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SIMULTANEOUS DETERMINATION OF VANILLYLMANDELIC AND HOMOVANILLIC ACIDS IN URINE USING LC WITH COULOMETRIC ELECTROCHEMICAL DETECTION BASED ON SPE SAMPLE PREPARATION

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The aim of the study was to develop a simple and selective LC method for the simultaneous measurement of vanillylmandelic acid (VMA) and homovanillic acid (HVA) in human urine. A method for the measurement of VMA and HVA using LC with coulometric electrochemical detection and investigation into the extraction techniques with regard to stability and recovery are described. The analytical performance of presented method is satisfactory. The presented method uses a relatively rapid SPE procedure. It can be used both for research and for clinical testing purposes.

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Introduction

Vanillylmandelic acid (VMA) and homovanillic acid (HVA) are the major metabolites of catecholamines. Epinephrine and norepinephrine are metabolized to VMA by the combined action of catechol-*O*-methyltransferase and monoamino-oxidase. HVA represents the main metabolite of dopamine [1-3]. Urine VMA and HVA are often measured in diagnostics of neuroblastoma, pheochromocytoma and neurological diseases [4, 5].

Numerous analytical methods for the measurement of VMA and HVA in urine have been described [6-13]. With regard to the presence of other metabolites with similar structures and properties, it is necessary to use purification steps prior to their determination. These steps have been based on either SPE or liquid-liquid extraction (LLE). SPE and LLE are often time-consuming and a source of errors. Final quantification is most often performed using GC and LC. GC methods need a sample derivatization step, which is time-consuming [7,14]. Different LC methods have been described, mainly with electrochemical [8,15,16] or fluorescence detection [17]. LC with electrochemical detection, using either amperometric or coulometric electrodes, can measure VMA and HVA directly. Moreover, these techniques avoid typical problems associated with derivatization procedures. Companies Chromsystems (Munich, Germany) and Recipe (Munich, Germany) have marketed commercial LC reagent sets for the determination of VMA and HVA. Although LC methods for the simultaneous determination of VMA and HVA have been substantially improved and simplified, they are still expensive hence unsuitable for routine analyses. Recently the application of LC-MS or LC-MS-MS methods for the determination of VMA and HVA has been reported [12,13]. The advantage of LC-MS methods is a rapid assay without sample pretreatment; the disadvantage is mainly their expensiveness. For screening of large numbers of samples are available enzyme immunoassay (EIA) methods (6). The immunoassays are problematical in cross-reactivity, and monoclonal antibodies are expensive.

In this paper, we describe a sensitive LC with coulometric electrochemical detection for simultaneous determination of VMA and HVA using an internal standard and a relative rapid and accuracy SPE procedure. Moreover, this method is inexpensive and applicable to routine analyses.

Materials and Methods

Reagents and Chemicals

Vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid, VMA), *iso*-vanillyl-mandelic acid (3-hydroxy-4-methoxymandelic acid, iVMA), homovanillic acid (4-

hydroxy-3-methoxyphenylacetic acid, HVA), 5-hydroxyindoleacetic acid (5-HIAA), hydrochloric acid, sodium hydroxide, orthophosphoric acid, acetic acid, sodium acetate, ammonium acetate and sodium hydrogenphosphate were obtained from Sigma Chemical Company (St. Louis, MO, USA). AG 1-X8 resin, 100-200 mesh, chloride form, was purchased from Bio-Rad Laboratories (Hercules, CA, USA). HPLC gradient-grade methanol, ethanol, and acetonitrile were from Merck KgaA (Darmstadt, Germany). Lyophilized urine endocrine controls (normal range, Lot No.: 195; pathological range, Lot No.: 155) were from Chromsystems (Munich, Germany). All the other chemicals were of analytical grade.

VMA, iVMA and HVA solutions were prepared daily in 0.1 mol l^{-1} hydrochloric acid (5-HIAA in water) and stored at 4 °C until used.

