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Note

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

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Homovanillic acid (HVA) is a major circulating metabolite of dopamine and it is derived from both the peripheral and central nervous system. However, animal studies have shown that the HVA level in plasma follows the changes in brain level after neuroleptic administrations [1, 2]. It has been recently suggested that plasma HVA levels may be useful as a biochemical predictor of neuroleptic response in schizophrenic patients [3–6]. Several methods have been reported to measure HVA in biological fluids, including high-performance liquid chromatography (HPLC) with electrochemical detection [7–13] and gas chromatography–mass spectrometry (GC–MS) [14, 15]. Because a GC–MS facility is not readily available to us, we used the HPLC method to measure plasma HVA levels. Previously reported HPLC methods did not use an internal standard. In some reports [7, 10, 12] the authors used an internal standard, but used aqueous standards for the standard curve. However, ideally, in order to minimize experimental error, we preferred to use spiked standard curve samples made in plasma and an internal standard instead of using aqueous standards. In this note, we report a new HPLC method for the determination of plasma HVA levels in which both the spiked plasma HVA standards and the patients plasma samples were subjected to the same extraction procedure. The method described here is simple, not time-consuming and suitable for the routine monitoring of plasma HVA.

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EXPERIMENTAL

Chemicals and reagents

HVA and the internal standard, vanillic acid (VA), were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC-grade solvents were obtained from Altech Assoc. (Deerfield, IL, U.S.A.) and the reagent-grade chemicals were procured from Fisher Scientific (Los Angeles, CA, U.S.A.). Deionized water (Nanopure, Barnstead), chemicals and the solvents were used without further purification.

Samples

Venous blood samples were collected from normal volunteers and schizophrenic patients in heparinized tubes (Vacutainer). The blood samples were centrifuged immediately (1725 *g*) at 4°C and plasma was separated and stored at −40°C until analysis. The spiked standard curve and quality control samples for HVA were made in a single pool of plasma from healthy volunteers such that the blank HVA level in all standard curve and quality control samples is the same. The spiked samples for the standard curve consisted of 40, 20, 10, 5 and 2.5 ng of HVA per ml of plasma. Three quality control spiked samples were made in the same pool of plasma to check the reliability of the standard curve.

Extraction procedure

The plasma samples (spiked standards or from patients) were thawed at room temperature. To an aliquot of 0.5 ml of the plasma sample taken in a glass test tube (100 mm × 16 mm), 0.1 ml of a 2 μg/ml aqueous solution of VA (200 ng) as internal standard and 1 ml of saturated sodium chloride solution were added. Tubes were vortex-mixed for 10 s, and 0.1 ml of 17% (w/v) perchloric acid solution was added. The tubes were vortex-mixed again, left at room temperature for 10 min and centrifuged for 15 min (1725 *g*) at 18°C. The clear supernatant liquid was transferred to another glass tube (125 mm × 16 mm) and washed with 5 ml of pentane by shaking for 10 min. The tubes were centrifuged for 10 min at 18°C and the pentane layer was discarded. Then the aqueous layer was extracted with 6 ml of a mixture of ethyl acetate–pentane (1:1) by shaking for 10 min. After centrifuging for 10 min at 18°C the organic layer was transferred to a glass tube (100 mm × 16 mm) containing 1 g of anhydrous sodium sulfate. The tubes were kept at room temperature for 10 min. The dry organic layer was transferred to a clean glass test tube and evaporated to dryness at 55°C under a slow stream of nitrogen. The residue was reconstituted in 250 μl of mobile phase and a 25- or 50-μl aliquot was injected into the HPLC system.

