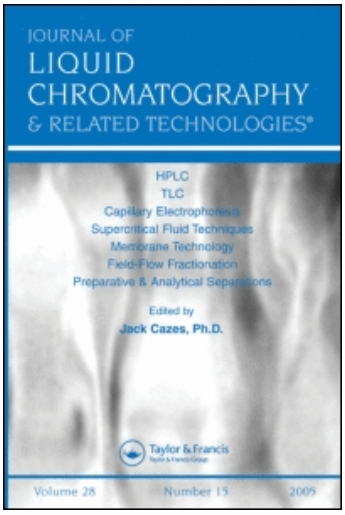

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AN ISOCRATIC CONCURRENT ASSAY OF FREE METABOLITES, 4-HYDROXY-3-METHOXY MANDELIC ACID, 3-METHOXY-4-HYDROXY-PHENYLGLYCOL, NORMETANEPHRINE, METANEPHRINE, AND 5-HYDROXY-INDOLEACETIC ACID IN SAME SAMPLE OF URINE EXTRACT USING HPLC-ECD

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ABSTRACT

This paper describes a method for simultaneous extraction and concurrent quantification of metabolites of norepinepohrine (NE) and 5-hydroxytryptamine (5-HT) in human urine using an isocratic HPLC-ECD technique. These metabolites include 4-hydroxy-3-methoxy mandelic acid (VMA), 3-methoxy-4-hydroxyphenylglycol (MHPG), normetanephrine (NMTN), metanephrine (MTN), and 5-hydroxyindoleacetic acid (5-HIAA). The samples of urine were passed through preconditioned alumina-B column and washed with diethylether to remove interfering substances. Metabolites were extracted in ethylacetate.

The extracts were evaporated under nitrogen, reconstituted in water and injected into a 5μ spherical, C_{18}, reverse phase column for HPLC-ECD analysis. The analytes were eluted with mobile phase containing citric acid, sodium acetate, sodium octyl sulfate, Na_2EDTA, methanol 10%, and isopropanol 0.5%. Isoproterenol was used as an internal standard. Electrochemical detection was carried out at a potential of +0.60 V and a flow rate of 0.5 mL/minute. Peaks were characterized by their retention times. Concentrations were determined by the method using ratio of the peak areas of metabolites to that of internal standard.

A linear relationship between the ratio of the areas and concentrations was obtained between 5.0-90 ng/mL of each VMA, MHPG, NMTN, and MTN. The sensitivity for 5-HIAA was found to be ten times higher with a linear relationship between 1.0-8.0 ng/mL. Total elution time for all the metabolites was less than 20 minutes.

INTRODUCTION

The role of biogenic amines including catecholamines and serotonin have been implicated in a number of physiological and psychiatric dysfunctions including stress, depression, autonomic reactivity, eating disorders, mania and neurogenic tumors.[1-3] Concentration of monoamines and their metabolites in the cerebrospinal fluid (CSF) represents, most likely, the approximate central activity of monoamines, however, obtaining CSF is an invasive procedure and is not performed routinely. On the other hand, plasma measurement of monoamines and their metabolites is not considered to be reliable since they have a very rapid turnover and are found in extremely low concentrations. The major metabolites of norepinephrine (NE), epinephrine (E) and serotonin (5-hydroxytryptamine, 5-HT) are 4-hydroxy-3-methoxy mandelic acid (VMA), 3-methoxy-4-hydroxy phenylglycol (MHPG), nor-metanephrine (NMTN), metanephrine (MTN), and 5-hydroxyindoleacetic acid (5-HIAA) respectively, and are excreted in urine in high concentrations. These metabolites reflect activity of the brain as well as that of the peripheral noradrenergic, adrenergic, and serotonergic systems. Recently, quantification of normetanephrine (NMTN) and metanephrine (MTN) in 24-hour urine has been used for the diagnosis of hypertension,[4,5] myocardial infarction,[6] and muscular dystrophy[7] whereas, urinary excretion of 3-methoxy-4-hydroxymethylglycol (MHPG) and 4-hydroxy-3-methoxymandelic acid (VMA) has been used for the diagnosis of carcinoid syndrome, neuroblastoma, and pheochromocytoma as well as, neurological and psychiatric disorders.[8-11] 5-HIAA, the major metabolite of 5-HT, has been associated with the diagnosis of behavioral problems[12] and

excessive urinary excretion has been used to diagnose carcinoid syndrome.[13,14] Analysis of these biogenic amines and their metabolites is also, increasingly, being used as markers of reactivity to field and laboratory challenges.[15] Studying the changes in the concentration of these metabolites of catecholamine as well as serotonin in 24 hours urine is therefore, of great importance and biologically more reliable not only for the diagnosis of diseases and behavioral problems but also for monitoring therapy.

