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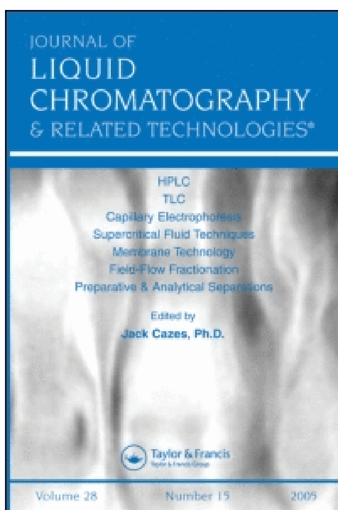

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SIMPLE HPLC METHOD FOR ROUTINE DETERMINATION OF URINARY PHENYLACETIC ACID EXCRETION

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ABSTRACT

A simple and reliable isocratic reversed-phase HPLC method for the determination of phenylacetic acid (PAA) in urine is described. After acid hydrolysis of its glutaminoconjugate, phenylacetic acid is extracted by diethylether ; chromatography is followed by UV detection at 210 nm. This rapid method is quite specific and sufficiently sensitive. We found satisfactory linearity up to 200 mg/l PAA urinary concentrations. Within-day and between-day assays showed variation coefficients below 10 %. Twenty-four hour urinary excretion of PAA might therefore be easily and routinely measured ; it could represent a non-invasive and suitable biological marker in psychiatry.

INTRODUCTION

2-Phenylethylamine (PEA) is an endogenous amine found in the mammalian brain [1] that may act as a neuromodulator on catecholamine synapses [2].

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This compound, structurally similar to amphetamine, induces the same behavioural effects, with a stimulant activity [3].

Many reports have suggested that abnormal regulation of PEA metabolism could be involved in psychic disorders affecting humour, either through relative lack as in unipolar major depression [2,4], or through excess as in paranoid schizophrenia [5,6].

Because of PEA high turn-over rates [7], authors have focused on its main metabolite [8], phenylacetic acid (PAA).

Several methods for the determination of PAA in biological fluids have been described ; assays have been performed on either cerebrospinal fluid (CSF) or plasma samples [9,10], even on 24-hour urine samples [11,12,13].

Twenty-four hour urine sampling appears to be more suitable , since it is non-invasive and gives results independently of circadian variations , contrary to plasma and CSF ; furthermore, PAA is excreted mainly in urine and thus found there in larger amounts than in other human fluids.

However, most of these techniques require expensive technology or tedious procedures, such as gas chromatography-mass spectrometry [10,13] or gas chromatography-flame ionization detection [12], while only a few methods are based on HPLC [14,15].

Nevertheless, these ones require lengthy sample preparation time, with preliminary derivatization prior to detection [14] or three-step extraction [15], and are less suitable in routine.

We described here a simple, reliable and rapid HPLC technique that could be routinely performed in most laboratories.

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MATERIALS AND METHOD

Chemicals

Phenylacetic acid (PAA) and Chloro-2-phenylacetic acid (internal standard) were purchased respectively from Sigma [St Louis, USA] and Aldrich [Steinheim, Germany]. All the organic solvents were of HPLC grade and obtained from Carlo Erba [Milan, Italy].
The analytical grade sodium acetate was purchased from Merck [Darmstadt, Germany].

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Chromatography

The HPLC system consisted of a Model LC-6A Shimadzu pump, a Model 231 Gilson sample injector, equipped with a 5μm Spherisorb ODS1 analytical column (30cmx4.2mm ID) and a Model 450 Waters variable-wavelength ultraviolet spectrophotometer set at 210 nm.
A C18 guard column was also used (packed with 30μm Spherisorb ODS) and data recording was performed by a BD 40 Touzart and Matignon recorder, with chart speed at 0.25 cm/min.
The mobile phase consisted of sodium acetate 0.01M with acetonitrile (94/6;V/V) adjusted to pH 4.8 and was filtered through a 0.45μm Millipore filter.
Flow rate was set at 1.0 ml/min.

Standard solutions

Stock solutions of PAA and internal standard were prepared in distilled water at a concentration of 1 g/l divided into 5ml aliquots and stored at - 20°C during one month.

Calibration standards were established by adding increasing amounts of PAA to a human urine pool containing low PAA concentrations.

Sample preparation

24h urines were collected in containers holding 5ml of HCl 5N. After measurement of total urine volume and determination of creatinine levels, 20ml aliquots were kept at - 20°C until the day of analysis.

For each sample, 1ml of centrifugated-urine was set into a temperature-resistant screw-capped 10ml glass tube ; then 200µl of internal standard solution were added and the mixture acidified by 1ml of 5N hydrochloric acid.

All samples were heated at 100°C for at least one hour to hydrolyze conjugated PAA and then cooled in fresh water.

Extraction was performed with 7ml of diethylether ; the organic layer was transferred into clean tubes and then evaporated to dryness under a nitrogen stream.

Dry residue was reconstituted in 500µl of mobile phase and an aliquot of 20µl was injected into the chromatographic system.

RESULTS AND DISCUSSION

Chromatograms

Chromatograms of standard PAA and Chloro-2-PAA solutions in distilled water are shown in Figure 1.

Chromatogram of the urine extract from healthy subject is shown in Figure 2.

