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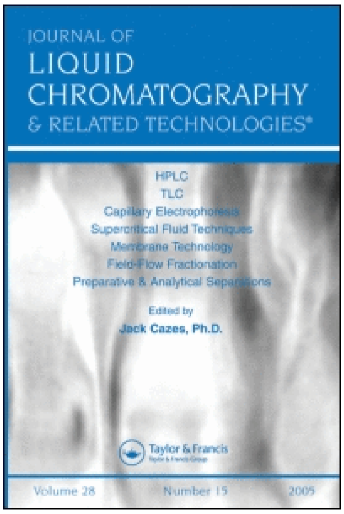

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An Improved Assay of Urinary Catecholamine Metabolites, 3-Methoxy-4-Hydroxyphenylglycol and Vanillylmandelic Acid, Using High Performance Liquid Chromatography With Electrochemical Detection

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AN IMPROVED ASSAY OF URINARY CATECHOLAMINE METABOLITES, 3-METHOXY-4-HYDROXYPHENYLGLYCOL AND VANILLYLMANDELIC ACID, USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

An improved method for the measurement of norepinephrine metabolites, 3-methoxy-4-hydroxyphenylgycol (MHPG) and vanillylmandelic acid (VMA), in urine using High Performance liquid Chromatography with Electrochemical Detector is described. An aliquot of urine was charged with an internal standard, dihydroxybenzylamine (DHBA), and the conjugated metabolites were hydrolyzed enzymatically. The hydrolysed and the free metabolites were extracted in ethyl acetate, dried, and dissolved in an aqueous buffer. An aliquot of appropriately diluted extract was injected into reverse phase C_{18} Nova-Pack, 4u column. Metabolites were eluted with a mobile phase containing citric acid-acetate buffer, sodium octyl sulfate and 5% methanol. Peaks were detected with an electrochemical detector. Integration and calculations were performed by a preprogrammed data module. The concentrations were determined using the ratio of the areas of peaks of metabolites to that of internal standard. A linear relationship was observed between of 20 - 100 ng VMA and 30 - 90 ng of MHPG. an intraassay coefficient of variance (%CV) was 8.6 and 3.2 for VMA and MHPG respectively. Interassay coefficient of variance was 10.3% for VMA, and 4.7% for MHPG. A total run time for each urine extract, including integration and calculations is less than 20 minutes. The method is specific, sensitive and therefore, can be used for assaying a number of samples for urinary metabolites of catecholamine.

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Introduction

The measurement of catecholamine metabolites in urine is important for evaluation of production of the whole body biogenic amines in the central and peripheral nervous system. For example, urinary excretion of norepinephrine metabolites, 3-methoxy-4-hydroxy phenylglycol (MHPG) and 3-methoxy-4-hydroxymandelic acid (VMA), may partially reflect the functional activity of the central noradrenergic neurons. Furthermore urinary output of norepinephrine and its major metabolite, MHPG, have also been found to be highly correlated (1,2,3). Quantification of these amine metabolites in urine is therefore, useful for the assessment of a variety of disease states characterized by abnormal secretion of catecholamine and include carcinoid syndrome, neuroblastoma and pheochromocytoma (4), as well as neurological and psychiatric disorders (5,6).

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Urinary excretion of metabolites of biogenic amines has been measured historically using spectrophotometric and fluorometric methods (7). These techniques, however, lacked sensitivity and specificity. More specific, sensitive, and reliable methods such as gas chromatography-mass spectrometry, are very expensive and involve tedious procedure for sample preparation, and, therefore, cannot be used when larger number of assays are to be performed.

In recent years techniques using the high performance liquid chromatography (HPLC) with reverse phase column packing have become available. These methods are more specific, sensitive and economical for quantifying biogenic amine metabolites as well as other compounds with related structures and having some hydrophobic character but soluble in aqueous buffers. However, detection with UV monitors attached to HPLC used earlier, lacks requisite sensitivity for the measurement of endogenous amine metabolites. HPLC with electrochemical detector (ECD) is found to increase the specificity and sensitivity for these metabolites. Although separate HPLC-ECD methods for VMA(8) and MHPG(5), have been described, there is scarcity of a simplified method to measure simultaneously both these metabolites in a single sample of urine. In this report we describe a convenient, sensitive and specific HPLC method for the simultaneous determination of both VMA and MHPG in the same sample of urine using dhydroxybenzylamine as an internal standard. Since the time for HPLC separation of these compounds is less than 20 minutes, a large number of samples can be assayed at a time.