In view of their importance, there is a constant search for developing simple, convenient, reliable, sensitive, and precise methods for quantification of these metabolites in a single sample. Earlier investigators had used separate methods for determination of each metabolite of catecholamines. VMA and MHPG were measured separately using gas chromatography.[16-18] Similarly, NMTN and MTN were each measured by separate methods. These procedures were quite reliable and specific but were complicated and time consuming since they involved derivatization of compounds and more than two metabolites could not be measured by one analytical method.[19] Recently, capillary gas chromatography has been used for measuring, simultaneously, a number of catecholamine metabolites in urine.[20] However, this method involves time consuming derivatization steps and is not convenient to use for monitoring changes in the activity of monoamines as a result of therapy. At our laboratories, we have earlier established HPLC-ECD methodology for quantification of 5-HT and 5-HIAA in platelets, plasma, and cerebrospinal fluid,[21] urinary free catecholamines,[22] catecholamine metabolites (MHPG and VMA) in urine,[23] as well as urinary normetanephrine and metanephrine, the methylated metabolites of norepinephrine and epinephrine respectively.[24] Although the techniques described in the above methods are very sensitive and reliable, they require separate HPLC-ECD analysis of the samples for different metabolites and, moreover, 5-HIAA could not be measured simultaneously with metabolites of NE and E. In the present report, we describe a simplified isocratic HPLC-ECD method for assaying, simultaneously, VMA, MHPG, NMTN, MTN, and 5-HIAA in a single sample of urine after purification on alumina B column, washing with diethylether and extraction with ethylacetate.

MATERIAL AND METHODS

Chemicals and Reagents

3-methoxy-4-hydroxy-mandelic acid, 4-hydroxy-3-methoxy phenylglycol, normetanephrine hydrochloride, metanephrine hydrochloride, 5-hydroxyindoleacetic acid, and isoproterenol hydrochloride, were purchased

from Sigma Chemical Co. (St Louis, MO). Octyl sulfate was obtained from Aldrich Chemical Co. (Milwaukee, WI). Alumina-B columns were purchased from BioRad Laboratories (Richmond, CA). Sodium acetate, citric acid, ethylenediaminetetraacetic acid (Na_2EDTA), and dibutylamine were obtained from Across Organics (New Jersey). All other HPLC grade chemicals, including diethylether and ethylacetate were obtained commercially. Alumina B columns were purchased from Waters Chromatography Division of Millipore (Millford, MA).

Mobile Phase

The solution used for eluting all the metabolites of our interest in the urine contained the following ingredients: citric acid 0.05M, sodium acetate 0.05M, sodium octyl sulfate 0.5mM, Na_2EDTA 0.075M, dibutylamine 0.5mM, methanol 10%, and isopropanol 0.5%. The pH of the solution was adjusted to 3.5 and solution was filtered through 0.45μm filter and degassed before use.

Standard Solutions

Standard solutions (stock) of VMA, MHPG, NMTN, MTN, 5-HIAA, and isoproterenol (ISOP) were prepared separately in HPLC grade water and each contained 1mg/mL of the metabolite. Each working standard solution was prepared just before use and contained 25ng VMA, 25ng MHPG, 50ng NMTN, 50ng MTN, 10ng ISOP (internal standard), and 3ng 5-HIAA in 50 μL of water.

The mixture of standard solutions was added for spiking either to water equivalent of urine volume or metabolite-free urine. Internal standard was added to the urine sample before extraction through Alumina-B column.

