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Rapid and reliable high-performance liquid chromatographic method for analysing human plasma serotonin, 5-hydroxyindoleacetic acid, homovanillic acid and 3,4-dihydroxyphenylacetic acid

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ABSTRACT

The simultaneous measurement of homovanillic acid, 3,4-dihydroxyphenylacetic acid, serotonin and 5-hydroxyindoleacetic acid in human plasma by an ultrafiltration and microbore high-performance liquid chromatography–electrochemical detection technique is established. Conventional preparation of blood is very tedious and time-consuming, but isocratic separation of the analytes in plasma ultrafiltrates using a microbore column could be achieved within 10 min. Hence, theoretically, over 140 analyses can be performed in a working day. The detection limit (signal-to-noise ratio = 3) of this method is about 0.1–0.5 pg per injection for all analytes. The required volume of plasma samples can be less than 100 μl. Hence, blood loss is minimal, especially in repeated blood sampling. This rapid, simple and sensitive method can, therefore, be used as a routine clinical tool in the simultaneous measurement of plasma homovanillic acid, 3,4-dihydroxyphenylacetic acid, serotonin and 5-hydroxyindoleacetic acid.

INTRODUCTION

Measurement of plasma and cerebrospinal fluid dopamine (DA) and serotonin (5-HT) and their metabolites, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), is perhaps the most direct method currently available to assess the changing activities of central DA and 5-HT neurons in a living human [1,2]. Plasma metabolites have been used as predictors of drug response or clinical response in neuropsychopharmacology and neuropsychiatry research [3–6]. Indeed, about 25% of plasma HVA is of central origin [7]. Many analytical procedures have been devised for these purposes [8–11]. In comparison with other methods, high-performance liquid chromatography–electrochemical detection (HPLC–ED) is considered to be one of most pop-

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ular and highly sensitive assay methods in the determination of these compounds, because of its reasonable simplicity, versatility, sensitivity and specificity [12–14]. Generally, traditional HPLC methods that exist for the determination of these compounds in plasma samples need extensive and time-consuming clean-up procedures [15–18]. This is because of the large number of substances in plasma which occur in traditional chromatography. Separation and different methods are therefore required for the measurement of these compounds in plasma samples. This goal was accomplished in our previous study by combining *in vitro* microdialysis (MD) and microbore HPLC (MHPLC)–ED [19]. Yet the clean-up procedures for plasma samples required by *in vitro* MD remain somewhat complex and recovery is not sufficient for some acid metabolites [19]. Therefore, a further simple, rapid and sensitive methodology for the determination of plasma HVA, DOPAC, 5-HIAA and 5-HT is of great interest.

Ultrafiltration (UF) techniques have been widely applied in plasma processing for drug monitoring assays [20,21]. To the best of our knowledge, purification by ultrafiltration has not been applied in assaying plasma catecholamines and serotonin. The role of the ultrafiltration membrane is to act as a selective barrier, enriching certain compounds and depleting others [22]. This is very attractive for isolation of small and hydrophilic molecules by discriminating against high-molecular-mass substances, such as proteins and suspended particles, on the basis of molecular size. In addition, like the MD sample, the ultrafiltration sample (the ultrafiltrate) can also be applied directly onto microbore HPLC without further purification. Nevertheless, an advantage of the ultrafiltration technique over *in vitro* MD is faster and more efficient separation of the plasma. In addition, the plasma volume required for ultrafiltration is relatively small compared with that required for *in vitro* MD. Thus, in the present study, ultrafiltration instead of *in vitro* MD was applied in pretreatment of the plasma. Several aspects of this modified assay are considered, including analytical precision, accuracy, liability and applications.

EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC system comprised a Beckman 126 pump (Beckman Instruments, Taiwan), a CMA-200 microautosampler (CMA/Microdialysis, Stockholm, Sweden), a BAS-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), a microbore reversed-phase column (BAS Sep-Stik, 5-μm octadecylsilane, 100 × 1.0 mm I.D., Bioanalytical Systems), a Beckman I/O 406 interface and Beckman System Gold Data Analysis Software (Beckman Instruments). The potential of the glassy carbon working electrode was held at +0.75 V with respect to an Ag/AgCl reference electrode. Glassy carbon working electrodes are maintained by polishing once every week or as needed in accordance with the manufacturer's manual. In order to increase the sensitivity of the microbore HPLC system, a very thin spacer (14 μm) was used instead of a conventional one (51 μm) to create a sub-microlitre thin-layer electrochemical cell. Low flow-rates were used to minimize pulse fluctuations.

