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# Short Communication

# Determination of plasma homovanillic acid by liquid chromatography with electrochemical detection

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ABSTRACT

We describe a simple method for extracting homovanillic acid (HVA) from plasma. An aliquot of 0.5 ml of the internal standard solution (3-hydroxy-4-methoxycinnamic acid in 0.2 mol/l phosphoric acid) and 0.5 ml of the sample are applied to a 1-ml Bond Elut $C_{18}$ column prewashed with methanol and 0.2 mol/l phosphoric acid. The sample is drawn through the column at low speed. The column is washed with water and eluted with dichloromethane. The eluate is evaporated under vacuum at ambient temperature and the residue reconstituted with 250 $\mu$l of the mobile phase. A 10-$\mu$l aliquot of the resulting solution is injected onto a 150 mm × 4.6 mm I.D. column packed with 5-$\mu$m octadecylsilyl silica particles (Beckman). Peaks are detected coulometrically in the screening-oxidation mode with $E_1$ = −0.25 V and $E_2$ = +0.38 V. In the resulting chromatogram, HVA and the internal standard give sharp peaks and are well separated from solvent and other endogenous electroactive acids. The extraction recovery is 90–95% which allows the determination of 0.5 $\mu$g/l analyte.

## INTRODUCTION

Homovanillic acid (HVA) is the main circulating metabolite of dopamine. A number of reports published since the mid-1980s suggest that plasma HVA concentration may be an index of dopaminergic activity in the brain [1] since a large proportion of plasma HVA originates from the brain. Determination of plasma HVA has been claimed to be particularly useful to monitor the response to neuroleptics [2,3] in the treatment of schizophrenia. However, there is as yet no well defined consensus about the role of HVA in the diagnosis or monitoring of treatment in schizophrenia [4]. It is obvious that additional data about the role of HVA needs to be gathered in different centres, and for this purpose a simple and reliable analytical procedure for the determination of plasma HVA is required. Column liquid chromatography (LC) with electrochemical detection (ED) is now the most practical approach commonly used for the determination of

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catecholamines and their metabolites. Several LC–ED procedures for the determination of plasma HVA have been described [5–12]. These procedures differ primarily in the choice of the internal standard and in sample preparation. Here we describe an alternative simple and economical procedure for extracting HVA from plasma and evaluate the use of a novel, commercially available compound as the internal standard.

## EXPERIMENTAL

### *Reagents*

All reagents were of analytical grade. Deionized water was distilled in an all-glass still.

*Stock HVA solution (1.0 g/l).* A 10-mg amount of 4-hydroxy-3-methoxyphenylacetic acid (Sigma, St. Louis, MO, USA) was dissolved in 10 ml of methanol. This solution was stored at −20°C in 1-ml aliquots; the solution was stable for at least six months.

*Working HVA solution (1 mg/l).* A 25-μl volume of stock HVA was diluted to 25 ml with water.

*Plasma HVA standards.* Thoroughly mixed pooled fasting plasma was centrifuged. Aliquots of 250 and 500 μl of working HVA solution were diluted to 50 ml with pooled plasma in volumetric flasks. Unspiked pooled plasma and the two standards were stored frozen at −20°C in 1-ml aliquots. They were stable for at least four months.

*Stock internal standard solution (0.1 g/l).* A 10-mg amount of 3-hydroxy-4-methoxycinnamic acid (Aldrich, Milwaukee, WI, USA) was dissolved in 100 ml of methanol. The solution was stored at −20°C in 1-ml aliquots and was stable for at least four months.

*Working internal standard solution (20 μg/l).* A 10-μl volume of stock solution was diluted to 50 ml with stabilized phosphoric acid reagent.

*Stabilized phosphoric acid reagent.* A 1.4-ml volume of phosphoric acid was diluted to 100 ml with water and 5 mg each of reduced glutathione and $Na_2EDTA$ were added. The solutions was stored at 4°C.

### *Sample collection*

The samples were collected in 7-ml Vacutainer tubes (Cat. No. 6450, Becton Dickinson, Rutherford, NJ, USA) containing EDTA in the morning after an overnight fast and prior to any physical activity. Ambulatory patients were made to rest for 30 min prior to blood collection. The plasma was separated from the cells within 1–2 h and stored frozen till analysis.

