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Note

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

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The level of the dopamine metabolite homovanillic acid (HVA) in blood is of interest as an index of dopaminergic function in the brain [1, 2]. Plasma and serum HVA have been determined by means of gas chromatography—mass spectrometry (GC—MS) [1, 3, 4]. GC—MS is a sensitive method, but is available to only a limited number of workers.

Reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been shown to provide high selectivity and sensitivity for the determination of monoamine metabolites in urine [5—9], cerebrospinal fluid [10, 11] and brain tissue [12, 13]. The oxidation products of HVA and vanillylmandelic acid were identified by us using ED [14]. The application of HPLC with ED to the determination of monoamine metabolites in plasma, however, is difficult in comparison with that in urine, cerebrospinal fluid or brain tissue because of the much lower level and various interfering substances. The use of a reversed-phase octadecylsilane column [15] or organic extraction [16] has been attempted in order to overcome these difficulties for the determination of plasma HVA, but further purification before HPLC is desirable.

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In this work, a procedure for the clean-up of HVA was elaboratored by using both a reversed-phase octadecylsilane column (Sep-Pak C_{18} cartridge) and an anion-exchange gel (QAE-Sephadex A-25) column, and a selective and reliable HPLC procedure was established for the determination of HVA in human plasma.

EXPERIMENTAL

Materials

HVA was purchased from Tokyo Kasei Kogyo (Tokyo, Japan), Sep-Pak C_{18} cartridges from Waters Assoc. (Milford, MA, U.S.A.) and QAE-Sephadex A-25 strongly basic anion-exchange gel from Pharmacia (Uppsala, Sweden). Ethyl acetate was of LC grade from Wako (Osaka, Japan) and purified by passage through a column (180 × 20 mm I.D.) of neutral aluminium oxide (aluminium oxide 90 for column chromatography, activity I; E. Merck, Darmstadt, F.R.G.) prior to use. All other chemicals were of analytical-reagent grade from Wako. The water used was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

A working reference solution of 300 ng/ml of HVA in 10 m*M* hydrochloric acid was prepared. The solution was stable for 3 months at −80°C. The solution was thawed and used daily.

Apparatus

A Model LC 3A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) was equipped with a Rheodyne 7125 injection valve with a 20-μl sample loop, a LiChrosorb RP-18 column (5-μm particle size, 300 × 4.0 mm I.D.) from E. Merck and an EC-8 electrochemical detector (Toyo Soda, Tokyo, Japan). The column temperature was maintained at 50°C with a water-jacket. For injections into the liquid chromatograph a Hamilton No. 701 10.0-μl syringe (Hamilton, Reno, NV, U.S.A.) was used.

Preparation of the Sep-Pak C_{18} cartridge

Before use, the cartridge was washed with 10 ml of acetone, 10 ml of methanol, 10 ml of water, 10 ml of 10 m*M* ammonium acetate solution, 10 ml of water and 5 ml of 1 *M* phosphoric acid buffer adjusted to pH 2.0 with sodium hydroxide, with the aid of an injection syringe.

Preparation of the column of QAE-Sephadex A-25

QAE-Sephadex A-25 (acetate form) was rinsed and decanted ten times with water. The gel slurried in water was poured into a glass tube (I.D. 9.5 mm) up to a height of 35 mm. The column was washed with water prior to use.

Plasma samples

Venous blood was collected in a tube containing 1 mg of EDTA, potassium salt, per 1 ml of blood. Plasma was separated by centrifugation at 1000 *g* for 20—60 min at 4°C, and stored at −80°C until taken for analysis.

Sample preparation

A 1-ml volume of plasma mixed with 2 ml of 1 *M* phosphate buffer (pH 2.0)

was passed through the Sep-Pak C_{18} cartridge. The cartridge was washed with 5 ml of 1 m*M* hydrochloric acid and the adsorbed HVA was eluted from the cartridge with 12 ml of 10 m*M* ammonium acetate solution. The effluent was then introduced into the QAE-Sephadex A-25 column. After the column had been washed with 8 ml of 1 m*M* hydrochloric acid, the adsorbed HVA was eluted from the column with 5 ml of a 0.2 *M* solution of sodium chloride in 10 m*M* hydrochloric acid.

To the effluent were added 1 g of sodium chloride and 1 ml of 1 *M* phosphoric acid buffer adjusted to pH 1.0 with sodium hydroxide. After the resulting mixture had been extracted with 10 ml of ethyl acetate by shaking for 2 min, 8 ml of the organic phase were taken and evaporated to dryness at 30°C under reduced pressure. The residue was dissolved in 0.2 ml of the HPLC eluent and 10 μl of the solution were injected into the chromatograph.

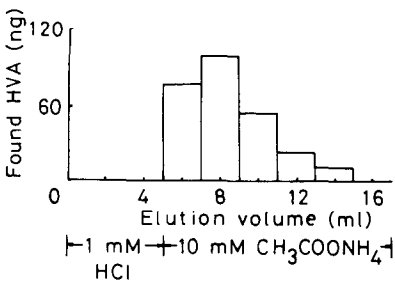
HPLC

Isocratic elution of the reversed-phase HPLC system was employed: column, LiChrosorb RP-18 (300 × 4.0 mm I.D.); eluent, 20% methanol in 0.05 *M* potassium dihydrogen phosphate buffer adjusted to pH 2.2 with phosphoric acid, degassed under reduced pressure. The flow-rate of the eluent was 0.8 ml/min and the column temperature was maintained at 50°C. The inlet pressure was about 180 kg/cm^2. An oxidation potential of +0.70 V with respect to an Ag—AgCl reference electrode was applied and the sensitivity was set at 4 nA full-scale.

