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Note

Measurement of homovanillic acid in human plasma by high-performance liquid chromatography with electrochemical detection

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Several recent reports have discussed the possible use of plasma homovanillic acid (HVA) as an index of brain dopaminergic function [1—6]. Except for one study [6], determination of plasma HVA has been by means of gas chromatography with mass spectrometry (GC—MS). High-performance liquid chromatographic (HPLC) methods found to be suitable for measuring HVA in cerebrospinal fluid (CSF), have not been considered suitable for plasma or serum because compounds having long retention times have made repeated injections at short intervals impossible [7]. Because GC—MS methods were not readily available to us, we developed an HPLC method to measure HVA in human plasma.

EXPERIMENTAL

Samples were obtained from normal controls and psychiatric inpatients before and after administration of neuroleptic medication [6]. Informed consent was obtained from all subjects. Morning fasting blood was drawn into

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heparinized Vacutainer tubes. Plasma was separated within 1 h of collection and 2—4 replicate aliquots of 0.5—2.5 ml were placed in glass scintillation vials. For each 0.5 ml of plasma, 0.25 ml of 1 mol/l hydrochloric acid, 20 μl of 10% EDTA and 20 μl of 10% sodium metabisulfite were added. Samples were stored at −70°C until the time of assay, which was generally within one month after collection. Samples could be stored for six months without apparent loss of HVA.

A modification of the HVA extraction method described by Bacopoulos et al. [1] was used. To determine percent recovery, 50 μl of 0.001 mol/l hydrochloric acid containing 10 ng HVA was added to replicate patient samples and/or to replicate control samples run simultaneously. To determine linearity of extraction from plasma, recoveries of 1, 5, 10, 15, 20, and 25 ng of HVA added to a pooled plasma sample were determined. Volumes of plasma of 0.5, 1.0, 1.5, 2.0, and 2.5 ml were extracted using each amount of HVA standard. Recovery was measured as the difference between HVA in the sample containing standard and the same sample without added HVA.

Lipids were extracted at room temperature using two hexane washes of 5 ml (for 0.5- and 1.0-ml plasma samples) or 10 ml (for 1.5—2.5 ml plasma samples). Following extraction of lipids, samples were transferred to centrifuge tubes and proteins were precipitated by adding perchloric acid to achieve a final concentration of 0.3 mol/l. The precipitate was removed by centrifugation at 22,000 *g* for 15 min. A measured aliquot of supernatant (0.5—2.7 ml depending on original sample size) was added to glass scintillation vials containing potassium chloride to saturate. HVA was extracted into 6 ml of ethyl acetate added to each vial by shaking the samples for 15 min at high speed using a mechanical shaker. After a 15-min centrifugation at low speed to separate the organic and aqueous phases, a 4.5-ml aliquot of the ethyl acetate extract was evaporated to dryness at 28°C under nitrogen. Dried samples were capped and stored frozen until the time of injection into the chromatograph.

A Bioanalytical Systems liquid chromatography system consisting of an M-45 solvent delivery system, an LC-4A amperometric detector connected to a glassy carbon (TL-5) working electrode, and a RYT recorder were used. The Biophase ODS, 5-μm column, 25 cm × 5 mm I.D., was protected by a guard column of the same material. An oxidation potential of 0.72 V with respect to an Ag/AgCl reference electrode was applied. The mobile phase consisted of 50 m*M* sodium acetate containing 100 mg/l EDTA and 12% methanol, with an apparent pH of 4.4. Flow-rate was 1.1 ml/min at ambient temperature. Elution was isocratic.

Samples were reconstituted with 300 μl of mobile phase and filtered through a 0.45-μm RC 45 filter (Bioanalytical Systems) before injection into a 100-μl sample loop. A sensitivity of 2 nA full scale was used for 0.5- and 1.0-ml samples; for larger samples a 5-nA sensitivity was used.

