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Note

Determination of plasma homovanillic acid by two-step solid-phase extraction and high-performance liquid chromatography with electrochemical detection

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The clinical importance of plasma homovanillic acid (HVA) has been emphasized since plasma HVA was first suggested to reflect central dopamine metabolism [1–3]. Plasma HVA has been determined by gas chromatography–mass spectrometry (GC–MS) because of its low level and various interfering substances [1, 4, 5]. However, this requires expensive equipment and the technique is rather tedious. Recently, the use of high-performance liquid chromatography with electrochemical detection (HPLC–ED) has led to the development of sensitive and selective assay methods for plasma HVA [6–10]. These methods, however, require lengthy column separation [9], organic solvent extraction [6, 9, 10] or a dual electrochemical detector [7], and an appropriate internal standard is lacking [6, 9, 10]. In recent years, bonded-silica extraction columns, which have many advantages, including high selectivity, high recovery and simplicity, have attracted wide interest [11–13].

This paper describes a new HPLC–ED method for plasma HVA, involving two bonded-silica extraction columns for purification. HVA was extracted from plasma with a Bond Elut C_8 column followed by a Bond Elut SAX column. This method is simpler and less time-consuming for extracting HVA from plasma than other HPLC methods, and can be employed on a routine basis.

EXPERIMENTAL

Materials

HVA was purchased from Nakarai (Kyoto, Japan). A Bond Elut C_8 column prepacked with 500 mg of octylsilica and a SAX column prepacked with 100 mg

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of trimethylaminopropylsilica were purchased from Analytichem (Harbor City, CA, U.S.A.). 3-Hydroxy-4-methoxyphenylacetic acid (iso-HVA) was a gift from Hoffmann-La Roche (Basel, Switzerland). The water used was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus

The liquid chromatographic system consisted of a Model L-4000 W pump, a Model VMD-101A electrochemical detector (Yanagimoto, Kyoto, Japan) and a reversed-phase column (Senshu Pack 5-ODS-H, 5 μm particle size, 150 mm $\times$ 4.6 mm I.D.; Senshu Scientific, Tokyo, Japan). The mobile phase consisted of 0.05 *M* phosphate–citrate buffer containing 13% (v/v) acetonitrile and 0.1 m*M* EDTA (pH 4.2). The mobile phase was degassed and filtered through 0.45-μm membrane filters before use. The flow-rate was maintained at 1.0 ml/min. The applied potential was set at +0.7 V vs. Ag/AgCl electrode.

Activation of the Bond Elut columns

Prior to extraction, the Bond Elut C_8 columns were activated with 5 ml of methanol followed by 5 ml of water under a vacuum water aspirator. To achieve complete ion exchange in the Bond Elut SAX columns, they were washed successively with 1 ml of methanol, 5 ml of 1.0 *M* sodium acetate buffer (pH 6.0) and 1 ml of water.

Extraction

To 1.0 ml of plasma, 50 μl of 0.1 *M* EDTA, 30 μl (30 ng) of iso-HVA in 0.01 *M* hydrochloric acid and 200 μl of 1.0 *M* hydrochloric acid were added. The acidified plasma was diluted with 2 ml of water and then passed through the Bond Elut C_8 column. The column was washed with 2 ml of distilled water, and then the adsorbed HVA was eluted with 2 ml of 50% methanol. The eluate was diluted with 1 ml of 0.1 *M* sodium acetate buffer (pH 6.0) and applied to the Bond Elut SAX column. Then, HVA was eluted with 300 μl of 1 *M* hydrochloric acid. The flow-rate of the solvent from this column should not exceed 0.5 ml/min. Usually, a 50-μl portion of the eluate was injected.

RESULTS AND DISCUSSION

Representative chromatograms of an extract from human plasma and a standard solution are shown in Fig. 1. The peaks of HVA and iso-HVA are clearly separated from those of interfering substances. For plasma samples, a late-eluting peak appeared at ca. 22 min. Identification of the HVA peak was based on its chromatographic behaviour and electrochemical characteristics (Fig. 2). Iso-HVA proved to be an ideal internal standard, since it behaves like HVA during analysis and has the same electrochemical activity. Although HVA and iso-HVA showed plateaux at 0.8 V in the voltammograms, we selected 0.7 V as an adequate applied potential, since a high potential increases noise and interfering peaks.

The main advantage of our extraction method is the use of Bond Elut extraction columns, which require only minimal handling, and also HVA can be directly

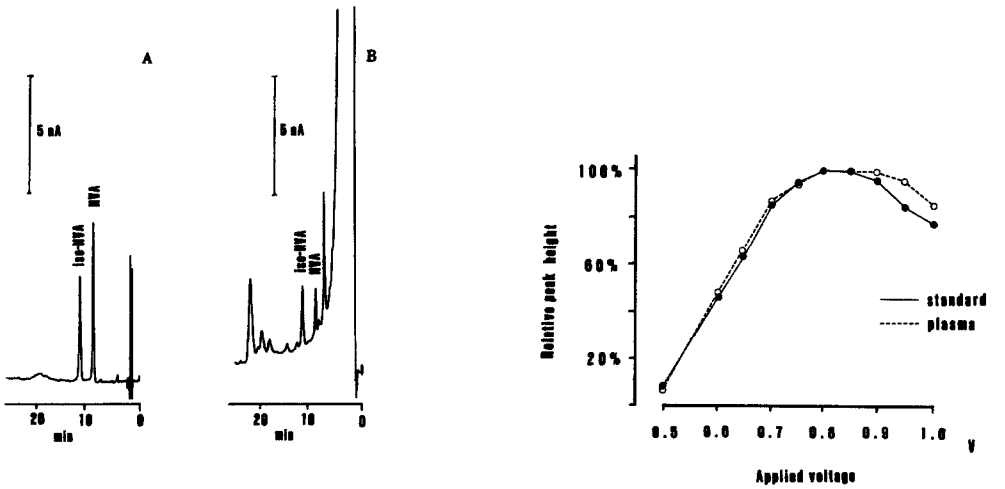

Fig. 1. Representative chromatograms of (A) a standard solution containing 5 ng each of HVA and iso-HVA and (B) a plasma extract containing 12.0 ng/ml HVA.