### *Extraction*

The required number of 1-ml Bond Elut $C_{18}$ columns (Varian, Harbor City, CA, USA) was placed on a Vac Elut system. The columns were washed twice with 1-ml aliquots of methanol and once with 0.5 ml of phosphoric acid reagent, each time aspirating the liquid using mild suction. The suction was stopped completely, and 0.5 ml of working internal standard solution and 0.5 ml of the sample (standard or unknown) were applied. After about 2 min mild suction was applied so that the samples passed through the columns at a rate of less than 1 ml/min. The suction was increased briefly to expel all the trapped liquid. The columns were washed at a low rate once with 0.5 ml of water and once with 0.25 ml of water each time increasing the suction for a few seconds to drain the columns completely. Any drop of liquid adhering to the top or the bottom tip of the column was removed with tissue paper. The columns were placed on correspondingly labelled disposable glass tubes which had been washed with water and methanol. The columns were eluted twice with 1-ml aliquots of dichloromethane by centrifugation at a low speed (100 *g*), finally draining the columns by centrifugation for 10 s at a high speed (700 *g*). The eluate was evaporated in a vacuum evaporator (Savant Instrument, Farmingdale, NY, USA) keeping the heater switched off. The residue was dissolved in 250 μl of the mobile phase, and a 10-μl aliquot of the resulting solution was injected into the liquid chromatograph.

### *Chromatography*

We used a Model 100A pump (Beckman Instruments, Berkeley, CA, USA), a 20-μl loop in-

jector (Model 7125, Rheodyne, Cotati, CA, USA), a reversed-phase 150 mm × 4.6 mm I.D.) column of Ultrasphere ODS (5-μm particles, Beckman) protected by an RP-18 guard cartridge (7-μm particles, Brownlee Labs., Santa Clara, CA, USA), and a Model 5100A coulometric detector equipped with a Model 5010 analytical cell (ESA, Bedford, MA, USA).

The chromatography was performed at ambient temperature and the peaks were recorded with a plotter integrator (Model C-R3A, Shimadzu Scientific Instruments, Columbia, MD, USA). We used a mobile phase (800 ml of water + 100 ml of methanol + 100 ml of acetonitrile + 3 g of citric acid + 1 g of dibasic ammonium phosphate + 5 mg of $Na_2EDTA$) at a flow-rate of 1.5 ml/min and an operating pressure of 14 MPa (2800 p.s.i.). The electrodes I and II of the analytical cell were set at +0.25 and +0.38 V, respectively. The signal from electrode II was monitored.

## RESULTS AND DISCUSSION

Harris *et al.* [6] and Zumàrraga *et al.* [7] did not use any internal standard for the determination of plasma HVA although both of these procedures required multi-step extraction. Hovevey-Sion *et al.* [8] and Lambert *et al.* [12] have used 5-fluorohomovanillic acid, a compound which is not commercially available. Similarly, iso-HVA used as internal standard by Semba [9] is not readily available. Javaid *et al.* [5] and Aravagiri *et al.* [11] have used vanillic acid as the internal standard. This compound is commercially available and is not normally present in fasting human plasma. However, vanillic acid elutes close to a number of other endogenous acids and a long run time is required for adequate separation of vanillic acid from HVA and other endogenous acids. We screened a number of commercially available hydroxy and methoxy aromatic acids and found 3-hydroxy-4-methoxycinnamic acid the most suitable internal standard because of its similarity in electrochemical detection response to that of HVA and its easy separation from HVA. Analysis of 25 different plasma samples without the addition of this compound as internal standard did not show the presence of any peak which could interfere with the internal standard peak.

Javaid *et al.* [5] used a simple protein precipitation approach for the determination of plasma HVA. However, such an approach has been abandoned because of very long chromatographic run times required after the injection of such samples. In a number of procedures, protein-free filtrates were prepared which were then extracted with ethyl acetate [6,7] or a mixture of pentane and ethyl acetate in equal proportions [12]. Hovevey-Sion *et al.* [8] purified the protein-free filtrate by adsorbing the protein-free filtrate on a Sep-Pak $C_{18}$ cartridge which was then eluted with ethyl acetate to recover the acids. Lambert *et al.* [12] followed a similar procedure. However, the acids were eluted from the extraction column with 0.5 ml of 50% methanol rather than with 5 ml of ethyl acetate. Semba *et al.* [9] did not precipitate the proteins but applied the diluted sample directly to a 300-mg $C_8$ extraction column which was then eluted with 2 ml of 50% methanol. This eluate was further purified with the use of a second solid-phase (strong anion-exchange) extraction column. The acids from this anion-exchange column were eluted with 300 μl of 1 *M* hydrochloric acid. A 50-μl aliquot of this eluate was injected directly. However, such an injection gave a large solvent peak.

