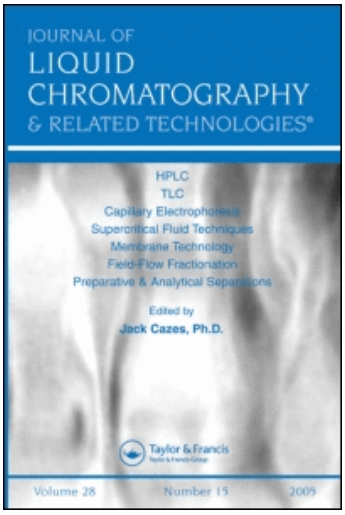

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ABSTRACT

A sensitive and a rapid procedure is described using high performance liquid chromatography combined with electro-chemical detection (HPLC-ECD), for quantification of whole blood biogenic amines and their metabolites. Serotonin (5-hydroxytryptamine, 5-HT), its precursor 5-hydroxytryptophan (5-HTRP), and metabolite 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA) and its metabolite homovanillic acid (HVA), were extracted from an aliquot of whole blood.

After precipitation of proteins with 20% zinc sulfate and centrifugation the clear supernatant was treated with 10% sodium hydroxide and centrifuged. The supernatant was filtered and injected into the HPLC system. Separation of the analytes of interest was carried out with a mobile phase containing sodium acetate, citric acid, sodium octyl sulfate, disodium-EDTA, dibutylamine, and 15% methanol, at a flow rate of 0.5mL/min and at a potential of +0.60 V. Peaks of each compound were identified by their respective retention times established separately with each standard, as well as with a mixture of all standards. Calibration was carried out by injecting the extract prepared by spiking 0.5 mL of 5% bovine serum albumin (BSA) solution with known concentrations of standards (8.0 ng/mL for each, except that of HVA with a higher concentration of 20 ng/mL) and a fixed concentration of internal standard (8.0 ng/mL).

Concentration in the samples were calculated by the data module using the ratio of the detector response (area under the peak) of each compound relative to that of the internal standard. The lower limits of sensitivity for 5-HTRP, DA, 5-HIAA and 5-HT was 0.5 ng/mL. For HVA, the lower limit of sensitivity was 2.0 ng/mL. Recovery of all compounds ranged between 80.0-96.0% and was tested by adding the known concentration of standards to either a solution of BSA or to an aliquot of whole blood. All components from the extract were eluted within 25 minutes. This procedure has been found to be rapid, simple and reliable for investigating simultaneously the status of biogenic amines, 5-HT and DA and their metabolites, in whole blood.

INTRODUCTION

Biogenic amines, particularly serotonin (5-hydroxytryptamine, 5-HT) and dopamine (DA), have been implicated in regulating various functions in the central as well as the peripheral nervous systems. Various lines of investigations also provide convincing evidence of functional interactions among these neurotransmitters.[1,2] Alterations in the turnover rates of these neurotransmitters have been associated with various physiological dysfunctions as well as behaviors. Thus, 5-HT dysfunctions have been associated with depressive disorder,[3] aggressive behaviors,[4] and sleep disorders[5] and low levels of 5-hydroxyindoleacetic acid (5-HIAA), the metabolite of 5-HT, have been reported in cerebrospinal fluid of patients with depression and those who attempt or commit suicide.[4,6] 5-HT metabolism is also altered in individuals with elevated distress levels[7] and in mental disorders, as demonstrated by an increased concentration of 5-HT in whole blood and platelet in patients with schizophrenia.[8,9] On the other hand, deficiency of DA in the brain has been

associated with Parkinson's disease, a motor disorder characterized by rigidity, weakness, and tremors. Functions of these biogenic amines, 5-HT and DA, are also greatly influenced by the drugs of abuse such as LSD, cocaine, and opioids, leading to craving and addiction and ultimately to cognitive deficits,[10] locomotor, and behavioral dysfunctions.[11]

5-HT is biosynthesized from the aminoacid tryptophan (TRP), obtained from diet. TRP is hydroxylated in the body to 5-hydroxytryptophan (5-HTRP), the immediate precursor of 5-HT by the enzyme tryptophan hydrtoxylase, and regulating the synthesis of 5-HT in the central as well as peripheral systems. A simultaneous measurement of 5-HT along with the immediate precursor 5-HTRP as well as its major metabolite, 5-HIAA, in the same sample of blood is important for investigating the turnover of 5-HT in health and disease. Similarly, DA, and its metabolite HVA measured simultaneously in the same sample of blood can provide information about dopamine turnover. Furthermore, simultaneous measurement of both neurotransmitters, 5-HT and DA, as well as their metabolites, in the same sample is important for assessing concomitant changes in the functional state of these neurotransmitters for the diagnosis and treatment of various mental, behavioral, as well as, physiological disorders including those occurring because of drug abuse.