Materials and Methods

Chemicals

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3-methoxy-4-hydroxyphenylgycol (MHPG), vanillylmandelic acid (VMA), dihydroxyphenylacetic acid (DOPAC), β-glucuronidase (bacterial type, V11-A) were obtained from Sigma Chemical CO. (St. Louis, MO). Arylsulfatase was purchased from Boehringer (Indianapolis, IN) and dihydroxybenzylamine-hydrobromide (DHBA.HBr) was obtained from Waters (Millford, MA). Sodium acetate, citric acid, dibutylamine, ethylendediaminetetraacetate (Na_2EDTA) were obtained from Eastman Kodak Co. (Rochester, NY). Sodium octyl sulfate was purchased from Aldrich Chemical Co. (Milwauki, WI). Ethyl acetate and methanol, HPLC grade (Mallinkrodt) were obtained from the Baxter Health Care Corp. Biorex-70 ion exchange column were purchased from the Biorad Laboratories.

Standard Solution

Stock solutions containing VMA, 1.0 mg/ml, and DHBA, 1.0 mg/ml were prepared in distilled water. MHPG 1mg/ml was prepared in mobile phase to which were added EDTA (0.05%) and sodium metabisulfite (Na_2S_205, 0.5%) to enhance the stability of the solution. All stock solution were stored at 4°C for not more than two weeks. The stock solutions were diluted with mobile phase to prepare aqueous standard mixture containing 250 ng/ml VMA, 1 ug/ml MHPG and 5 ng/ml DHBA.

Sample Collection

Urine samples were collected in containers to which 6M hydrochloric acid (100 ul acid/10 ml urine) was added as a preservative. Urine samples could be stored for up to six months at -70°C without loss of metabolites.

Metabolite Free urine (MFU)

Urine was rendered free of metabolites by adjusting pH to 10.6 and exposing it to air and light for 5 days. After adjusting its pH to 6.5, the urine sample was centrifuged at 15,000 rpm for 10 minutes. The supernate was passed through an ion exchange column, Biorex-70. Aliquots of the eluate collected were stored at -20°C. These eluates when processed for assaying the metabolites, did not show any discernable peaks on HPLC. These eluates were used as MFU and were spiked with known standards for calibration of the instrument.

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Sample Preparation

The conjugated metabolites in urine were enzymatically hydrolysed before extraction for their quantification. To a 0.5 ml aliquot of urine were added 80 ul of 1.0 M Tris acetate buffer, pH 6.0, and 0.5, ug of DHBA as internal standard. The mixture was incubated with 50 ul β-glucuronidase (25,000 units/ml) and 15 ul arylsulfatase (4500 units/ml) for 16 hours at 37°C. At the end of incubation the pH was adjusted to 1.0 with HC1 and the metabolites were extracted twice with 1.0 ml ethyl acetate. The extract was pooled and solvent evaporated under nitrogen gas. The dry residue was constituted in 1.0 ml distilled water and extract stored at -20^0C until analyses. The extract was thawed and diluted 1:100 with mobile phase and 20 ul was injected for HPLC analysis of metabolites.

MFU Spiked with Standards for Calibration

MFU (0.5 ml) was spiked with 50 ul of a mixture containing 50 ug VMA, 500 ug MHPG, and 0.5 ug DHBA. After adding 50 ul of Tris-acetate buffer pH 6.0 the mixture was vortexed and incubated with β-glucuronidase and aryulsulfatse for 16 hours at 37°C and extracted with ethyl acetate, following procedure similar to the one used for urine samples as described above. A 20 ul aliquot of 1:100 diluted extract was injected to obtain discrete peaks for calibrating the integrator for calculation of metabolites in the sample of urine.

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HPLC Analysis

Mobile Phase

Deionized HPLC grade water was used to prepare the mobile phase, and contained 0.05M sodium acetate, 0.05M citric acid, 0.56 mM sodium octyl sulfate (SOS), 0.075 mM disodium ethylenediaminetetraacetate (Na_2EDTA), 1.0 mM dibutylamine and 5% methanol. The solution was adjusted to pH 3.5 with dilute HC1. The solution was filtered under vacuum using a 0.45 um filter and was sonicated for 30 seconds at room temperature to degas before use. To achieve the optimal percentage of methanol and pH of mobile phase for controlling retention times and sensitivity respectively, varying concentrations of methanol ranging between 5-20% and pH 3.5 to 6.0 were tried. The best separation of metabolites into discrete peaks without any overlap and stable retention time was achieved with mobile phase containing 5% methanol and at pH 3.5. A minimal flow of 0.2 ml/minute of mobile phase was always maintained to prevent any clogging of column and electrode and to facilitate stability and reproductibility in the separation of metabolites.