HPLC Equipment

HPLC-ECD system by Waters (Milford, MA) was used. The system consisted of electrochemical detector (ECD, Model 460) with a glassy carbon and auxillary electrodes and a Ag/AgCl reference electrode. Other parts of the equipment included an injector (U6K), a dual pump solvent delivery system (Model 590), and Waters data module, model 740. The chromatography column used for separation of metabolites was 5μ spherical, C_{18}, reverse phase, 3.9x150mm.

Metabolite-Free Urine (MFU)

A sample of urine (20mL) adjusted to pH 10.6 was exposed to light and air for a few days for metabolites to be degraded. Urine was then centrifuged at 3500 rpm for 15 minutes and the supernate adjusted to pH 6.5. Aliquots, 1.0 mL portions, were stored at -80°C until used.

Before calibration, MFU was spiked with known concentration of standards and extraction was carried out by the same procedure used for extraction of urine sample described below.

Identification of Individual Peaks

After equilibration of the system with elution buffer for a few hours at a flow rate of 0.5mL/minute, a 20 μL of working standard solution of the individual metabolites was injected sequentially to identify the peaks according to their respective retention times. A mixture of the working standards of all five metabolites and the internal standard was then injected for separating the peaks, and the HPLC system was calibrated with the aqueous standards.

Calibration of HPLC: Spiking of Standards

Water, 0.5 mL, was spiked with 50 μL solution containing 25 ng VMA, 25 ng MHPG, 50 ng NMTN, 50 ng MTN 10 ng ISOP, and 3 ng 5HIAA. The mixture was extracted from alumina B column by the procedure described below for urine sample. The extract was diluted appropriately and 20 μL was injected into the HPLC system.

Ratio of the areas under the individual peaks, with respect to internal standard, was calculated by the data module and concentrations were obtained as per mL urine. Similar procedure was used for calibration with MFU spiked with standards.

Standard curves of each metabolite were prepared with concentrations ranging as follows: VMA 2.5-80 ng, MHPG 10-80 ng, NMTN 10-90 ng, MTN 10-90 ng, 5-HIAA 1-10 ng, and a constant concentration, 10 ng of ISOP (internal standard) per milliliter. A 20 μL mixture of standard solutions was injected to validate the assay conditions, as well as to obtain coefficient of variations.

Extraction Procedure and HPLC Analysis

Preparation of alumina B columns

4.0 mL of distilled water was allowed to flow through the column followed by 4.0 mL of ethylacetate and 4.0 mL of diethylether. The column was allowed to be completely dry (20 minutes).

Extraction procedure

Urine, 0.5 mL containing internal standard or MFU, or water (pH 6.5, spiked with standards) was loaded on column and allowed to flow slowly. The fluid was not allowed to reach to the bottom of the column. The column was washed with 2.0 mL diethyl ether and metabolites were eluted with 3.0 mL ethylacetate into a glass tube. The eluate was dried at 37°C under N_2 and the residue was reconstituted in distilled water. After filtering through 0.2 μm filter appropriate dilution was made and 20 μL was injected into the HPLC system. Peaks of metabolites were separated at a flow rate of 0.5 mL/min and at sensitivity set at 5nAFs and potential at +0.60 V. Total elution time for all six eluents was set at twenty five minutes however, all the analytes were eluted within eighteen minutes.

RESULTS AND DISCUSSION

Chromatographic elution profiles of standards of VMA, MHPG, NMTN, MTN, ISOP (ISTD), and 5-HIAA are shown in Figure 1. A typical chromatogram obtained with aqueous standard mixture is shown in Figure 1A, with retention times of each metabolite. VMA was eluted at 3.680 minutes, MHPG at 5.220 minutes, NMTN at 7,325 minutes followed by MTN at 9.892 minutes. Internal standard ISOP was eluted at 13.532 minutes and the last eluent was 5-HIAA at 17.274 minutes. Although within 18 minutes all the analytes were eluted, running time of the chromatogram was kept at 25 minutes in order to allow for any electrical or room temperature fluctuations which may cause changes in retention times. The chromatogram in Figure 1B represents that of an extract of a spiked standard mixture with some changes in retention times of each eluent but, there was no disturbance in the order of elution of each metabolite. Figure 1C shows the profile obtained with a urine extract.