Instrumentation

The liquid chromatograph consisted of LC-10ADvp solvent delivery system (Shimadzu, Kyoto, Japan), injection valve Rheodyne 9725i (Rheodyne, L.P., Rohnert Park, CA, USA), with 10-μl loop and Coulochem® III electrochemical detector (ESA Laboratories, Inc., Chelmsford, MA, USA). The data were collected digitally with Clarity chromatography software (DataApex, Prague, the Czech Republic).

Subject and Samples

Samples of urine were obtained from a group of donors (n = 40, 20 women in the age 23-49 years, mean age 36 years, and 20 men in the age 21-52 years, mean age 38 years). None of the studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary or oncological diseases. A written informed consent was obtained from all the participants before starting the protocol and the Hospital Committee on Human Research (Regional Hospital of Pardubice, the Czech Republic) approved the study.

Sample Preparation

iso-Vanillylmandelic acid was used as an internal standard because it has similar properties to measured VMA and HVA.

Twenty-four-hour urine samples, protected against daylight, were acidified with concentrated hydrochloric acid.

One milliliter of acetate buffer (0.1 mol l^{-1}, pH 6.1 ± 0.1) containing internal standard (1.5 μmol l^{-1} of iVMA) was carefully added to 50 μl urine or spiked urine

standard. After vigorous mixing (20 s) and pH adjustment (between 5 and 7), the mixture was applied on the SPE column.

Solid-Phase Extraction

We have used SPE procedure for removal of interfering compounds.

Polypropylene SPE tube with a frit and a column volume of 3 ml (Supelco, Bellefonte, PA, USA) were filled with 0.5 ml of AG 1-X8 anion-exchange resin. The sorbent was tightened with another frit and washed with deionized water until a negative reaction to chloride ions, which was tested by the addition of 300 μl 0.3 mol l^{-1} $AgNO_3$ to 1 ml effluent. The column was equilibrated stepwise (by 2 ml) with 4 ml acetate buffer (0.1 mol l^{-1}, pH = 6.1 ± 0.1) at a flow-rate of 1 ml min^{-1}. This flow-rate was achieved by centrifugation (780 × g, 2 min, room temperature). Then the mixture (1 ml) was applied on the equilibrated column. The SPE procedure was performed according to the sequence shown in Table I. Collected effluent was diluted with a mobile phase (50 μl of effluent and 450 μl of mobile phase) and filtered through a 0.20-μm nylon filter (4 mm diameter, Supelco, Bellefonte, PA, USA). Columns were regenerated stepwise (by 2 ml) with 6 ml HCl (1.0 mol l^{-1}), which removed bound anoins. After about 20 ml washing with deionized water and testing of negative reaction to chloride ions, the columns were ready for the next run.

Table I The solid-phase extraction procedure

Our SPE	Commercial SPE	
Column equilibrated with acetate buffer (0.1 mol l^{-1}, pH = 6.1±0.1) [2×2 ml]	-	-
Diluted urine applied to column	Diluted urine applied to column	Discard effluent
Column washed with ethanol-2 mol l^{-1}, acetic acid (1:1) [2×2 ml]	Column washed with Buffer I [3 ml]	Discard effluents
Column washed with acetate buffer (0.1 mol l^{-1}, pH = 6.1±0.1) [2×2 ml]	Column washed with Buffer II [2×3 ml]	Discard effluents
VMA, iVMA and HVA eluted with 0.5 mol l^{-1} H_3PO_4 [2×1 ml]	VMA, iVMA and HVA eluted with Elution Buffer [2 ml]	

Solid-Phase Extraction Using Set from Chromsystems

We have compared our SPE method with a commercial SPE method developed by firm Chromsystems (Munich, Germany). After addition of internal standard (1.0 ml) to urine or spiked urine standard (50 μl) and vigorous mixing (20 s), the mixture (1 ml) was applied on the SPE column. The SPE procedure was performed according to the sequence shown in Table I and the flow-rate (1 ml min^{-1}) was achieved by centrifugation (720 × g, 2 min, room temperature). Collected effluent was diluted with a mobile phase (50 μl effluent and 450 μl mobile phase) and filtered through a nylon filter.