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HPLC Apparatus: High performance liquid chromatography system of Water's (Division of Millipore, Milford, MA), used in the analysis of norepinephrine metabolites was the same as described earlier by Kumar et al. (9). The system consisted of U6K injector, solvent delivery system model 590, and an electrochemical detector (model 460) consisting of a glassy carbon working and auxiliary electrodes and a silver/silver chloride reference electrode. Data module 740 was used for the integration of the peak signal and for calculation of the results based on the ratio of the ares of peaks of the anaytes to that of the internal standard. The chromatography column, Nova-Pak (3.9 mm x 15 cm), steel column containing C_{18} hydrocarbon chain bonded to 4 um spherical silica particles packed by end capping process obtained from Waters (Part #86344) was used. This column was found to provide stable and reproducible separation of VMA and MHPG in a standard mixture as well as in urine samples extracts. The flow rate of mobile phase was kept at 0.4 ml/minute at a potential of +0.55V versus Ag/Agcl reference electrode and the detector sensitivity at 5nAFS.

Calibration and Calculation:

An elution profile of the metabolites was obtained by injecting a 20 ul solution of a mixture of VMA, MHPG and DHBA. The instrument was equilibrated and retention times were stabilized by another subsequent injection of 20 ul of the standard mixture. Total elution time for all peaks was less than 20 minutes.

Calibration of the instrument was achieved by injecting 20 ul aliquot of 1:100 diluted extract of 0.5 ml of MFU spiked with 50ug VMA, 500 ug MHPG, and 0.5 ug DHBA. For analysis of urine samples a 20 ul aliquot of extract (1:100) diluted) containing 0.1 ng DHBA (internal standard) was injected.

The method was validated by standard curves obtained by including a range of concentrations of standards of each analyte. Only one concentration (0.5 ug) of DHBA was used to determine the ratio of the area of peaks of various concentrations of VMA (20-200ng/20 ul) and MHPG (30-90ng/20ul) to that of DHBA.

Concentration of individual metabolites in 1.0 ml of urine were calculated by the preprogrammed data module (model 740, Waters) using the ratio of the integrated areas of individual analytes to that of the internal standard, DHBA, of known concentration. As low as 0.2 ng of VMA and 2.0 ng of MHPG could be detected under the conditions described.

Results and Discussion

Chromatograms, Fig 1, represent the typical elution profiles of VMA, MHPG and the internal standard, DHBA. The retention time of VMA was found to be 4.5 minutes, that of MHPG to be 5.7 minutes and that of DHBA was 8.9 minutes as shown in figure 1A. A profile from an extract of urine, rendered free of metabolites (MFU) is shown in figure 1 B. The profile obtained from extract of MFU was similar to the baseline showing no discernable peaks of either VMA, or MHPG. Figure 1C shows the profile of MFU spiked with known amounts of VMA, MHPG and DHBA. A typical chromatographic profile of VMA and MHPG obtained from an extract of a sample of human urine is presented in figure 1D. The last peak in each chromatogram was that of DOPAC (retention time 12.6 minutes), a metabolite of dopamine and was characterized using a known concentration of DOPAC standard.

The calibration curves were found to be linear over a wide range of concentrations of VMA and MHPG. In Fig. 2 is shown a calibration curve obtained between various concentrations of VMA (20-100ng/20 ul) and MHPG (30-90ng/20 ul) and the ratio of the corresponding areas of each of the analytes to that of DHBA. As is evident from the slopes of VMA and MHPG (fig 2), the detector sensitivity for VMA was always three times greater than that of MHPG for the same concentration.

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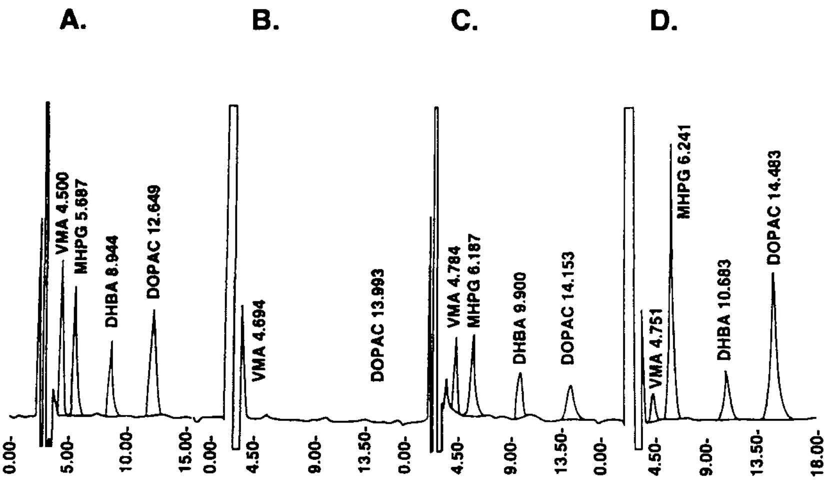