RESULTS AND DISCUSSION

Clean-up of plasma

HVA was extracted directly from plasma into ethyl acetate, but not separated from the other constituents in the plasma by reversed-phase HPLC with ED. Before the organic extraction, we adopted a purification method using both the Sep-Pak C_{18} cartridge for retention of undissociated HVA in an acidic medium and the QAE-Sephadex A-25 column for cation exchange-ability of dissociated HVA in a neutral or alkaline medium. Fig. 1 shows the elution profile of 300 ng of authentic HVA applied as a solution in a mixture



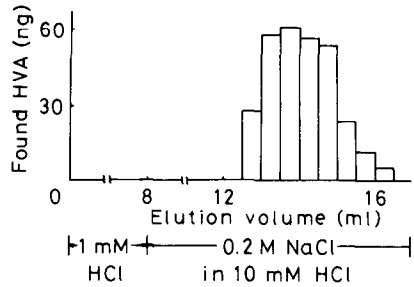


Fig. 1. Elution profile of 300 ng of HVA from the Sep-Pak C_{18} cartridge. Conditions as described under Results and Discussion.

Fig. 2. Elution profile of 300 ng of HVA from the QAE-Sephadex A-25 column. Conditions as described under Results and Discussion.

of 1 ml of plasma and 2 ml of 1 *M* phosphate buffer (pH 2.0) to the Sep-Pak C_{18} cartridge. The reversed-phase cartridge adsorbs HVA at lower pH. During washing with 5 ml of 1 m*M* hydrochloric acid, HVA remains adsorbed on the cartridge. Subsequently, HVA was eluted with 12 ml of 10 m*M* ammonium acetate. Under the HPLC conditions described, however, the remaining constituents in the plasma still interfered with HVA extracted from this effluent into ethyl acetate. Therefore, further purification was necessary. The recovery of HVA from the Sep-Pak C_{18} cartridge was 90 ± 2% (mean ± S.D., n = 3) of the amount applied.

Fig. 2 shows the elution profile of 300 ng of authentic HVA applied as 12 ml of a solution in 10 m*M* ammonium acetate to the column of QAE-Sephadex A-25. HVA retained on the gel was not desorbed by washing the column with 8 ml of 1 m*M* hydrochloric acid and was eluted with 0.2 *M* sodium chloride in 10 m*M* hydrochloric acid. The recovery of HVA from the QAE-Sephadex A-25 column was 96 ± 1% (mean ± S.D., n = 3).

HPLC

Although the authentic HVA showed the highest sensitivity at an oxidation potential of +0.80 V, an adjoining peak interfered with the quantitation of HVA. Monitoring the current at a potential of +0.70 V provided a selective and sensitive assay for HVA in plasma.

Under the conditions described, HVA was eluted at 14.6 min and was well separated from the other constituents in plasma.

Calibration graph, recovery and precision

A graph of peak height against the amount of authentic substance added to plasma was linear over the range 5—60 ng/ml. The detection limit in plasma, based on a signal-to-noise ratio of 4, was 2 ng/ml. The recovery was 86 ± 2% (mean ± S.D., n = 5) over the same range.

The reproducibility of the method was evaluated by multiple analyses of a pooled plasma containing 7.5 ng/ml of HVA. The coefficient of variation for the within-day precision was estimated to be 5.3% from six measurements and that for the between-day precision was 11.9%.

TABLE I

LEVELS OF HVA IN PLASMA FROM NORMAL SUBJECTS

Subject No.	HVA (ng/ml)
1	6.7
2	8.4
3	8.5
4	8.1
5	6.6
6	6.1
7	7.7
8	6.7
9	8.6
10	10.5
Mean ± S.D.	7.8 ± 1.3

Method validation

The results for the determination of HVA in plasma from ten normal subjects are given in Table I. The values are roughly compatible with those recently obtained with GC-MS [3, 4] and HPLC [15—17].

In summary, liquid chromatographic analysis combined with the procedure using the Sep-Pak C_{18} cartridge and the column of Sephadex anion-exchange gel provides a reliable method for the determination of HVA in human plasma. The method proved useful for the further investigation of the relationship between HVA and antipsychotic drug levels in plasma, e.g., in view of the dopaminergic function in the central nervous systems. These results will be reported elsewhere.

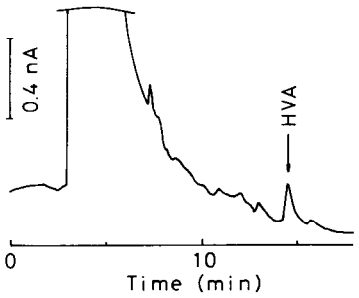


Fig. 3. Typical chromatogram of 1 ml of plasma containing 7.3 ng of HVA from a normal subject.

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