phenylacetic acid before extraction with an organic solvent: washed ethyl acetate saturated in sodium chloride. The recovery of extraction was estimated by internal standardization with phenylpropionic acid, and validated by addition of phenylacetic acid standards. A preliminary application to plasma phenylacetic acid in patients suffering from depression is described.

INTRODUCTION

Phenylacetic acid (PAA) is considered to be the main metabolite of brain 2-phenylethylamine (PEA), an endogenous amine, structurally and pharmacologically similar to amphetamine and having behavior-stimulating activity [1,2]. PAA was first identified in the mammalian brain, where it could be an indicator of PEA activity (see ref. 3 for review). In man, many reports have suggested that depression could be associated with a relative lack of PEA, while mania could be the result of its abundance [4,5].

An abnormal regulation of PEA has also been implicated in some mental diseases, including hysteroid dysphoria [6] and paranoid schizophrenia [7], as well as in migraine [8] and stress [9]. Many trace amines have been shown to possess high turnover rates, and the study of PEA metabolites may therefore be of great interest. However, only a few methods for determining human fluid PAA levels are available. Most of them are based on expensive technology, such as gas chromatography–mass spectrometry (GC–MS) [10], other on tedious procedures of low sensitivity, such as gas chromatography–flame ionization detection (GC–

0378-4347/91/$03.50

FID) [11]. Furthermore, assays have mostly been performed on either 24-h urine samples or cerebrospinal fluid [12], but rarely on plasma samples [13,14].

Because of its easy access, human plasma has increasingly become the medium of choice for investigation of many neurotransmitters and modulators thought to act centrally, such as catecholamines. In addition, high-performance liquid chromatography (HPLC) has become popular because of its low cost and high sensitivity, and it is interesting to note that in the literature only a few papers have reported plasma PAA analysis using this technique. Yamaguchi and Nakamura [14] have used fluorimetric detection, a technique which requires the derivatization of PAA, whereas Gusovsky *et al.* [13] have utilized a radioactive isotope of PAA ([^{14}C]PAA) as an external standard for estimation of recovery.

We have therefore developed a simple and reliable method based on HPLC analysis with ultraviolet detection which does not require derivatization. Various chromatographic conditions were assayed and a preliminary application to plasma PAA in patients suffering from depression is described.

EXPERIMENTAL

Chemicals

PAA was purchased from Aldrich (Steinheim, Germany). PPA and organic solvents (methanol and acetonitrile) were from Merck (Zurich, Switzerland). All chemicals and solvents were of analytical grade and deionized, bidistilled water was used.

Chromatography

The HPLC system consisted of a Model 510 solvent-delivery system, a Model U6K injector, equipped with a C_{18} 4-μm Nova-Pak analytical column (150 mm $\times$ 3.9 mm I.D.) and a Model 460 variable-wavelength, ultraviolet spectrophotometer set at 210 nm. All these instruments were from Waters Assoc. (Milford, MA, USA). A C_{18} guard column was always used (ODS-5S from BioRad, Richmond, CA, USA), and peak integration was performed with a Model 470 Spectra-Physics integrator with chart speed set at 0.5 cm/min.

Different mobile phases were assayed, based on 0.5 *M* sodium acetate (pH 4.8) with increasing concentrations of either methanol (5–30%) or acetonitrile (10–30%); the mobile phase which yielded optimal conditions was chosen for routine use and consisted of 0.5 *M* sodium acetate with acetonitrile (15%). The mobile phase was filtered through a Millipore membrane (0.22 μm), and degassed before use. Flow-rate was set at 1.0 ml/min.