FIGURE 1: Chromatograms shown represent typical profiles of peaks for VMA, MHPG and internal standard, DHBA. The last peak in the chromatograms is that of dihydroxy phenyl acetic acid (DOPAC). Standard mixture (aqueous solution) (A), metabolite free urine (MFU) extract (B), MFU spiked with known standards of metabolites (C), a typical profile of a urine extract (D). Chromatograms B, C and D were obtained from the extracts after enzymatic hydrolysis of the urine samples.

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Mobile phase containing 5% methanol and at pH 3.5 was most effective in maintaining the stability of retention times and sensitivity of the detector. Presence of 0.56 mM sodium octyle sulfate was found to be critical to both stability and sensitivity of chromatographic separation of analytes. When 0.1M phosphate buffer was substituted for citrate-acetate buffer in mobile phase, it had adverse effects on HPLC elution profile.

To evaluate reproducibility of the method, aliquots of extract obtained from the same sample of urine were injected repeatedly. Intraassay coefficient of variation (CV) with six injections was 8.6% for VMA and 3.2% for MHPG. The interassay variation was obtained

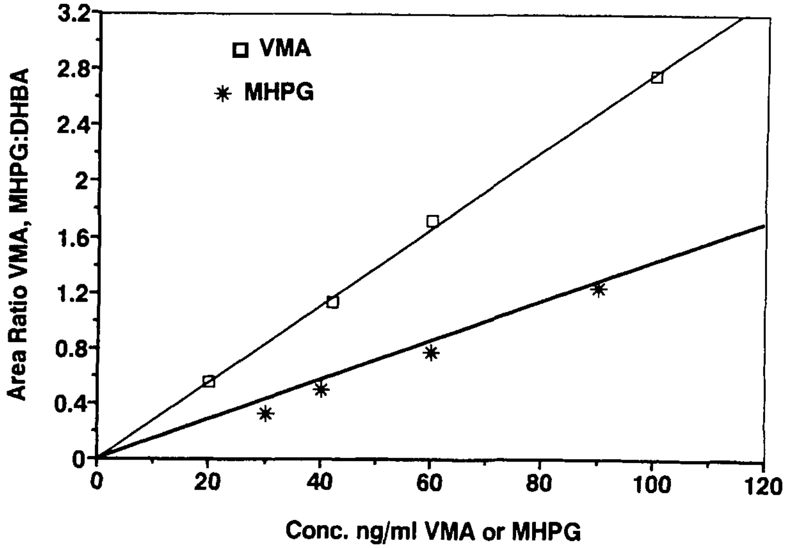

Figure 2: A standard curve showing a linear relationship between various concentrations of VMA and MHPG, and the ratio of the areas of peaks of VMA and MHPG to that of DHBA.

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by injecting extracts of 6 aliquots (0.5. ml) of a single urine sample which gave a CV of 10.3% for VMA and 4.7% for MHPG.

Samples of urine obtained randomly from 12 subjects with no dietary and drinking restrictions were analyzed. Urinary catecholamine total metabolite concentrations in 24 hour urine samples, were, VMA 0.03 ± 0.02 mg (mean ± SD), and MHPG, 2.46 ± 1.80 mg. The enzyme hydrolysis of the conjugated metabolites was carried out under the optimum conditions described by Dekirmenjian and Mass (10) and by Joseph et al (5). After hydrolysis the extraction of metabolites from the incubate at pH 1.0 was best achieved by ethylacetate. Attempts were made to further purify the urine samples using *ion-exchange* columns (BIOrex-70) which lead to a substantial loss of both the analytes and did not show any improvement in the chromatographic profile (data not presented).

The working potential of 0.55V was found to be suitable for detector sensivity of 5nAFS at a flow rate of 0.4 ml/min. the baseline was stable and the back ground signal was very low.