Chromatographic Analysis

Chromatography of VMA and HVA was accomplished using an isocratic elution on a Discovery® HS C_{18}, 250 × 4 mm i.d., 5 μm analytical column (Column#: 54951-01; Bonded Phase Lot#: 5084; Silica Lot#: 020419BQ) fitted Discovery® HS C_{18}, 20 × 4 mm i.d., 5 μm guard column (Supelco, Bellefonte, PA, USA) at the temperature of 30 °C. The mobile phase consisted of 5 % methanol in 25 mmol l^{-1} Na_2HPO_4 (v/v), pH = 6.0 ± 0.1. The flow-rate was kept constant at 0.5 ml min^{-1}. VMA and HVA were detected following LC separation with a Coulochem® III detector equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). The guard cell was connected in line before the injector and was used to remove oxidizable impurities in the mobile phase. The dual analytical cell contains two porous graphite electrodes in series. A carbon filter was placed before a guard cell and between the injector and guard column, a PEEK filter between the analytical column and analytical cell. For optimum detection of VMA and HVA, the electrode potentials for the guard cell, *E*1, and *E*2 were set at +450 mV, +180 mV, and +400 mV, respectively. The gain range was 200 nA. The hydrodynamic voltammogram analysis was performed to optimize conditions for the accurate determination of VMA and HVA. It was developed by injection of 10 μl mixed solution of VMA and HVA (0.1 mmol l^{-1}) and measuring the current produced by VMA and HVA at the electrodes. Before injection of the first sample, the potential at the electrodes (except the guard cell) was increased stepwise from +100 mV in about 40 mV increments to the final working potential, and LC system was equilibrated for approximately 2 hours with the mobile phase at a flow-rate of 0.5 ml min^{-1}. When not running samples overnight, flow-rate of mobile phase was set at 0.1 ml min^{-1} with voltages +100 mV, +200 mV, and +250 mV, respectively. After about 50 injects, the electrode potentials were set first at +600 mV, then at –400 mV, at each electrode for 1 hour with the mobile phase at a flow-rate of 0.5 ml min^{-1}, followed by 20-min water rinse and by 60-min methanol rinse at a flow-rate of 0.5 ml min^{-1} (electrodes off). This procedure was used for removal of

impurities from electrodes and achieving electrode sensitivity and baseline stability.

Statistical Analysis

The data are presented as mean values ±*S.D*. Differences between women and men were analyzed with the use of the Mann-Whitney Rank Sum Test and analysis of correlation were carried out using Spearman Rank Order Correlation (software QCexpert, Trilobyte, Pardubice, the Czech Republic). A $p < 0.05$ value was considered statistically significant.

Results and Discussion

The Effectiveness of SPE, Stability

We have tested the effectiveness of our and commercial SPE procedure. Although commercial SPE procedure is as rapid and simple as our method, it has less recovery and VMA was with difficulties separated from interfering compounds. SPE of the samples was studied using a strong AG 1-X8 anex. The volume, flow-rate and composition of solutions were studied with the aim of removing the interferences with minimum loss of VMA, iVMA and HVA. The volumes assayed were from 1 to 3 ml at a flow-rate of 1-3 ml min^{-1}. The optimum volume and flow-rate were 2 ml and 1 ml min^{-1}, respectively. The column was equilibrated with acetate buffer (0.1 mol l^{-1}, pH = 6.1 ± 0.1); it has similar composition as the diluted urine. Different mixtures of ethanol and acetic acid (2.0 mol l^{-1}) were assayed within clean-up step . The 1:1 ethanol-acetic acid mixture provided the best results, removing the interferences and causing practically no loss of VMA, iVMA and HVA. A solution of 0.5 mol l^{-1} orthophosphoric acid provided the best elution of the analytes. The VMA and HVA content in samples extracted using SPE was stable at 4 °C for at least 12 hours.

In comparison with the commercial SPE procedure, our SPE procedure is inexpensive. With careful regeneration of resin, we used the same SPE column more than ten times (intra-assay with CV below 5 % and inter-assay with CV below 10 % both VMA and HVA).