Extraction

A sample of 2 ml of plasma was diluted with 50 μl of a PPA internal standard solution, or with the same volume of the different concentrations of PAA in the case of the standard added method. A 1-ml aliquot of 7% perchloric acid was

added, and the mixture was vortex-mixed then centrifuged at 12 000 *g* for 30 min. The supernatant (deproteinized plasma) was transferred in temperature-resistant 10-ml glass tubes fitted with screw caps, acidified with 1 ml of 3 *M* hydrochloric acid, then heated in a water bath at 95°C for at least 1 h to hydrolyze conjugated PAA. After hydrolysis, samples were cooled on ice, and 1 g of sodium chloride was added before the organic phase extraction.

For extraction, ethyl acetate was washed successively with 50 ml of 1.0 *M* hydrochloric acid, 50 ml of 1.0 *M* sodium hydroxide and four times with 50 ml of water per litre of solvent and saturated with sodium chloride as described by Oishi *et al.* [15]. The sample was extracted with three times 10 ml of this washed ethyl acetate. The solvent was then evaporated at 30°C under reduced pressure. Then the sample was reconstituted with 1 ml of mobile phase, and 50 μl were injected into the HPLC system.

For monitoring the sample, the detector was set at 210 nm, and for peak computing a calibration curve, prepared with increasing quantities of PAA or PPA, was registered in the integrator memory.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms of standard PAA and PPA solutions and of plasma

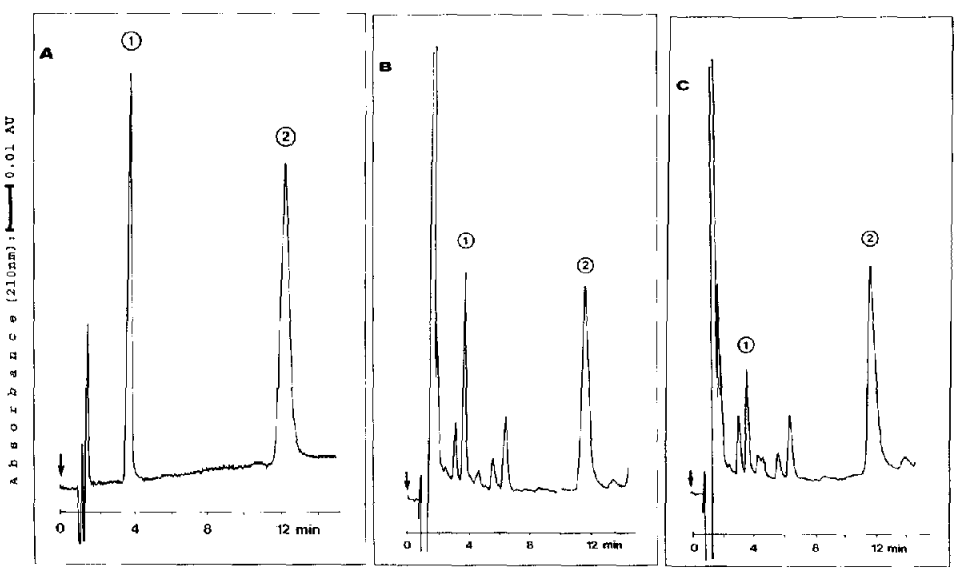

Fig. 1. Typical chromatograms obtained using the reversed-phase HPLC system. (A) Standard solutions of 125 ng of PAA (peak 1) and 250 ng of PPA (peak 2); actual plasma extract was carried through the hydrolysis and extraction procedure, and previously spiked with 2 μg/ml PPA (internal standard: peak 2). (B) Plasma from healthy subjects. (C) Plasma from patients with depression. See Experimental section for chromatographic conditions.

extracts previously spiked with PPA (2 μg/ml of plasma) of healthy subjects and depressed patients are presented in Fig. 1A–C. Different concentrations of organic solvents (methanol and acetonitrile) had previously been assayed with the same analytical column (4-μm NovaPak), and the optimal conditions for eluting plasma PAA and the internal standard PPA were retained. Acetonitrile afforded a faster separation than methanol, and was therefore chosen for subsequent experiments. However, high concentrations of organic solvent (> 30%) resulted in PAA peaks which were poorly resolved from the solvent front. The PAA peak had been previously characterized on the basis of co-elution with the reference compound.