A number of assay procedures have been described for determination of 5-HT and DA along with their respective metabolites in the brain, blood, plasma, and CSF. Most of the assay procedures including radioenzymatic[12,13] or gas chromatography mass spectrometry[14] are highly sensitive and specific but are time consuming and involve the use of expensive equipment. On the other hand, less expensive fluorometric detection combined with HPLC separation of biogenic amines from the body fluid extracts used in various recent studies[9,15-17] lacks sensitivity required for small quantities of sample. However, recent developments of the extremely sensitive procedure of electrochemical detection (ECD) combined with HPLC separation of these compounds from the body tissues and fluids has become the state of the art method for quantification of biogenic amines and their metabolites. Because of high sensitivity as well as specificity, HPLC-ECD has become an extremely useful technology for measuring, separately, alterations in nanogram and picogram quantities of specific biogenic amines and their metabolites in various psychiatric conditions using small quantities of various body tissues and fluids including the brain,[18,19] platelet, plasma, CSF,[20,21] as well as, urine.[22-24] Since biogenic amines including 5-HT and DA interact with each other and undergo changes concomitantly in many behavioral disorders, procedures involving simultaneous extraction and quantification of both these amines and their metabolites from the same sample of body fluid or tissue can provide valuable information about their functional status. In human subjects, obtaining blood is a less invasive procedure compared with obtaining CSF, and assessment of various functions in the body can be made by the analysis of whole blood, plasma or serum.

Although biogenic amines have been measured in separate assays using platelets and/or plasma, use of whole blood, particularly for 5HT is important since it provides the combined level in both platelets and plasma since most of 5-HT is stored in the platelet and a small amount is also present in plasma. The status of 5-HT in blood as well as brain is based upon the availability of TRP, and it is important that the levels of TRP and 5-HT are simultaneously determined in the same sample of blood.

However, extraction of TRP from plasma has met with difficulties and in earlier studies TRP has been determined directly in plasma without extraction by using spectrofluorometric detection, which had lower sensitivity,[25] than the recently developed HPLC-ECD method. But injecting plasma directly into the HPLC-ECD system for quantification of TRP, as reported earlier,[26] may have deleterious effects on the performance and the life of the HPLC column as well as on that of the electrode due to the presence of proteins and many other contaminants. Since 5-HTRP, the immediate precursor of 5-HT can be extracted simultaneously from whole blood, it may be used as an index for TRP availability.

We describe here a method for simultaneous extraction and quantification of 5-HTRP, 5-HT, and 5-HIAA as well as DA, and HVA, in the same sample of whole blood with recovery ranging between 80.0%-96% for all compounds. All compounds can be eluted within 25 minutes and a number of samples can be analyzed in one day.

EXPERIMENTAL

Chemicals

5-Hydroxytryptophan, dopamine, 5-hydroxyindoleacetic acid, homo-vanillic acid, 5-hydroxytryptamine, N-ω-methyl-5-hydroxytryptamine, bovine serum albumin, zinc sulfate, and sodium hydroxide were purchased from the Sigma Chemical Co (St Louis, MO, USA). HPLC grade chemicals for mobile phase including sodium acetate, citric acid, disodium-EDTA (Na_2EDTA), dibutylamine, sodium octyl sulfate (SOS), and methanol were purchased from the Across Organics Co. (New Jersey, USA).

Apparatus

HPLC system (Waters, Milford, MA, USA) equipped with electrochemical detector (ECD) model 460, was used for these assays. The ECD system is comprised of a glassy carbon working electrode, an auxiliary electrode, and a Ag/AgCl reference electrode. The other components of the system include an injector (model U6K), a dual pump solvent delivery system (model 590), and

Waters data module (model 740). Chromatographic separations were performed using a 3.9x150mm stainless steel resolve column packed with octadecylsilane (C_{18}) on microparticulate (5μm) spherical silica gel.