Standard curves were generated for each metabolite using a different range of concentrations. The area under the curve (AUC) obtained for the same concentration of different metabolites at the same sensitivity (5nAFS) was found to be a characteristic of each metabolite.

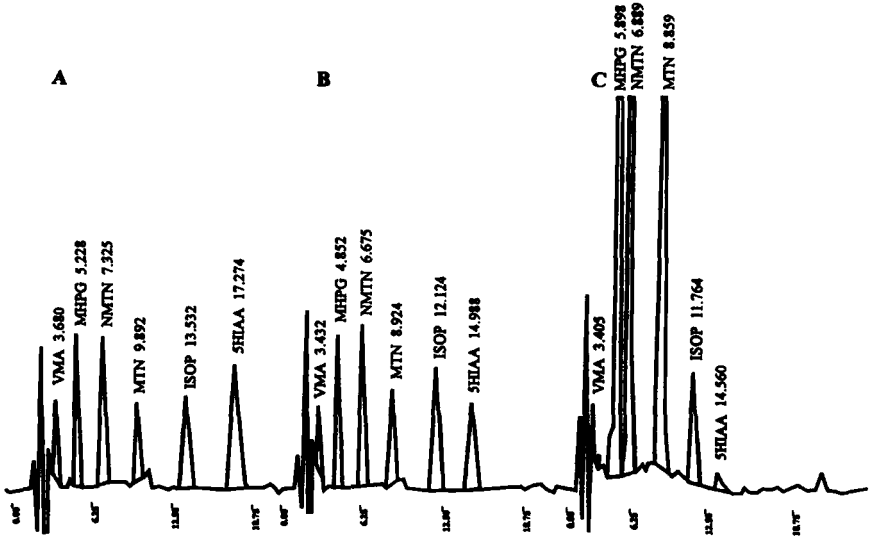

Figure 1. Chromatographic profile of standards of VMA, MHPG, NMTN, MTN, ISOP and 5-HIAA are shown in order of their retention times in 1A. Spiked standards of metabolites are shown in fig, 1B. Chromatogram 1C shows profile of metabolites in a urine extract.

For instance, AUC obtained for 5-HIAA was much greater than those of NMTN or MTN at the same concentration. Similarly, AUC for VMA and MHPG was greater than that of NMTN or MTN. Thus, the concentration required for 5-HIAA was much lower to achieve peak size similar to that of VMA, MHPG, NMTN, or MTN.

A standard curve, showing concentration as a function of ratio of AUC of the analytes to that of internal standard, for each metabolite is given in Figures 2 and 3.

The intra-assay coefficient of variation (CV) was determined from the ratio of the areas of curves of samples to that of ISOP (std) by injecting, repeatedly, seven times the same extract prepared from a single sample containing all five metabolites. CV for VMA was 6.92 %, for both MHPG and NMTN it was 4.5 %, for MTN it was 5.9 %, and for 5-HIAA the CV was 5.3 %.

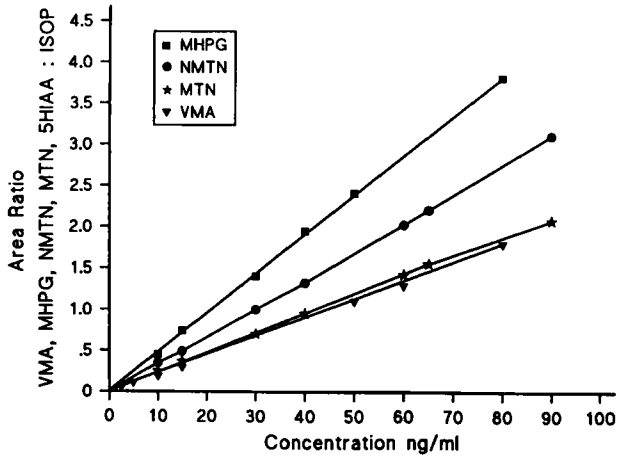

Figure 2. Standard curves of VMA, MHPG, NMTN and MTN show linear relationship between various concentrations and ratios of the areas of peaks of individual metabolites to that of the internal standard (ISOP).

