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Note

Measurement of homovanillic acid in small volumes of plasma using liquid chromatography with electrochemical detection

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Homovanillic acid (HVA) is the main metabolite of dopamine in the brain. Since a large proportion of plasma HVA is derived from brain, HVA levels may reflect dopaminergic activity in the central nervous system [1–8]. Whereas high-performance liquid chromatography with electrochemical detection (LC–ED) has been used to measure HVA in cerebrospinal fluid, urine, or brain tissue [9–11], LC–ED has not been applied widely to measurement of HVA in plasma because of the relatively low HVA levels and the presence of substances which can interfere with the chromatographic separation. Plasma HVA has been determined mainly using gas chromatography with mass spectrometry (GC–MS), which is a cumbersome technique requiring expensive equipment [2, 12, 13]. Attempts to apply two reported methods for measurement of HVA in plasma using LC–ED [14, 15] were unsuccessful in this laboratory, even with large volumes of plasma.

Repeated sampling of blood from rodents or small primates requires small amounts of plasma to avoid volume depletion. A convenient, sensitive method for measurement of HVA in small amounts of plasma was needed.

Here we describe a rapid procedure for extraction of HVA from small volumes of plasma, with quantification using LC–ED. The sensitivity and reliability of the procedure were assessed using GC–MS.

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EXPERIMENTAL

Blood sampling

Jugular venous or vena cava blood of ten rats anesthetized with pentobarbital (Somnifer, Richmond Veterinary Supply Co., Richmond, VA, U.S.A.) was collected into heparinized tubes. In eight rats blood was sampled at 16.5 h after administration of nialamide (75 mg/kg intraperitoneally, obtained from Sigma) or saline (8 ml/kg intraperitoneally). Plasma was separated within 1 h by refrigerated (4°C) centrifugation at 3000 *g*. Plasma was removed and stored at −70°C until assayed. Antecubital venous plasma from three normal volunteers was pooled and aliquoted into 1-ml plastic test tubes (Eppendorf, Brinkmann Instruments, Westbury, NY, U.S.A.) and stored at −70°C until assayed. Antecubital venous plasma samples from 36 other volunteers were assayed using both LC–ED and GC–MS.

Deproteinization and extraction

After thawing, 400 μl of plasma (or 200 μl with 200 μl Type I water) were transferred to a polypropylene test tube and kept on ice. HVA (Behring Diagnostics, La Jolla, CA, U.S.A.), 5–15 ng as an external standard in 30 μl of acetic acid (0.2 *M*), or 5-fluorohomovanillic acid (F-HVA), 5–15 ng as an internal standard (kindly provided by Dr. K.I. Kirk, Laboratory of Chemistry, NIDDK, National Institutes of Health, Bethesda, MD, U.S.A.) was added. Plasma proteins were precipitated by adding 40 μl of 60–62% perchloric acid and vortex-mixing for 10 s. Samples were kept on ice for 15 min, vortex-mixed for 5 s, and centrifuged at 17 000 *g* for 10 min at 4°C. An aliquot (300 μl) of the clear supernatant was transferred into a disposable, sterilized, polyethylene/polypropylene 2-ml syringe (Aldrich, Milwaukee, WI, U.S.A.) and 500 μl water were added. This solution was loaded onto a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) which had been activated using 6 ml methanol and 12 ml water. The cartridge was washed with 3 ml water using the same syringe used to tansfer the supernatant fluid to the column. Ethyl acetate (5 ml) was then flushed through the cartridge. The first milliliter of the effluent was discarded and the next four milliliters collected into a glass tube. Aliquots (2 ml) were evaporated in a stream of nitrogen at room temperature. The dry residue was dissolved in 250 μl acetic acid (0.2 *M*) and 40 μl were injected onto the chromatographic column.

Assays of plasma HVA by GC–MS were carried out as previously described [12].

Apparatus

The chromatographic apparatus included a Waters Model 590 solvent delivery system (Millipore, Milfod, MA, U.S.A.), an Ultrasphere XL-ODS 3-μm, 7.0 cm×4.6 mm stainless-steel column (Beckman Instruments, Berkeley, CA, U.S.A.), a Waters Model 710B automated sample processor, and an Environmental Sciences Assoc. Model 5100A electrochemical detector with Model 5011 and 5021 electrodes (ESA, Milford, MA, U.S.A.). The mobile phase contained (per liter): 6.8 g sodium acetate, 100 mg EDTA, 80 ml methanol, and 10 ml acetonitrile. The pH of the mobile phase was adjusted to 3.55 with 85% phosphoric

acid. The mobile phase was filtered and degassed before use and pumped at a flow-rate of 1 ml/min.