Chemicals and reagents

HVA, 5-HT, 5-HIAA, DOPAC, 3,4-dihydoxybenzylamine (DHBA), isoproterenol (IPT), ethylenediaminetetraacetic acid (EDTA), diethylamine, sodium 1-octanesulphonate (SOS), monosodium dihydrogen orthophosphate, sodium citrate and 3-methoxytyramine (3-MT, as an internal standard) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and tetrahydrofuran (THF) were purchased from Merck (Merck–Schuchardt, Darmstadt, Germany). Unless otherwise stated, all reagents were of analytical quality.

Sample preparation and assay

Standard stock solutions of HVA, 5-HT, 5-HIAA and 3-MT were prepared at a concentration of 2 ng/ml in 0.1 *M* perchloric acid and stored at −70°C in the dark and thawed at 4°C prior to preparation of a standard mixture. In our routine assays, two internal standards, DHBA and IPT, were used. Unfortunately, DHBA and IPT coeluted with DOPAC and HVA (or 5-HIAA), respectively. Modification of

the mobile phase did not result in satisfactory separation. In our previous study, 3-MT was very well separated from other analytes in brain homogenate samples. In addition, 3-MT could not be detected in plasma from patients with schizophrenia [23] and plasma ultrafiltrates ($n = 9$) in our preliminary study. Therefore, 3-MT was chosen as the internal standard. If sufficient 3-MT does occur in plasma ultrafiltrates under some circumstances, the peak heights of 3-MT in chromatograms will increase significantly. In fact, this was not found to be the case. The internal standard 3-MT and the standard mixture were prepared every day from a portion of these stock solutions after appropriate dilution with Ringer solution containing 10^{-7} *M* ascorbic acid in 0.1 *M* hydrochloric acid.

Venous blood samples from patients with schizophrenia were collected into prechilled polypropylene tubes containing heparin as an anticoagulant and centrifuged (10 min, 700 *g* at 4°C) to separate the plasma. Frozen plasma samples were kept at −70°C prior to assays. After thawing at 4°C, 100 μl of plasma sample were transferred into a Millipore Ultrafree-MC unit [PLACE, Ultrafree-MC with 10 000 nominal molecular mass cut-off (NMWC), Millipore, Bedford, MA, USA] and then centrifuged at 15 000 *g* for 15 min. The Ultrafree-MC unit is a 400-μl sample cup with a regenerated cellulose membrane sealed to the bottom, which sits inside a 1.5-ml microcentrifuge tube. Under these conditions, typically more than 60% ($n = 9$, 61 ± 1%) of the sample volume is ultrafiltered. A 50-μl aliquot of the ultrafiltrate and 10 μl of Ringer solution containing 1.4 ng of 3-MT (as an internal standard) were mixed; 5 μl of the mixture were injected onto the MHPLC–ED system.

Plasma concentrations of HVA, DOPAC, 5-HT and 5-HIAA were calculated by determining each peak-area ratio relative to the internal standard, 3-MT, and corrected by recovery volume. The identity of the peaks in the plasma filtrate was confirmed by their retention times, standard addition and a superimpose–alignment technique, which was provided by Beckman (System Gold Data Analysis Software, Version 6.01). In order to ascertain the accuracy of the present method, plasma HVA was also assayed according to Chang *et al.* [24].

Mobile phase preparation

The mobile phase was slightly modified from our previous study [19] by adding 60 ml of acetonitrile, 8 ml of THF, 0.48 g of SOS (2.6 m*M*), 2.00 g of monosodium dihydrogen orthophosphate (14.7 m*M*), 8.82 g of sodium citrate (30 m*M*), 10 mg of EDTA (0.027 m*M*) and 1 ml of diethylamine in double-distilled water. The solution pH was adjusted to 3.2 with concentrated orthophosphoric acid and its final volume was adjusted to 1 l. The mixture was filtered with a 0.22-μm nylon filter under reduced pressure and degassed by purging with helium for 20 min. The flow-rate was 120 μl/min maintaining column pressure at *ca.* 10.3 MPa.

RESULTS AND DISCUSSION

Fig. 1A shows a typical chromatogram of a standard mixture containing DOPAC, HVA, 5-HIAA, 3-MT (internal standard) and 5-HT. An analysis was completed within 10 min. All components under study were well resolved. Fig. 1B shows a typical chromatogram of the ultrafiltrate of a human plasma sample. Peaks of DOPAC, 5-HIAA, HVA and 5-HT in Fig. 1B were identical to those in Fig. 1A. For each peak was verified if the addition of a standard mixture increased its peak height but not its peak shape. In addition, a superimpose–alignment technique is also useful if chromatographic peaks differ slightly in elution times between runs or coelute with other unknown interfering substances. These chromatograms are adjusted to align according to the differences between selected peaks and the internal standard, 3-MT, by the Beckman Gold system. Sometimes superimposition of these chromatograms is nice to confirm alignment of peaks.