One of the important features of our procedure is the use of DHBA as a suitable internal standard. When known amounts of VMA, MHPG and DHBA were added to MFU for spiking and calibration of the instrument, or when DHBA was added as an internal standard to a sample of urine, a recovery of 79-92% for DHBA was always obtained. It is therefore evident that through the procedure of hydrolysis and extraction most of DHBA could be recovered. In some of the earlier studies (11,12) MHPG itself has been used as an internal as well as an external standard. When MHPG is used as an internal standard, the values obtained may not represent the true content of MHPG and VMA since the content of these metabolites in different samples of urine is variable, and MHPG when added as the internal standard, to a sample with a higher content of MHPG, may overshoot the detection limits and introduce a larger error in the final values of its urinary content. The use of DHBA as an internal standard eliminates such errors, and in addition, corrects for volumetric errors in extraction as well as in injection of samples and provides a convenient and a consistent method of calculations of the final results. The internal standard such as DHBA with similar detection characteristics as the analytes (VMA and MHPG) will also reduce the variability due to transient changes in the detector sensitivity as well as column performance since both the internal standard and the analytes (MHPG and VMA) will exhibit parallel responses at the same time.

Another salient feature of this method is the calibration of the instrument using MFU spiked with known concentrations of analytes. The absence of metabolites from MFU was

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ensured by obtaining a chromatogram of the extract of enzyme treated MFU before calibration with spiked MFU and the analysis of urine samples. Finally, another important feature of this method is the short run time of 20 minutes for one urine sample extract.

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References

1. Roy A, Guthrie S, Karoum F, Pickar D, & Linnoila M: High intercorrelations among urinary outputs of norepinephrine and its metabolite. Arch Ghen Psychiatry 45(2):158-161, 1988.

2. Linnoila M, Karaim F, Potter WZ: Correlatio of norepinephrine and its major metabolite excretion rates. Arch Gen Psychiatry 39:521-523, 1982.

3. Blombery PA, Kopi IJ, Gordon EK, Markey SP, Ebert MH, Conversion of mHPG to Vanillylmandelie acid (VMA) Implications for the importance of Urinary MHPG. Arch Gen Psychiatry 37:1095-1098, 1980.

4. Krstulovic AM, Zakaria M, Lohse K, Bertani-Dziedzic L: Diagnosis of neural crest tumors by reverse phase high performance liquid chromatographic determination of urinary catecholamine metabolites. J Chromatography, 186: 733-748, 1979.

5. Krstulovic AM, Matzara CT, Bertain-Diedzic, Cerqueria S, ad Gitlow SE: Endogeous levels of free and conjugated urinary 3-methoxy-4-hydroxyphenylglycol in control subjects and patiets with pheochromocytoma determined by high performance liquid Chromatography with electrochemical detection. Clin Chim Acta 103:109, 1980.

6. Joseph MH, Kadam, VB, and Risby D: Simple high performance liquid chromatographic method for the concurrent determination of the amine metabolities vanillymandelic acid, 3-methoxy-4-hydroxyphenylglycol, 5-hydroxyindoleacetic acid, dihydroxyphenlylacetic acid and homovanillic acid in urine using electrochemical detection. J Chromatography, 226:361-368, 1980.

7. Shildkraut JJ, Orsulak PJ, Schatzberg AF, Guderman JE, Cole JO, Phode WA, LaBride RA: Towards a biochemical classification of depressive disorders: I Difference in urinary excretion of MHPG and other catecholamine metabolities in clinically defined subtypes of depression. Arch Gen Psychiatry *35:1427, 1978.*

8. Rainer F: Methods of determination of catecholamines and their metabolities In: Methods of Neurochemistry. Marcel Dekker Inc. New York, p. 83-127, 1971.

9. Kumar AM, Kumar M, Fernandez JB, Mellman TM, Eisdorfer CE: A simplified HPLC-ECD technique for measurement of urinary free catecholamines. J Liquid Chromatogr, 14 (19):3547-3557, 1991.

10. Dekirmenjian H and J.W. Maas: An improved procedure of MHPG determination by gas liquid chromatography. Anal Biochem 35:113-122, 1970.

11. Fellows L, P Riederer and M Sandler: A rapid assay of 4-hydroxy-3-methoxyphenylglycol in urine. Clinica Chimica Acta 59:255-257, 1975.

12. Taylor J, S. Freeman and P. Brewer: Liquid chromatography of 3-methoxy-4-hydroxyphenylglycol in urine with Fluorescence Detection. Clinical Chemistry, 27:173-175, 1981.

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