The first electrode in the post-column series of electrodes was set at +0.15 V, the second at +0.15 V, and the third at +0.35 V (in preliminary experiments the electrode settings were +0.15, +0.15, and −0.35 V).

Data analysis

Recoveries of HVA and F-HVA through the sample preparation steps were calculated using eqns. 1 and 2 below.

$$\text{F-HVA recovery} = \frac{\text{sample F-HVA peak height} \times \text{ng F-HVA in standard}}{\text{standard F-HVA peak height} \times \text{ng F-HVA in sample}} \quad (1)$$

$$\text{HVA recovery} = \frac{\text{ng HVA injected directly}}{\text{ng HVA added to sample}} \times \frac{\text{HVA peak height in spiked sample} - \text{HVA peak height in unspiked sample}}{\text{peak height of standard HVA injected directly}} \quad (2)$$

The linearity of curves relating calculated HVA concentrations to amounts of added standard HVA was assessed by linear regression analysis. Inter- and intra-assay coefficients of variation were calculated at HVA concentrations in a physiological range. Assay validity was determined by comparison of LC–ED results with results using GC–MS on split plasma samples (1) from human volunteers, (2) from rats treated or not treated with nialamide to block monoamine oxidase, and (3) from pooled plasma to which various amounts of HVA standard had been added.

RESULTS AND DISCUSSION

Extraction of the plasma through Sep-Pak C_{18} minicolumn after protein precipitation resulted in clean chromatograms where HVA and F-HVA could be detected without interference. Fig. 1A shows a chromatographic recording after injection of a standard solution of HVA and F-HVA and Fig. 1B shows a recording after injection of supernatant of a deproteinized plasma sample to which F-HVA had been added as an internal standard. A large contaminating peak was observed between the peaks corresponding to HVA and F-HVA. When the supernatant was extracted through the Sep-Pak cartridge, eluted with ethyl acetate, evaporated to dryness, and reconstituted in acetic acid prior to injection, the contaminating peak was removed (Fig. 1C). Changing the voltage of the third post-column electrode to +0.35 V enhanced chromatographic resolution and sensitivity for HVA and F-HVA in both human (Fig. 2A) and rat (Fig. 2B) plasma. Under these conditions, there was a linear correlation ($r = 1.00$) between the injected amount of HVA or F-HVA (20–5000 pg) standards and peak height.

Using pooled standard plasma, 27.1 ± 1.04% of added HVA (5–15 ng) was re-

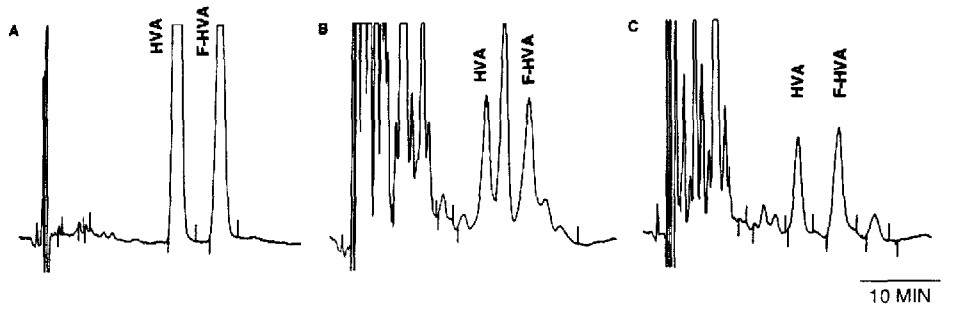

Fig. 1. Chromatogram of (A) standard HVA (10 ng) and F-HVA (50 ng), (B) deproteinized plasma, and (C) deproteinized plasma after extraction through Sep-Pak C_{18}. The chromatograms were obtained using a potential of −0.35 V at the second electrode. Peak height is the digital electronic relative height using the data module.