Fig. 2. Voltammograms of the HVA standard and of the assumed HVA from a plasma extract. The relative peak heights of HVA as a function of the electrode potential are shown. The peak height observed at 0.8 V for each compound was taken as 100.

extracted from a plasma sample with a good recovery. Since HVA is a weakly polar substance, HVA in an acidic medium is initially retained on a Bond Elut C_8 column through non-polar interaction. Thus, proteins, lipids and other highly hydrophilic substances can be eliminated. In the second step, plasma HVA was further purified and enriched on a Bond Elut SAX column through anion exchange.

In our preliminary experiments, we evaluated columns of Bond Elut octyl (C_8), octadecyl (C_{18}) and phenyl (Ph) silica as reversed-phase columns and columns of trimethylaminopropyl (SAX) and aminopropyl (NH_2) silica as anion-exchange columns. Of these columns, the C_8 and SAX proved to be the most suitable for the extraction and separation of HVA from other interfering substances.

We next examined monoamine-related compounds that may be extracted with our assay method (Table I). Although some acidic compounds were also extracted, they were all eluted before HVA and thus did not interfere with the HVA assay.

The pH of the buffer applied to the Bond Elut SAX column was examined. HVA and iso-HVA were adsorbed efficiently between pH 6.5 and 8.5 (Fig. 3). Since HVA and iso-HVA are stable in an acidic medium, we adjusted the pH of the elution buffer to 6. Moreover, we found that among the counter-ions with which HVA is eluted from the SAX column, 1 *M* hydrochloric acid was the most satisfactory.

The linearity of the assay was verified by adding known amounts of HVA to the pooled plasma samples. The curve was linear over the range 0.5–50 ng/ml. The detection limit for HVA was found to be ca. 20 pg (signal-to-noise ratio greater than 3). The recoveries of HVA from the C_8 and SAX columns were

TABLE I

RETENTION TIMES AND EXTRACTION EFFICIENCIES OF SOME CATECHOLAMINES, INDOLEAMINES AND RELATED SUBSTANCES

Dihydroxyphenylmandelic acid and tyrosine could not be detected at 0.7 V.

Compound	Retention time (min)	Adsorption	
		C_8	SAX
Uric acid	<1.80		
Dopamine	1.87		
5-Hydroxytryptamine	2.61	+	–
3-Methoxytyramine	2.92	+	–
3-Methoxy-4-hydroxyphenylethyleneglycol	3.23	–	
Tryptophan	4.09	+	–
5-Hydroxytryptophan	4.45	+	–
3,4-Dihydroxyphenylacetic acid	6.89	+	+
5-Hydroxyindoleacetic acid	7.34	+	+
Homovanillic acid	8.81	+	+
Isohomovanillic acid	11.21	+	+

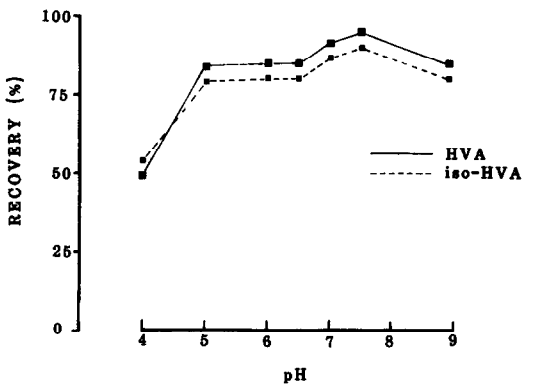

Fig. 3. Effect of pH of the elution buffer on the recovery of HVA and iso-HVA from a Bond Elut SAX column. The recoveries of HVA and iso-HVA from a Bond Elut SAX column were determined in buffers of different pH of 0.1 *M* sodium acetate (pH 4-7.5) and 0.1 *M* Tris-HCl (pH 8.8).

96.7 ± 4.0% and 82.5 ± 6.2% (mean ± S.D., $n=5$), respectively. Thus, the total recovery was 80.0 ± 8.3% ($n=5$). The results for human plasma from normal healthy subjects were in the region 10.8 ± 3.3 ng/ml (mean ± S.D., $n=13$). These values are compatible with those recently obtained by HPLC [6–10] or GC–MS [4, 5, 14]. The intra- and inter-assay coefficients of variation were 3.1% ($n=6$) and 6.4% ($n=8$), respectively.

In summary, this two-step solid-phase extraction with Bond Elut C_8 and SAX columns and HPLC–ED analysis constitutes a rapid and simple method for the routine assay of plasma HVA. This method may be useful for studies on plasma HVA in neuropsychiatric disorders in which central dopaminergic dysfunction has been implicated [15, 16].

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