To minimize the use of disposable glass or plasticware, we avoided the protein precipitation step and applied the sample directly to the extraction column packed with only 100 mg rather than 300 mg of the sorbent. After washing, the column was eluted with dichloromethane which is more volatile and less polar than both ethyl acetate or 50% methanol. Initially, we tried to back-extract the eluate with 0.2 mol/l dibasic ammonium phosphate. HVA and the internal standard were efficiently recovered in 0.25 ml of the base. However, traces of methylene chloride injected with the aqueous phase gave a negative dip between the HVA and internal standard peaks which in some cases made the integration of peaks unreliable. Evaporation of the eluate in a 50°C water

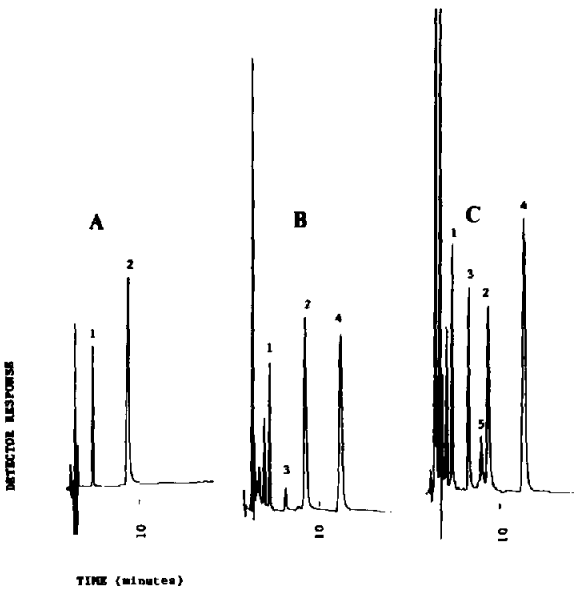


Fig. 1. Representative chromatograms of 10-μl injections of (A) an aqueous unextracted standard, (B) an extract of a fasting plasma sample, and (C) an extract of a non-fasting plasma sample. Peaks: 1 = HVA; 2 = 3-hydroxy-4-methoxycinnamic acid; 3 = probably 4-hydroxy-3-methoxyphenylpropionic acid; 4 = probably indole-3-acetic acid; 5 = probably 4-hydroxy-3-methoxycinnamic acid. Detector I = +0.30 V; II = +0.38 V; gain = 1 × 10; output = 1 V; integrator attenuation = 3.

bath gave inconsistent HVA recovery. The eluate was conveniently and rapidly evaporated under vacuum in the absence of any heat.

Fig. 1A shows a chromatogram of a 40 μg/l standard each of HVA and the internal standard. The baseline remains stable even when the sensitivity of detection (gain) is increased by a factor of 4. Detector response for HVA is optimal when the potential of electrode II is set at +0.35 V. However, the response for the internal standard at this voltage was about 60% of that of HVA. The response for both HVA and the internal standard are similar at the selected voltage for electrode II of 0.38 V.

Fig. 1B shows a chromatogram of a 10-μl injection of an extract of a fasting plasma sample spiked to a total concentration of 20 μg/l. The recovery of HVA is between 90 and 95% and there is no change in the HVA/internal standard ratio. The final solution, being in the mobile phase, is especially compatible with ED. Aliquots of up to 50 μl of the final solution were injected without producing a large solvent peak or a negative dip in the baseline. The final extract is quite stable. There was no change in the peak areas of HVA or the internal standard even after storage for three days at 4°C.