Mobile Phase

The solution used for eluting all the components individually, in the standard mixture as well as blood sample extracts contained sodium acetate 0.1M, citric acid 0.1M, SOS 0.75mM, sodium-EDTA 0.15mM, dibutylamine 1.0 mM, and methanol 15%. The solution was adjusted to pH 4.0 and was filtered through a 0.2μm filter using glass filtration system and was degassed with ultrasonic agitation before use. All separations were performed isocratically at a flow rate of 0.5mL/min and at a potential of +0.60 V.

Standard Solutions

Standard stock solutions containing 1.0 mg/mL of 5-HTRP, DA, 5-HIAA, HVA, and the internal standard, N-ω-methyl-5-HT (NM), were prepared in HPLC-grade water and stored for one week at 4°C. Working standard solutions were prepared freshly before use and concentrations of 5-HTRP, 5-HT, 5-HIAA, DA, and HVA, were based on the sensitivity of detection (ng/mL), 0.5 at lower limits and 25.0-180.0 at higher limits. The concentration of internal standard (NM) was kept constant (8.0 ng/mL).

Peak Identification and Calibration

Each working standard solution (20 μL) was injected separately for identification of its individual peak, with respect to its retention time. An aliquot of the mixture of all standard solutions along with the internal standard (NM) was then injected in order to establish area of the peak of each standard compound in relationship to its concentration. Calibration was achieved by spiking 0.5 mL of 5% BSA with a known concentration of each standard (ng/mL; 5-HTRP 8.0, DA 8.0, 5-HIAA 8.0, HVA 40.0, 5-HT 8.0 respectively, and NM 8.0) and the final volume was made up to 1.0 mL with HPLC- grade water. To this was added 0.2 mL of 20% zinc sulfate and the mixture was vortexed and centrifuged at room temperature for 10 minutes. The supernate was treated with 10 μL of 10% sodium hydroxide, vortexed and centrifuged as above and the supernatant from the last step was filtered through a 0.2 μm filter. An aliquot of 20 μL was injected into the HPLC system. Before spiking BSA with a known concentration of standards for calibrating the system, an extract (20 μL) of only BSA was injected in order to establish the baseline as well as to confirm the absence of any interfering peaks.

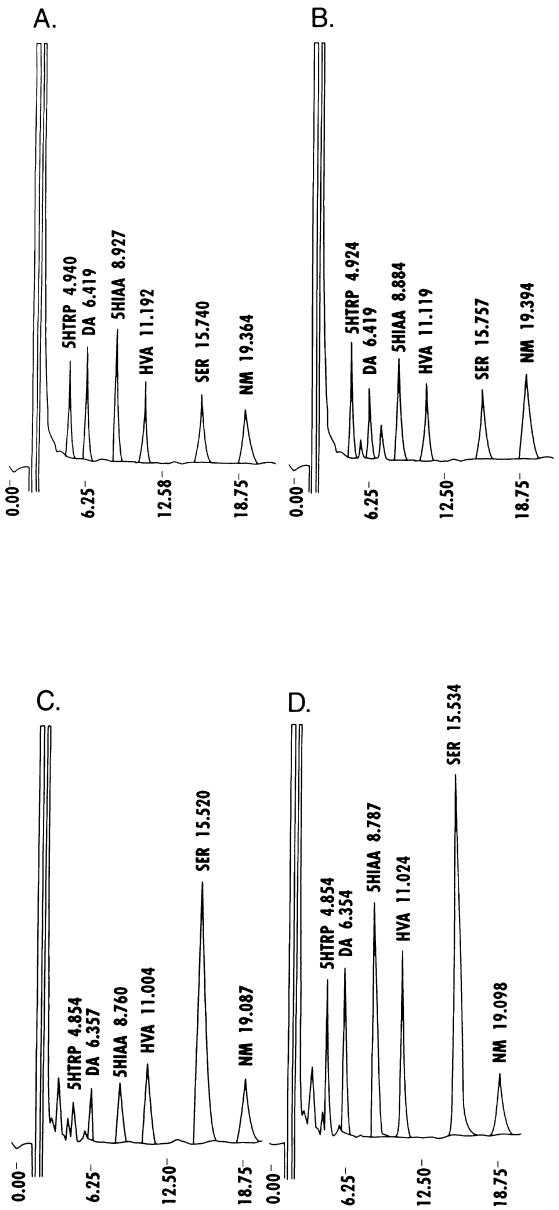

Figure 1. Chromatographic profiles of standards of 5HTRP, DA, 5HIAA, HVA, SER and internal standard, NM, are shown in the order of their retention times (1A). The profile of spiked standards is shown in 1B. Separation of whole blood extract is shown in 1C, and that of whole blood spiked with standards is shown in 1D.