The known amount of analytes (x), which ranged from 5 pg to 20 ng in standard mixtures, was linearly correlated with the chromatographic area (y) obtained from standard mixtures by the

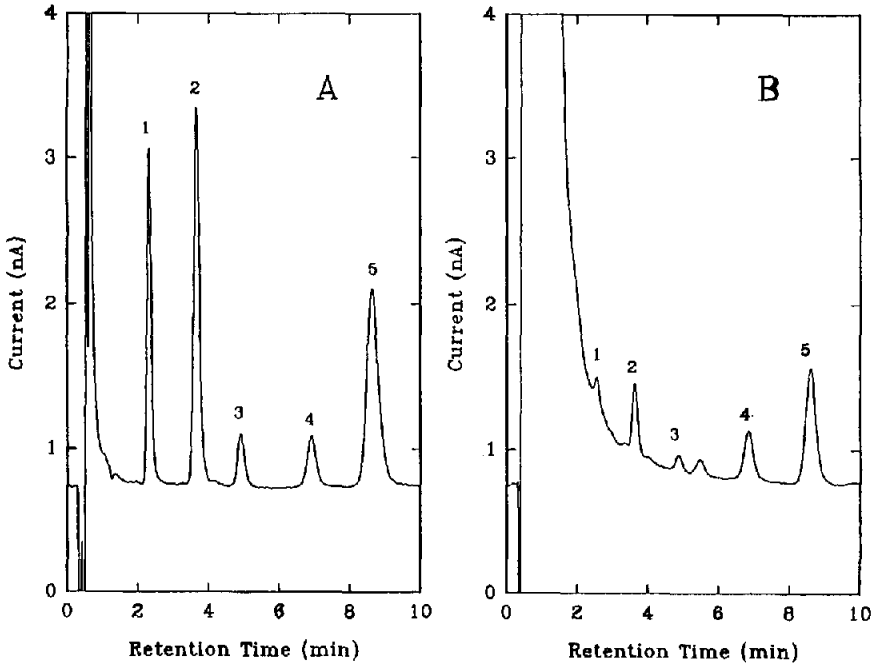

Fig. 1. Typical chromatograms of (A) a standard mixture containing: (1) DOPAC, 113 pg; (2) 5-HIAA, 59 pg; (3) HVA, 129 pg; (4) 3-MT (as an internal standard), 115 pg; (5) 5-HT, 146 pg; and (B) an ultrafiltrate of human plasma.

MHPLC–ED system. The standard curves and correlation coefficients (r^2) of the analytes were as follows: DOPAC: $y = 5.95\ x - 0.42$, $r^2 = 1.00$; HVA: $y = 6.53\ x - 7.07$, $r^2 = 1.00$; 5-HIAA : $y = 9.70\ x - 14.98$, $r^2 = 1.00$; 5-HT : $y = 8.02\ x - 8.29$, $r^2 = 1.00$. The precision of the assays was tested using pooled plasma samples and a standard mixture dilution with Ringer solution containing 10^{-7} *M* ascorbic acid. The intra- and inter-assay variabilities were assessed and expressed as coefficients of variation (C.V.), shown in Table I. Comparing the present ultrafiltration method with the *in vitro* MD method [19], recoveries of DOPAC and 5-HIAA between consecutive fractions (at 10-min intervals) by *in vitro* MD are less stable than those of others. The coefficients of variation (C.V.) ($n = 9$) of DOPAC and 5-HIAA ranged from 37 and 41% for the first fractions to 14 and 18% for the fifth fractions of DOPAC and 5-HIAA, respectively.

TABLE I

ANALYTICAL PRECISION OF THE INTRA-ASSAY OF THE STABILITY OF A STANDARD MIXTURE ($n = 9$, AT 1-h INTERVALS) AND POOLED PLASMA ($n = 9$) AND INTER-ASSAY STABILITY ($n = 6$) OVER SIX CONSECUTIVE WORKING DAYS) BY THE UF–MHPLC–ED METHOD

Standard mixtures consisted of *ca.* 100 pg of each analyte.