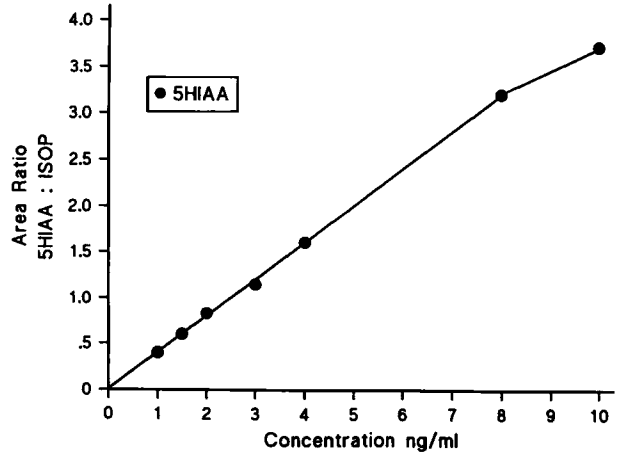

Figure 3. A standard curve showing a linear relationship between various concentrations of 5-HIAA and the ratio of the area of 5-HIAA peaks to that of ISOP.

The inter-assay coefficient of variation was determined from the percentage recovery after spiking seven different concentrations of each metabolite. The inter-assay coefficient of variation for VMA, MHPG, NMTN, MTN, and 5-HIAA was found to be 7.5 %, , 11.9 %, 8.69 %, 13.9 % and 16.17 %, respectively.

The percentage recoveries of all the metabolites were carried out using seven replicates and were found to range between 83 to 91. For VMA the recovery was 91.5±6.86 %, for MHPG it was 83.4±9.9 %, NMTN had a recovery of 88.3±7.7 %, MTN had 90.8±12.7 %, and 5-HIAA had 88.4±14.3 %. Validation of the procedure for urine samples was achieved by analysis of spot samples of urine obtained from normal subjects, which gave the values of free metabolites (ng/mL) VMA=22.7, MHPG=470.5, NMTN=293.3, MTN= 795.1, and 5-HIAA= 34.8.

Alumina B ion exchange columns used in this study for urine sample cleanup was found to be an important step for eliminating many interfering compounds which would otherwise interfere during the extraction procedure, as well as during HPLC analysis. Care was taken to condition the columns before loading the sample and to maintain a constant flow rate for proper elution of the metabolites of our interest. Extraction with two different solvents (diethylether and ethylacetate) sequentially improved recovery of metabolites. To the best of our knowledge, Alumina B columns have not been used earlier for urine sample clean up before solvent extraction and HPLC analysis of catecholamine and serotonin metabolites.

Addition of 0.5% isopropanol in mobilphase was found to increase the ion-pairing property, as well as decrease air bubble formation and stabilize the baseline. Isoproterenol was found to be a better internal standard compared with dihydroxybenzylamine (DHBA) and monohydroxybenzylamine (MHBA), with respect to its higher sensitivity and well separated peak from the analytes of our interest. There was no overlap of peak of ISOP with peaks of MTN or 5-HIAA.

Earlier studies have attempted to quantify simultaneously, VMA, MHPG, 5-HIAA, HVA, and DOPAC in urine using HPLC-ECD[25] but the recovery of all these metabolites was lower (56-80 %) compared to that achieved by the present method (83-91%) and the metabolites NMTN and MTN were not included in the analysis. The purification steps with Alumina B columns and diehylether wash in order to eliminate the interfering compounds, as well as an isocratic HPLC analysis in the present method were helpful for better recovery, as well as high sensitivity. Since the role of dopamine and its metabolites (DOPAC and HVA) in behavioral problems and in reactivity to challenges is

not clear, we attempted to include all major metabolites of NE, E, and 5-HT, that have been implicated in behavioral problems, so that total activity of these monoamines can be assessed in response to field stressors, or for diagnosing behavioral problems, as well as for follow-up for successful intervention in *different groups of patients. NMTN and MTN, together with VMA and MHPG analyzed simultaneously, should provide full profiles of NE, and E activity whereas, 5-HIAA levels reflect the activity of 5-HT system.

Analysis of these major and important five metabolites in a single sample of urine by one relatively simple method, is economical with respect to time, as well as expense since the method described can be used for the analysis of several urine samples in one day.

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