Preparation of Whole Blood Extract

Frozen whole blood (0.5 mL) was thawed and diluted with an equal amount of HPLC-grade water and kept on ice. To the diluted blood was added 0.2 mL of 20% ZnSO4 and vortexed, and then centrifuged for 10 min at 4°C. The supernatant was treated with 10 μL of 10% NaOH, vortexed, and centrifuged as above. The supernatant from the second centrifugation was filtered through a 0.2 μm syringe filter, and 20 μL was injected into the HPLC system for separation and quantification of the desired compounds.

Calculations

Standard curves were prepared with mixtures of standard solutions containing various concentrations of 5-HTRP, DA, 5-HIAA, HVA, 5-HT, and a fixed amount (8.0 ng/mL) of NM. Based on the calibration of the HPLC system with BSA solution spiked with standards, concentration in the samples were calculated by the data module using the ratio of the area under the individual peak relative to that of NM. Concentrations were expressed as ng/mL of whole blood.

RESULTS AND DISCUSSION

Figures 1A-1D show the chromatographic profile of 5-HTRP, 5-HT, DA, and the 5-HT and DA metabolites, 5-HIAA and HVA, in a standard mixture (A), BSA spiked with a mixture of all standards (B), that of a whole blood sample extract (C), and that of the whole blood spiked with a mixture of standards (D), respectively. The procedure described allows simultaneous extraction and quantification of all the compounds.

Retention times, in the order of peak appearance as shown in Figure 1, are of 5HTRP, DA, 5HIAA, HVA, SER (5HT), and NM, in typical chromatographic profiles obtained by injecting an aqueous standard mixture. The chromatograms show the peaks representing each compound in the aqueous mixture (Figure 1A), extract of spiked standards in BSA (Figure 1B), extract of whole blood sample (Figure 1C), and an aliquot of whole blood spiked with a mixture of all standards (Figure 1D).

Peaks representing each compound are clearly separated from that of the other with a substantial difference in retention time of > 1.5 minutes (5HIAA and DA), and > than 6.5 minutes (HVA and SER). Similarly, the differences in retention time between 5HTRP and DA, and HVA and 5HIAA was > 2.0 and 3.0 minutes respectively.

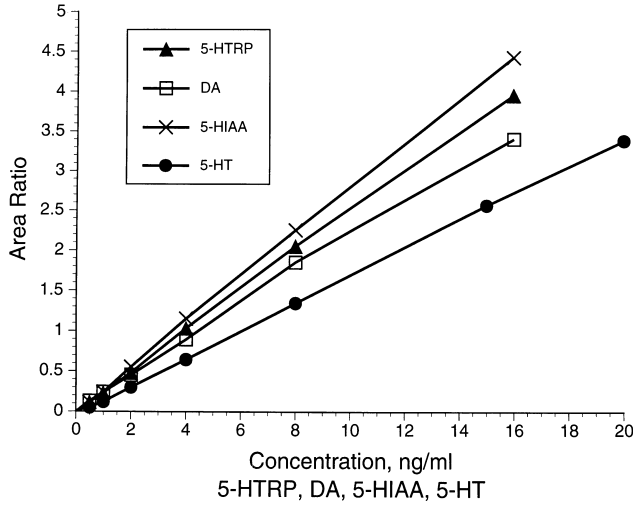

Figure 2. Standard curves showing a linear relationship between various concentrations of 5-HTRP, DA, 5-HIAA, and 5-HT, and the ratio of the area of the peaks of respective standards to that of the internal standard, NM.

Standard curves were prepared for each compound. A linear relationship was obtained between the ratio of the area under the curve (AUC) for each of the standards of 5-HTRP, DA, 5-HIAA, and 5-HT, with concentrations ranging between 0.5 and 20.0 ng/mL, to that of a constant concentration (8.0 ng/mL) of NM (Figure 2). Standards of 5-HT at concentrations ranging between 8.0-60 ng/mL and higher, also maintained a linear relationship (Figure 3). Although sensitivity of the detector response for HVA was found to be lower under these conditions, there was a linear relationship between the ratio of the area (HVA to that of NM) and various concentrations of HVA, ranging between 2.0 and 160 ng/mL (Figure 3).