RESULTS AND DISCUSSION

Using HPLC with electrochemical detection, we have measured HVA levels in 342 plasma samples (Fig. 1). Excluding the extreme high values (> 45 ng/ml), the mean (± standard error) plasma HVA levels were: controls, 9.8 ± 0.5 ng/ml

(n = 22), unmedicated subjects, 9.2 ± 1.8 ng/ml (n = 25), subjects receiving neuroleptics, 17.3 ± 1.1 ng/ml (n = 234). These values compare favorably with previously reported values for plasma HVA determined using GC—MS [5].

Peak identity was based on retention characteristics (Fig. 2) and a comparison of hydrodynamic voltammograms for reference HVA and the HVA peak in plasma (Fig. 3). The closeness of the voltammograms demonstrates that no interfering substances were co-eluting with plasma HVA [8] and allows quantitation of the plasma peak. The standard curve for HVA was

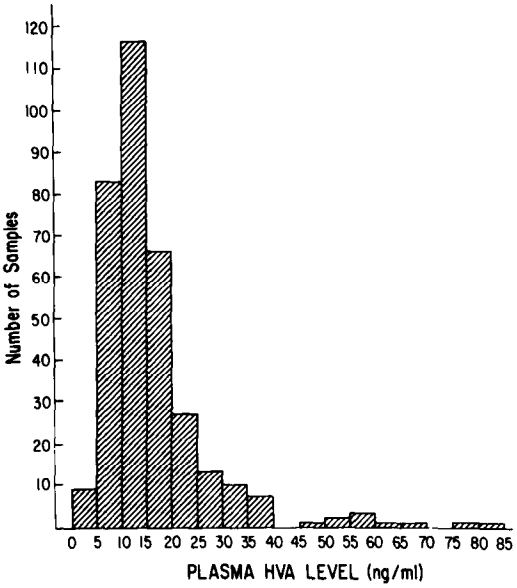

Fig. 1. Levels of homovanillic acid (HVA) measured in 342 plasma samples using HPLC with electrochemical detection.

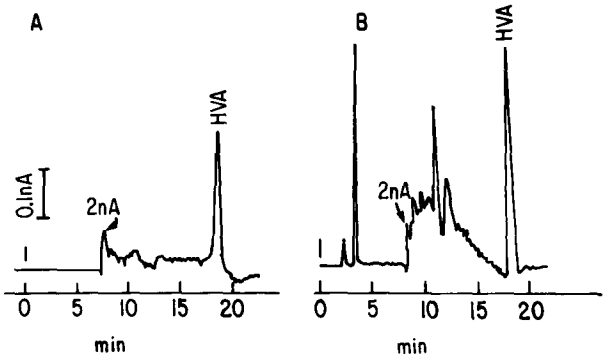

Fig. 2. Comparison of chromatograms for a 500-pg HVA standard (A) and a 0.5-ml plasma sample (B). Retention time for the plasma peak was identical with that for reference HVA and changed in an identical manner when the pH or methanol concentration of the mobile phase was changed. Reference HVA added to plasma produced an increase in the size of the plasma HVA peak with no evidence of a shoulder or split-peak. Mobile phase: 50 m*M* sodium acetate containing 100 mg/l EDTA and 12% methanol, pH 4.4; retention time for HVA: 17 min 50 sec.

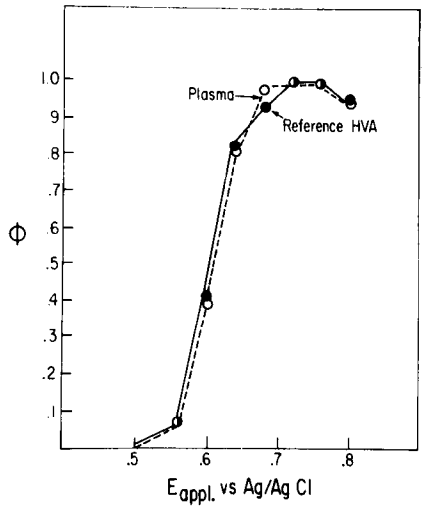

Fig. 3. Hydrodynamic voltammograms for the HVA reference compound and the peak in human plasma. Abscissa: oxidation potential vs. Ag/AgCl reference electrode. Ordinate: ratio of response at a particular potential to the maximal response. The close identity of the two curves demonstrates the purity of the plasma HVA peak.

linear to 100 ng, the highest concentration tested, and passed through zero. Therefore, no HVA was being retained by the column and there was no baseline interference with the peak of interest. As little as 100 pg HVA could be measured.