Extraction and recovery

To estimate the extraction efficiency of different metabolite concentrations, seven plasma samples were spiked with increasing levels of PAA and PPA (range 0–2000 ng/ml) before extraction was performed. Table I shows percentages of recovery with ethyl acetate over a large range of PAA and PPA levels. The mean recovery (±S.D.) was 42 ± 2 and 45 ± 3% for PAA and PPA, respectively. First, both values are close to each other, which validates the use of PPA as a good internal standard for PAA. Second, solvent extraction with ethyl acetate yielded a recovery value comparable to the one previously reported in the literature using different analytical techniques [13,14]. Yamaguchi and Nakamura [14], using diethyl ether as extraction solvent, reported a recovery of 50.2 ± 3% for PAA, and Gusovsky *et al.* [13], who used benzene to extract the metabolite, reported a value of 45% estimated by [^{14}C]PAA. Lastly, no decrease in extraction recovery was observed up to 2 μg/ml plasma PAA. The principal losses probably occurred in the protein precipitation and liquid–liquid extraction steps.

TABLE I

PERCENTAGE RECOVERY OF PHENYLACETIC ACID (PAA) AND PHENYLPROPIONIC ACID (PPA) FROM SPIKED PLASMA USING ETHYL ACETATE AS EXTRACTION SOLVENT

Sample No.	PAA and PPA levels added to plasma (ng/ml)	Recovery (%)	
		PAA	PPA
1	–	42.2	
2	250	39.4	46.3
3	625	45.3	48.0
4	1250	43.3	48.4
5	2500	40.8	44.6
6	3750	42.8	42.2
7	5000	41.2	40.5
Mean ± S.D.		42.0 ± 2	45.0 ± 3

Linearity, precision and dectection limit

As in the above experiment, different plasma samples were spiked with increasing concentrations of PAA. The extraction procedure and chromatography were performed as in the standard technique. The calibration curve was linear up to 2000 ng/ml of plasma PAA ($y = 0.538x + 330$) ($r^2 = 0.992$; $P < 0.001$). The analytical imprecision was determined from analyses of five different samples followed during up to ten days; the within-day and day-to-day coefficients of variation (C.V.) were 2.0 and 3.1%, respectively.

To determine the lower limit of detection, diluted solutions of PAA or PPA were also injected into the HPLC system, and concentrations as low as 40 ng/ml PAA and 100 ng/ml PPA could be detected with a good signal-to-noise ratio (3:1). This could lead to the use of this method for free PAA assay or for small plasma samples in animal experiments.

Plasma PAA in patients suffering from depression

A preliminary application of this method to plasma PAA from untreated endogenous patients with depression was performed. The mean levels (± S.D.) were 538 ± 130 and 310 ± 96 ng/ml of plasma for controls and patients, respectively. A net decrease of plasma PAA was observed in patients as compared with healthy subjects (Student t-test, $P < 0.05$; $n = 9$).

CONCLUSION

We have investigated a method for rapid determination of plasma PAA using HPLC and ultraviolet detection. The interest of this method lies in the use of a popular and relatively inexpensive technique such as HPLC to work on plasma samples (not frequently encountered in the literature), and also in the use of PPA, a substance which behaves like PAA during plasma extraction and not naturally found in plasma, as an internal standard. Ethyl acetate was used for PAA extraction rather than benzene, which is most frequently used. The latter solvent is suspected of inducing tumors in humans [16], although the mechanism by which benzene produces its toxicological effects is not known [17]. This method is comparable to previously published ones and reproducibility was satisfactory.

Lastly, this experiment has validated the use of internal standard and presented chromatographic conditions which allow relatively fast separation with good resolution. This method should therefore be useful for routine determination of plasma PAA levels in mental and nervous diseases.

ACKNOWLEDGEMENT

The authors thank Mrs. J. Mezger for her kind secretarial assistance.

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