	Coefficient of variation (%)			
	DOPAC	5-HIAA	HVA	5-HT
Intra-assay				
Standard mixture in 10^{-7} *M* vitamin C and Ringer solution	2.14	1.79	2.09	1.82
Pooled plasma	2.01	2.29	1.79	1.76
Inter-assay				
Standard mixture in 10^{-7} *M* vitamin C and Ringer solution	3.96	3.89	4.56	3.45

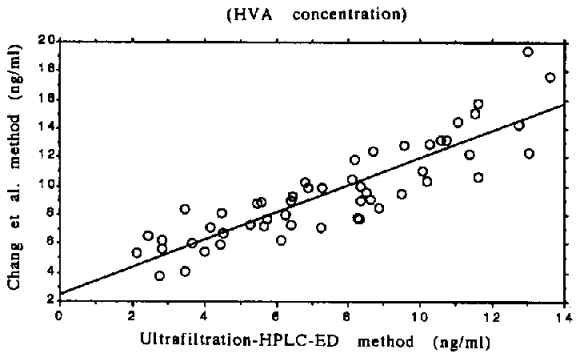

Fig. 2. Correlation of HVA concentrations of 53 human plasma samples measured by the present ultrafiltration–MHPLC–ED method and by the Chang *et al.* [24] extraction method. $y = 0.949\,x + 2.46$; a good correlation was obtained ($r = 0.882$).

The C.V. values of other analytes averaged 10% for the second or third fractions. Thus, measurements of DOPAC and 5-HIAA in the second or third fraction may lead to a great experimental error [19]. Good volume recoveries (>98%) of these analytes were achieved by the ultrafiltration of standard mixtures (data not shown). The C.V. values for determination of these analytes were on average 2% in both standard mixtures ($n = 9$) and pooled plasma samples ($n = 9$). The inter-assay variabilities assessed with a standard mixture over six consecutive working days were less than 5% for all analytes. The detection limits (signal-to-noise ratio = 3) of the analytes per injection were between 0.1 and 0.5 pg.

Comparison of the present method (x) with the procedure of Chang *et al.* [24] (y) resulted in a satisfactory correlation ($y = 0.949\,x + 2.46$, $r = 0.882$) (Fig. 2). In addition, the time required for plasma pretreatment in the present method averaged less than 30 min for sixteen samples, whereas the procedure of Chang *et al.* would take a few hours for six samples in our routine assays. The mean concentrations and standard deviations of HVA ($n = 53$) were 7.50 ± 3.12 ng/ml and 9.57 ± 3.36 ng/ml by the present method and the method of Chang *et al.* [24], respectively. These variations might be due to different methodologies or sample degradation in storage, since the latter assay was performed approximately three months prior to the present assay. It was reported that up to 12 and 39% degradation of these substances was observed after two and ten months, respectively (preservation at −70°C [25]). In addition, the present assay is advantageous in the simultaneous measurement of DOPAC, HVA, 5-HT and 5-HIAA (Table II). Our data are in agreement with those reported by others [14,26,27]. The life expectancy of the microbore column was also tested and recorded by frequent injection in our routine work. Generally, efficiency the same as for a new column was observed after 1000 injections.

Because of very low hold-up volume (< 5 μl) of Millipore Ultrafree-MC units and the precision of CMA-200 microautosampler, the plasma volume required is only 100 μl. However, a 30-μl plasma sample is sufficient to obtain precise data for the ultrafiltration assay in our preliminary study (data not shown). Measurements of plasma contents in such a small volume have great analytical potential in the field of repeated blood samplings or pharmacokinetic studies in small animals and clinical research. In this study, the automatic HPLC method is useful when many

TABLE II

THE MEAN LEVELS ($n = 53$) OF PLASMA CONTENTS (ng/ml) OBTAINED BY THE METHOD OF CHANG *et al.* [24] AND BY THE PRESENT UF–MHPLC–ED METHOD

N.D. = Not detectable.

Method	DOPAC	HVA	5-HIAA	5-HT
Chang *et al.*	N.D.	9.57 ± 3.36	N.D.	N.D.
Ultrafiltration	1.2 ± 0.62	7.50 ± 3.12	4.07 ± 2.60	43.86 ± 13.0

plasma samples have to be analysed in a short period of time. Hence, the present method applying ultrafiltration in the MHPLC–ED technique provides a rapid and simple method, since only a single-step ultrafiltration is necessary for sample pretreatment. Another advantage of this method is the very low detection limit (typically 0.1–0.5 pg/per injection). Furthermore, complete separation of these analytes within 10 min was achieved, hence over 140 analyses can be done in one working day by our automatic MHPLC–ED system.

CONCLUSION

Although compromises between economy and performance often have to be made in MHPLC and HPLC, we conclude that the method presented in this paper is rapid and simple compared with some rather complicated methods, such as liquid-phase or solid-phase extraction. We conclude that this simple and sensitive method is a suitable tool in basic research and routine clinical applications.

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