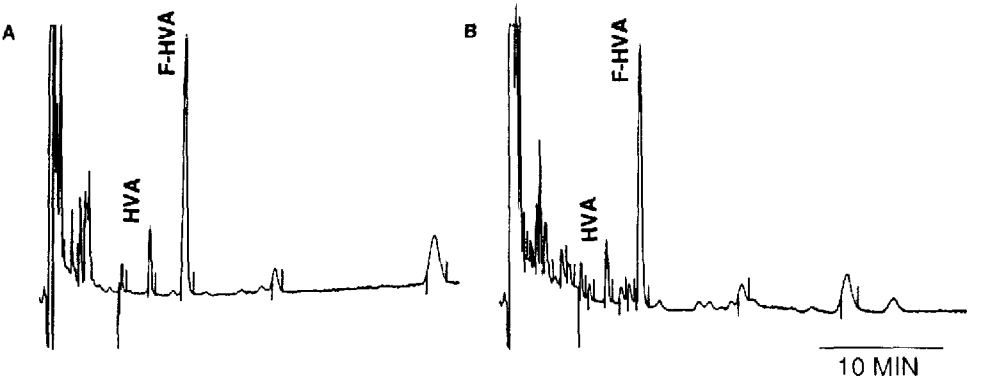

Fig. 2. Chromatogram of (A) human plasma and (B) rat plasma using 200 μl deproteinized plasma extracted through Sep-Pak C_{18} Peak height is the digital electronic relative height using the data module.

covered through the sample preparation steps ($n=19$). Recovery of F-HVA (5–15 ng) averaged $28.1 \pm 1.28\%$ ($n=19$). Thus, no differences were observed between HVA concentrations calculated using HVA as an external standard and using F-HVA as an internal standard. The calculated amount of HVA added to plasma correlated well ($r=0.99$) with the actual amount added to control plasma (results not shown) when F-HVA was used as internal standard. The amount of HVA in pooled plasma was independent of the amount of F-HVA added as an internal standard (5, 7.5, 10, or 15 ng, Table I).

Reconstituted samples were stable at room temperature for at least 24 h, and so the samples were not kept on ice until injection, in contrast with the procedure of Chang et al. [15]. At temperatures above about 35°C, the samples were not stable when reconstituted with acetic acid but were stable when reconstituted with the mobile phase. Extraction of plasma with ethyl acetate using the method of Harris et al. [14] gave rise to chromatographic recordings with many peaks which interfered with accurate measurement of HVA. Amicon filtration as done by Drebing et al. [16] resulted in unacceptable chromatography due to interfering peaks similar to those obtained using deproteinized plasma. Since late-eluting peaks sometimes were large, the method of Chang et al. [15] could not be applied.

TABLE I

EFFECT OF VARIOUS AMOUNTS OF F-HVA AS AN INTERNAL STANDARD ON RECOVERIES AND ON CALCULATED HVA LEVELS IN 400-μl ALIQUOTS OF POOLED PLASMA

F-HVA added (ng per 400 μl)	Recovery (%)	HVA level (ng per 400 μl)
5	27.7 ± 4.06	5.63 ± 0.49
7.5	29.5 ± 4.80	5.45 ± 0.29
10	28.2 ± 4.39	5.46 ± 0.16
15	26.7 ± 4.89	5.44 ± 0.37

The limit of detection was about 10 pg per injected sample at a signal-to-noise ratio of 3. Results using 400 μl plasma (HVA concentration 13.53 ± 0.22 ng/ml, $n=12$) were similar to those using 200 μl of the same plasma (13.76 ± 0.37 ng/ml, $n=15$). Using 200 μl of human (Fig. 2A) or rat (Fig. 2B) plasma, excellent chromatographic results were obained in which HVA could be detected easily. The simple, rapid clean-up step with Sep-Pak C_{18} cartridges resulted in clearcut improvement in the chromatographic results.

Since recoveries of HVA were found to be the same as for F-HVA, F-HVA can serve as an internal standard in the extraction procedure. With the mobile phase used in this study, each chromatograph required about 40 min, so that about 24 samples could be assayed overnight (16 h).

The validity of the LC–ED method was assessed by comparing the results for different human plasma samples ($n=36$) using the LC–ED procedure and using GC–MS (Fig. 3). A good correlation ($r=0.94$) was obtained for results obtained by the two methods, however, the results for most of the samples obtained using LC–ED were higher than those obtained with GC–MS. When given amounts of

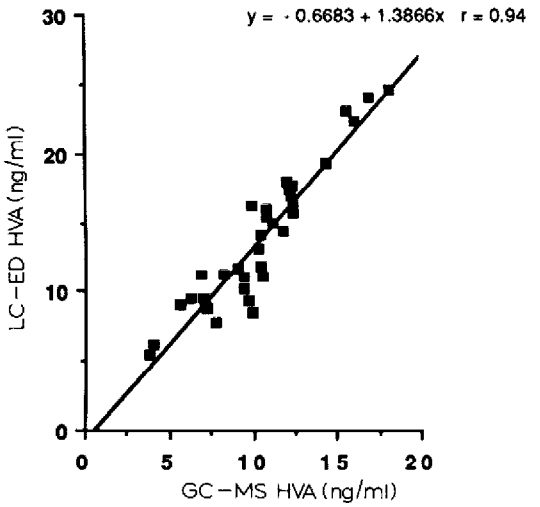

Fig. 3. Plasma HVA determination using LC-ED and GC-MS in different plasma samples.