Some of the polar acids, *e.g.* 3,4-dihydroxyphenylacetic acid, were recovered between 10 and 30%. They eluted prior to HVA and did not interfere with the HVA peak. Peak 3 matches with the retention time of 4-hydroxy-3-methoxyphenylpropionic acid. This peak becomes quite large in non-fasting samples (Fig. 1C). Peak 4 matches with the retention time of indole-3-acetic acid and is present roughly to the same extent in both fasting and non-fasting samples. Presence of this peak makes the chromatogram run time somewhat long. It has not been possible to eliminate this peak by any extraction approach. However, this peak can be totally eliminated from the chromatogram if the detector is operated in oxidation–reduction mode (electrode I = +0.38 V; II = −0.42 V) as used by Gerhardt *et al.* [10]. We have opted for the longer run time as we got a better precision in the screening–oxidation mode as described in this report than that obtained in the oxidation–reduction mode. Indole-3-acetic acid is present in fasting human plasma in quite high concentrations (up to 600 μg/l) [13]. Non-fasting samples produce additional peaks, *e.g.* peak 5 of Fig. 1C, which matches in its retention time with 4-hydroxy-3-methoxycinnamic acid (ferulic acid). Although such peaks are separated from HVA and internal standard peaks, only fasting samples should be analyzed for useful HVA data [1]. Capacity factors of some of the electroactive acids or acidic compounds likely to be present in human plasma are summarized in Table 1. Amines and amino acids are not recovered in any significant yield by the described extraction procedure because of their poor retention on $C_{18}$ extraction column at acidic pH.

There is a linear relationship between the peak-area ratios of HVA and internal standard and HVA concentration up to 100 μg/l. Analysis of ten plasma samples obtained from healthy ambu-

TABLE I

CAPACITY FACTORS ($k'$) OF SOME POTENTIALLY INTERFERING COMPOUNDS

| Compound | $k'$ |
|---|---|
| 4-Hydroxy-3-methoxymandelic acid (VMA) | 2.6 |
| Acetaminophen | 3.2 |
| 3,4-Dihydroxyphenylacetic acid | 3.4 |
| 5-Hydroxyindole-3-acetic acid | 4.4 |
| 3,4-Dihydroxycinnamic acid (caffeic acid) | 5.2 |
| Vanillic acid | 5.6 |
| HVA | 6.4 |
| 4-Hydroxy-3-methoxypropionic acid | 10.6 |
| 4-Hydroxy-3-methoxycinnammic acid (ferulic acid) | 13.2 |
| Salicylic acid[a] | 13.4 |
| Indole-3-lactic acid[a] | 14.6 |
| 3-Hydroxy-4-methoxycinnamic acid (internal standard) | 16.2 |
| Indole-3-acetic acid | 25.2 |
| Indole-3-propionic acid[b] | 61.2 |

[a] Poor electrochemical response under the conditions for HVA detection.

[b] Electrochemically inactive under the conditions for HVA detection.

latory volunteers, who were not taking any medication, showed HVA concentrations from 5.8 to 16.5 μg/l with a mean of 10.4 μg/l. This value is similar to HVA concentrations in healthy controls determined by LC–ED as observed by Gerhardt *et al.* [10]. The lowest value of plasma HVA of 2.5 μg/l was observed in the sample of a healthy male volunteer collected at 10:00 a.m. with diet and physical activity controlled. However, the procedure could detect as low as 0.5 μg/l due to high extraction recovery. Limits of detection may be further increased by injecting a larger than 10-μl aliquot of the final extract. In a majority of the LC–ED procedures described for the determination of plasma HVA, the concentration of an unknown sample is determined by the addition of a standard amount of HVA to each sample and then analysing multiple tubes with and without the addition of standard HVA [6–8,10]. We analysed ten different fasting and non-fasting samples with and without the addition of standard HVA. The coefficient of variation (C.V.) of the recovery of added HVA (5 ng) was 4.8%. We have concluded that different plasma samples do not have a significant matrix effect on the recovery of HVA. Therefore, we process one set of standards (blank, 5 and 10 μg/l HVA) prepared in pooled plasma and a control prepared from a different unspiked pool with each batch of unknown samples. However, standards prepared in water, buffers or albumin are not suitable as the recovery from these standards was not consistent. It appears that co-extracted acids from plasma act as carriers and minimize the adsorption of HVA on glassware.

Analysis of pooled plasma showed a within-batch C.V. of 2.8% ($n$ = 10, mean = 8.1 μg/l) and a between-batch C.V. of 5.9% ($n$ = 10, mean = 8.7 μg/l).

We conclude that the procedure for the determination of plasma HVA described in this report is simple, economical and reliable for use in routine clinical laboratories.

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