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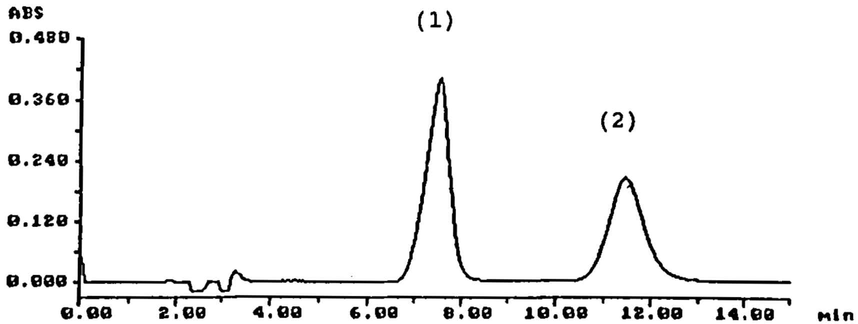

FIGURE 1: Chromatogram from mixed standard solution of phenylacetic acid(1) 200mg/l and internal standard(2) 200mg/l in distilled water.
Conditions: flow rate = 1.0ml/min, inj.volume = 20μl, wavelength = 210nm.

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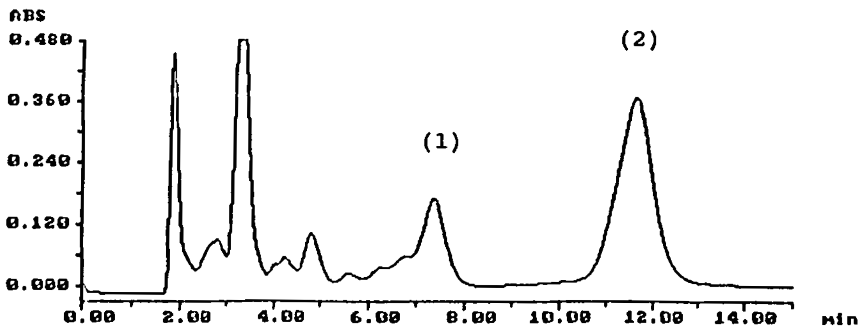

FIGURE 2: Chromatogram of urine extract from healthy subject ; peak(1) = phenylacetic acid [45mg/l] and peak(2) = internal standard [200mg/l].
Conditions: flow rate = 1.0ml/min, inj.volume = 20 μl, wavelength = 210nm.

Linearity

The calibration curve of urinary phenylacetic acid levels was obtained by plotting peak-height ratios against concentrations and we have found satisfactory linearity up to 200 mg/l ($y = 0.0079\ x + 0.127$; $n = 5$ $r = 0.9992$).

Variability and percentage recovery

High and low valued quality control samples (100 and 25 mg/l) were assayed five times on the same day and on several days during a two-week period, to evaluate the precision of the assay.
Results from within-day and day-to-day variability are grouped in Table 1 ; all the variation coefficients are below 10%.

Specificity

In order to obtain the highest specificity, we used a Merck L-3000 photo-diode array detector and modified the polarity of the mobile phase.

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TABLE 1: Within-day and day-to-day variability of measured phenylacetic acid concentrations in urine.

	Within-day variability (n = 5)		Day-to-day variability (n = 5)	
Theoretical PAA concentrations [mg/l]	25	100	25	100
Measured PAA concentrations [mg/l]	26.7	104.4	25.5	97.4
Percentage recovery [%]	106.8	104.4	102.0	97.4
Variation coefficients [%]	3.6	1.7	9.8	6.7

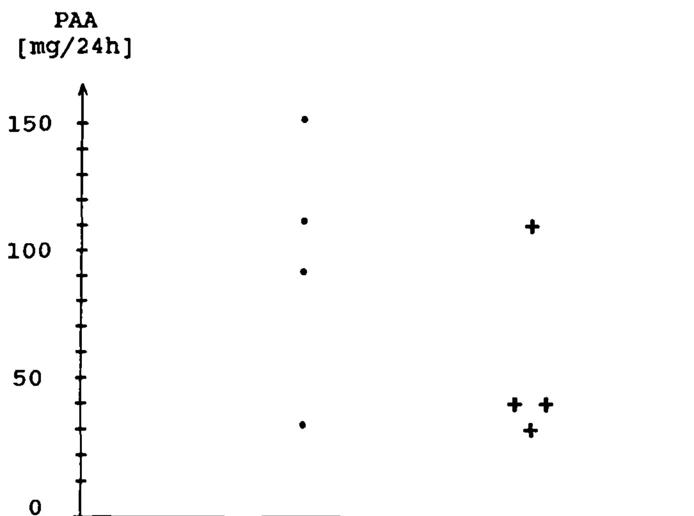

FIGURE 3 : Urinary excretion of phenylacetic acid in healthy volunteers (•) and depressed patients (+).

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That led us to select 210 nm as the optimal wavelength for detection and to determine a proportion of acetonitrile in the mobile phase equal to 6%.
We thus found no endogenous interference from urine, with a run time not exceeding 15 minutes.

Sensitivity

We have considered detection limit of the method as being lower PAA concentration what gives signal-to-noise ratio at least equal to 3. On spiked urinary extracts, we have found sensitivity around 1mg/l.

Urinary PAA levels in healthy and depressed patients

We have investigated PAA in 24h urine of 4 healthy volunteers and 4 untreated patients suffering from

major depression. PAA levels are expressed in mg/24h and results are given in Figure 3.
PAA urinary levels in depressed patients seem to be lower than in healthy volunteers according to previous reports [12,16].

CONCLUSION

We have developed a simple and reliable method for rapid determination of phenylacetic acid in human urine.
This HPLC procedure is sufficiently sensitive, specific and suitable for routine analysis in most laboratories. Determination of 24h phenylacetic acid excretion might therefore represent a non-invasive, inexpensive and interesting marker in the biological approach of psychiatric diseases.

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