Precision of the analytical procedure was determined by chromatographing aliquots of plasma samples. The day-to-day precision measurement produced a coefficient of variation of 3.9% (n = 4).

Extraction procedures described were highly reproducible with consistent results for a large number of extractions performed during a two-year period. Recovery of HVA, determined in over 300 different plasma samples, was generally 28—30%. It was the same for 1—25 ng of HVA added to a given volume of plasma and for a given amount of HVA added to different volumes (0.5—2.5 ml) of plasma. It was not altered by different amounts of endogenous HVA in different plasma samples or by neuroleptic medication. If samples are carefully prepared and stored, the standard deviation (S.D.) for four replicate samples can be as low as 0.1 to 0.5 ng/ml (Table I).

Some precautions should be taken in order to achieve consistent extraction of HVA from plasma. During the hexane washes, samples should be shaken only very gently because vigorous shaking will turn the sample into a gel [1]. In addition, incomplete separation of the ethyl acetate and aqueous layers may be a problem with 2.5-ml plasma samples and can be overcome by centrifuging the samples at low speed for 5 min, shaking the vials to break up the gel-layer that forms between the organic and aqueous phases, and recentrifuging.

Variation between replicate plasma samples depends on a number of factors other than reliability and reproducibility of extraction and chromatography procedures. Particular care should be taken that samples are not thawed and re-frozen prior to extraction. In addition, long-term storage of small sample

TABLE I

PLASMA HVA LEVELS

Plasma HVA levels are measured in four replicate samples obtained from ten subjects and demonstrate the reliability of the extraction, separation and quantitation methodes described in the Experimental section.

Sample		Plasma HVA (ng/ml, mean ± S.D.)
Control	A	10.2 ± 0.3
	B	8.5 ± 0.5
	C	12.8 ± 0.2
	D	11.0 ± 0.1
	E	10.4 ± 0.2
Subject	A	15.0 ± 0.5
	B	4.3 ± 0.4
	C	13.5 ± 0.3
	D	14.0 ± 0.2
	E	29.8 ± 0.5

volumes in scintillation vials is inadvisable because these samples have a tendency to dry down on the bottom of the vial.

One objection to the use of HPLC methods to measure HVA in plasma or serum has been that compounds in the blood are retained on the column for long periods of time, making repeated injections at reasonable intervals of time impossible [7]. Using the methods we have described, and an initial plasma volume of 0.5 ml, injections can be made every 19 min. There are peaks which elute after the HVA peak, but these are generally not a problem for small plasma samples. For samples larger than 0.5 ml plasma, we have found it necessary to stop injections and wash the column with mobile phase for 1—1.5 h after every six to eight injections. Samples larger than 0.5 ml may be desirable when plasma HVA levels are expected to be less than 3 ng/ml. However, an easier alternative is to reconstitute the ethyl acetate extract with less mobile phase and inject the entire sample.

In summary, we have described a reliable method for extraction of HVA from plasma, separation of HVA from other plasma constituents present in these extracts, and quantitation of amount of HVA in the isolated peak. Injections can be made at 19-min intervals without interference from compounds having longer retention times than HVA. Values obtained are consistent with previously reported values for plasma HVA obtained by GC—MS. HPLC is simpler, less expensive and more widely available than GC—MS. Therefore, measurement of plasma HVA using HPLC instead of GC—MS may facilitate further investigation of the relationship between plasma HVA and central dopaminergic function.

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