Chromatography

The liquid chromatographic system consisted of a Model 590 HPLC pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne Model 1725 injection valve equipped with a 500-μl sample loop and an Ultrasphere ODS analytical column (25 cm$\times$0.46 cm I.D., 5 μm average particle size, Beckman, San Ramon, CA, U.S.A.). A Model 5100A dual-cell Coulochem detector (ESA, Bedford, MA, U.S.A.) fitted with a Model 5011 analytical cell (ESA) and a Model 5100 guard cell (ESA) was used. The applied voltages were +0.2, +0.4 and +0.55 V, respectively, for cell 1, cell 2 and the guard cell. The mobile phase consisted of an aqueous solution of 0.05 *M* sodium acetate, 0.1 *M* citric acid and 0.27 m*M* disodium ethylenediaminetetraacetic acid–methanol (83:17, v/v). The pH of the mobile phase was adjusted to 4.0 by the addition of 8 *M* sodium hydroxide solution. The samples were eluted isocratically at ambient temperature with a mobile phase flow-rate of 1.5 ml/min (3500 p.s.i.).

Calibration

The standard curve was constructed by plotting the concentration of HVA on the *x*-axis and the peak-height ratio (PHR) on the *y*-axis. The PHR was calculated as peak height of HVA/peak height of internal standard for all patient plasma samples. However, the PHR of the spiked plasma HVA standards and quality control samples were calculated in a different way. Since the plasma HVA standards and quality control samples were made in a single pool of blank plasma from volunteers, it is assumed that the PHR of the blank plasma is the same in all spiked plasma HVA standards and quality control samples. Therefore the PHR of spiked standards and quality control samples were calculated as PHR of spiked plasma sample minus PHR of blank plasma. A set of three quality control samples were treated similarly to check the validity of the standard curve.

RESULTS AND DISCUSSION

The chromatograms of HVA and VA were identified by the retention behavior of the aqueous standards made from authentic samples of HVA and VA. Chromatograms of the aqueous standards, blank plasma, blank plasma spiked with internal standard and blank plasma spiked with internal standard and HVA are shown in Fig. 1. There was no peak observed in the chromatograms interfering either with HVA or with VA during the analysis of about 200 samples. However, the selection of a suitable reagent for the precipitation of plasma protein and suitable solvents to extract HVA and VA is critical for the reliability of the assay procedure. After many trials with reagents such as hydrochloric acid and perchloric acid at various concentrations, it was found that a 100-μl volume of perchloric acid at a concentration of 17% (w/w) was sufficient to precipitate the plasma proteins. Perchloric acid at this concentration,

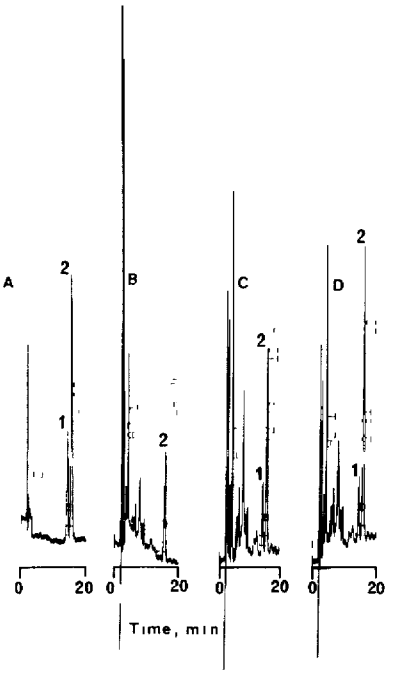

Fig. 1 Chromatograms of (A) a standard solution of a mixture of VA (40 ng) and HVA (2 ng), (B) a blank plasma, (C) a blank plasma spiked with VA (200 ng) and (D) blank plasma spiked with VA (200 ng) and HVA (5 ng). Peaks: 1 = VA; 2 = HVA.

a final concentration of approximately 1% (w/w), gave a better recovery of HVA and VA, approximately 30%, than using acids of higher concentrations. When ethyl acetate alone was used to extract HVA and VA from plasma water, there were peaks observed in the chromatograms interfering with HVA and the internal standard. However, there were no such interfering peaks observed when ethyl acetate–pentane (1:1, v/v) was used for the extraction and also there was no observable change in the recovery of HVA and VA. The absolute recovery of VA and HVA by this extraction procedure is $39.1 \pm 2.5\%$ ($n=27$) and $28.3 \pm 2.2\%$ ($n=27$), respectively.