The sensitivity of the assay for 5-HTRP, DA, 5-HIAA, and 5-HT at the lower limits of concentration was 0.5 ng/mL, and the detector response for HVA under these conditions was less sensitive, since a clearly identifiable peak was obtained with a minimum concentration of 2.0 ng/mL. The higher concentration of HVA for obtaining peaks of similar area under the curve (AUC) as that of the other compounds was 160 ng/mL (Figure 3).

Although it has been suggested that the sensitivity of HVA can be increased by using higher amplification with a two channel recorder,[27] in this study we used a single channel recorder with a setting at one amplification for all analytes of our interest.

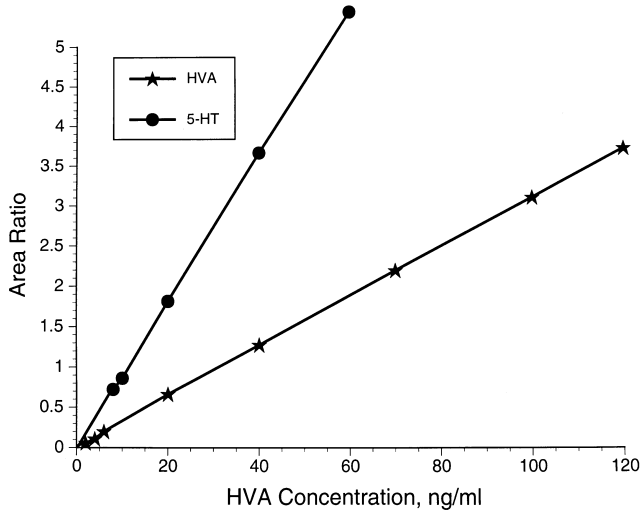

Figure 3. Standard curves of HVA and 5-HT at higher concentration, showing a linear relationship between various concentrations and the ratio of the area of peaks of each compound to that of a constant concentration of the internal standard, NM.

Percentage recovery of known concentration of all compounds added to a solution of BSA or to an aliquot of whole blood, ranged between 80.0% to 96.0% during multiple recovery experiments. Inter-assay coefficient of variation determined for each compound was 4.37% for 5-HTRP, 11.8% for DA, 10.5% for 5-HIAA, 7.02% for 5-HT, and 5.52% for HVA. Intra-assay coefficients of variation ranged between 5.92 to 9.7%.

The validity of the procedure was examined by analyzing aliquots of the whole blood samples obtained from a normal subject as well as from patients with major depressive disorder. The values (ng/mL) obtained in the normal subject for 5-HTRP, DA, 5-HIAA, HVA, and 5-HT were 2.5, 0.52, 1.82. 4.3, and 178.6 respectively, whereas in the samples of three depressed patients they were 8.7±0.55, 0.101±0.099, 2.41±0.45, 12.1±7.17, 108.8±12.06 respectively.

In order to obtain a clear extract after complete precipitation of proteins in whole blood we used 20% ZnSO4, followed by 10% NaOH for neutralizing excess ZnSO4, since we observed that the supernatant obtained after precipitation of proteins with 10% ZnSO4 as used in earlier studies[26,28] remained turbid, and the turbidity due to the presence of small proteins or other substances interfered with the proper functioning and life of our HPLC column as well as the working electrode.

The chromatogram (Figure 1C) shows the separation profile of all five compounds from the whole blood of a normal person. Analysis of five whole blood samples after freezing and thawing gave significantly higher values of 5-HT and 5-HIAA than when the freshly obtained blood sample was analyzed. However, freezing and thawing did not show any significant difference in the concentration of 5-HTRP, DA, or HVA. Stability of the extracts was examined in multiple experiments by storing the extracts at room temperature as well as at 4°C. When extracts of fresh or frozen-thawed blood were stored for one week at 4°C, there was no significant change in the concentration of HVA, but more than a 90% loss occurred in concentration of 5-HT and there was 10-40% loss in DA, 5-HIAA, and 5-HTRP, respectively. All five components in the extract were found to be fairly stable with no significant loss when extract was stored overnight at laboratory room temperature.

The procedure described in this report offers a simple and rapid method for simultaneous determination of whole blood 5-HT along with its precursor, 5-HTRP, and metabolite, 5-HIAA, as well as DA, and its metabolite HVA. Because of the rapid extraction procedure combined with a well defined separation and sensitive detection by HPLC-ECD, a number of samples can be analyzed in one day.

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