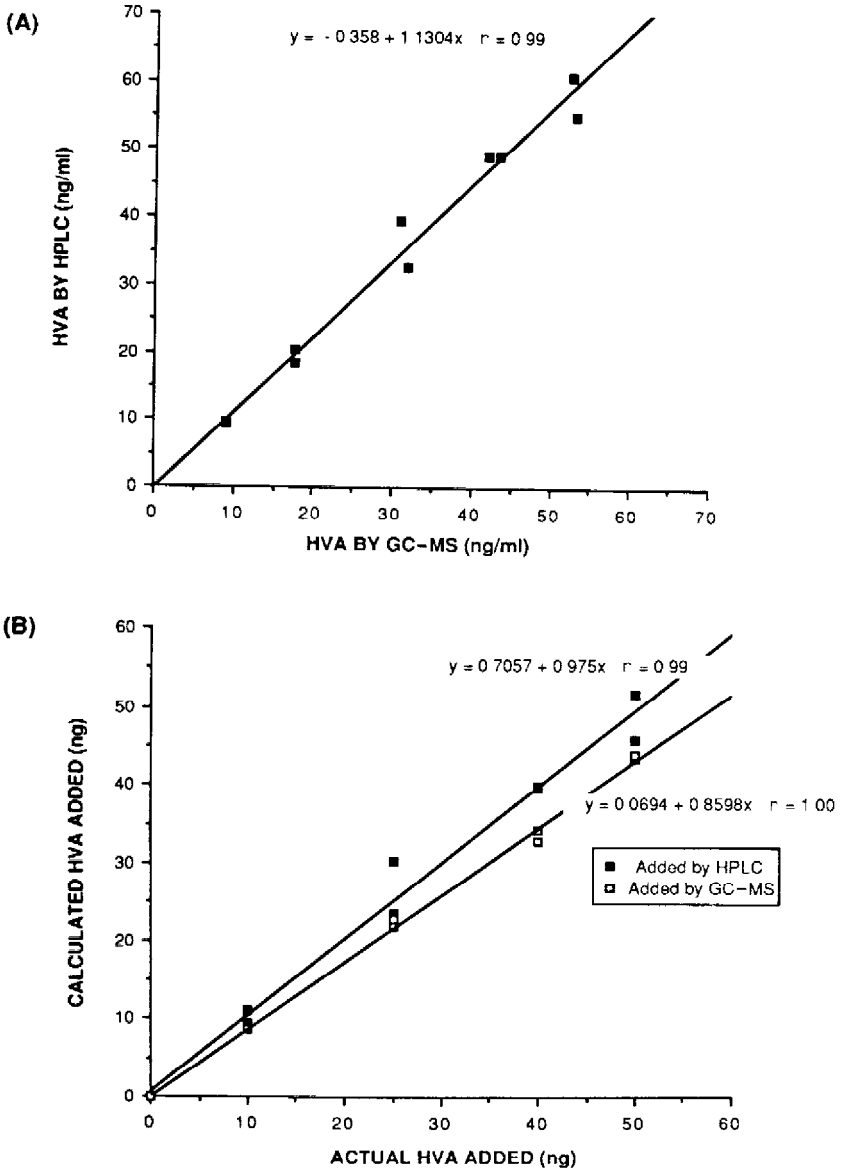

Fig. 4. Control plasma with added amounts of HVA standard. (A) HVA determination by LC-ED and GC-MS and (B) calculated added HVA by LC-ED and GC-MS versus actual amounts of added standard HVA.

HVA standard were added to aliquots of pooled plasma, agreement between the LC–ED and GC–MS results also was excellent ($r=0.99, p<0.01$, Fig. 4A), and the calculated amount added based on LC–ED was closely related to the actual amount added ($r=1.00, p<0.01$, Fig. 4B). In rats ($n=8$) treated with the non-specific monoamine oxidase inhibitor, nialamide, or with saline the LC–ED results agreed well with the GC–MS results ($r=0.95$); in this case the values ob-

tained by the GC–MS method were somewhat higher than by LC–ED (regression equation: $y = 0.0992 + 0.6882x$).

The strong correlations indicate the validity of the LC–ED approach. The results using LC–ED and GC–MS did not differ consistently, and LC–ED accurately quantified the amounts of HVA standard added to aliquots of plasma and is, therefore, valid for measuring plasma levels of HVA. The present method may be particularly useful in studies involving repeated blood sampling in rodents or small primates.

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