Some compounds in the plasma extracted along with HVA and VA are retained on the column for long periods of time such that repeated injections at reasonably short intervals of time become very difficult. We observed consistently a compound eluting 30 min after injection. Although the intensity of this peak is much lower when the plasma samples were extracted with ethyl acetate–pentane mixture than when extracted with ethyl acetate alone, it could prolong the time between injections. However, this compound was eluted faster by injecting 200 μl of HPLC-grade methanol soon after the elution of the HVA peak. The injection of methanol did not have any deleterious effect on the

analysis or on the equilibration of the analytical cell and the column. Thus, it takes only 20 min of the HPLC time to analyze a plasma sample for HVA by this method.

The standard calibration curve for HVA was made using concentrations from 2.5 to 40 ng per ml of plasma. This concentration range was adequate because we did not encounter a plasma sample from our patient population containing HVA at concentrations outside this range. The standard curve was linear and typically had a correlation of 0.9975, a slope of 0.18 and an intercept of 0.0988. The intra-assay variation was determined by analysing five aliquots of each of three plasma samples from patients in the same day. The inter-assay variation was determined by analyzing spiked quality control samples on twelve different days. In both cases the coefficient of variation was less than 7.4% (Table I).

The HVA concentrations determined by the procedure described in this note in the plasma of patients treated chronically with antipsychotic drugs varied from 3.91 to 25.25 ng/ml (Fig. 2) with a mean ± S.D. value of 9.4 ± 3.6 ng/ml. This mean value is comparable to the previously reported [11, 16] mean ± S.D. values of 11.3 ± 4.2 and 9.0 ± 8.4 ng of HVA per ml of plasma determined by the more specific GC–MS method. We have measured concentrations of HVA in more than 200 plasma samples using the HPLC method reported here without any major problem.

In summary, the method reported here is simple, sensitive and reliable for the routine monitoring of HVA levels in plasma. The procedure is designed for minimal experimental error because of the use of an internal standard and the spiked plasma standards which are subjected to the same extraction procedure

TABLE I

INTRA- AND INTER-ASSAY VARIATION OF DETERMINATION OF PLASMA HVA

Sample	Concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Intra-assay variation[a]		
I	14.81 ± 0.22	1.5
II	11.14 ± 0.25	2.2
III	13.4 ± 0.68	5.1
Inter-assay variation[b]		
I	25.50 ± 1.27	5.0
II	13.07 ± 0.78	5.9
III	6.53 ± 0.48	7.4

[a]Five aliquots of each three plasma samples from patients were analyzed on the same day

[b]Three plasma samples spiked with a known amount of HVA (25, 12.5 and 6.25 ng/ml) were analyzed on twelve different days.

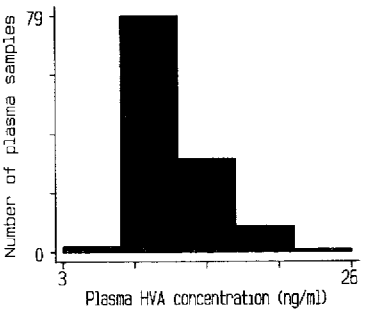

Fig. 2. Plasma HVA levels in schizophrenic patients measured by the HPLC method with dual-electrode electrochemical detection.

used for the preparation of the patients sample. The analysis is not time-consuming and it takes only 20 min of the HPLC time for each sample. The response of the analytical cell is stable over a long period of time enabling the analysis of a large number of samples without the frequent cleaning of the analytical cell. The plasma HVA levels obtained using this procedure is favorably consistent with the previously reported values determined by the more specific GC–MS method. This HPLC method is currently being used to investigate the correlation between the plasma HVA levels and the prediction of response of the patients to neuroleptic treatment.

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