Some LC methods use time-consuming extraction techniques [15] or analyze only diluted urine samples [16,18]. When crude samples are injected on an LC column, resolution and column life decrease. Compared to some published extraction techniques, our SPE procedure uses eco-friendly extraction reagents. Organic solvents such as ethyl acetate [7,16] or methanol [12,13] are considered as significant pollutants.

LC Assay of VMA and HVA

Representative LC chromatogram of VMA and HVA in human urine is shown in Fig. 1. The analytical parameters of VMA and HVA analysis are shown in Tables II and III. To determine the within-day precision, the urine samples were analyzed ten times on the same day under the same conditions. Similarly, the between-day precision obtained on the same urine samples, were analyzed for 12 consecutive days. Precision and recovery studies were carried out using normal urine for VMA and HVA, aliquoted and stored at the temperature of –20 °C. Samples of this urine were spiked with VMA and HVA to obtain levels of these analytes in the pathological range. The calibration curves were linear in the whole range tested: (2.0-200.0) μmol l^{-1}. The regression lines obtained from the combination of ten standard curves were $y = 0.0181x - 0.0066$ for VMA and $y = 0.0199\,x - 0.0094$ for HVA. The mean slope, intercept, and correlation coefficient (r) for the calibration curves were 0.0181 (95 % confidence interval, 0.0156–0.0207), 0.4 μmol l^{-1} (0.2–0.7 μmol l^{-1}), and 0.9992 for VMA, 0.0199 (95 % confidence interval, 0.0141–0.0258), 0.5 μmol l^{-1} (0.3-0.8 μmol l^{-1}), and 0.9993 for HVA. The lowest concentration that can be quantified with acceptable accuracy and precision was 2.0 μmol l^{-1} [47.6 fmol $inject^{-1}$] (intra-assay with CV = 7.8 %) for VMA and 2.0 μmol l^{-1} [47.6 fmol $inject^{-1}$] (intra-assay with CV = 9.7 %) for HVA. Furthermore, the limit of detection for VMA and HVA, defined as signal-to-noise (S/N) ratio of 3:1, was 0.4 μmol l^{-1} (9.5 fmol $inject^{-1}$) and 0.8 μmol

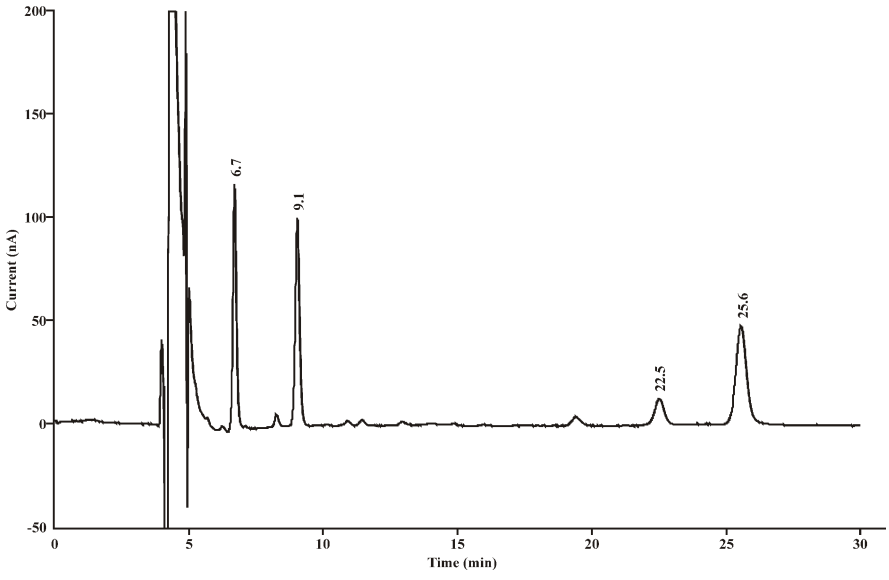

Fig. 1 Representative LC chromatogram of VMA (38.6 μmol l^{-1}) and HVA (40.2 μmol l^{-1}) in human urine. Peaks: VMA (6.7 min); iVMA (9.1 min) and HVA (25.6 min). Peak (22.5 min) corresponds to 5-hydroxyindoleacetic acid. LC conditions: isocratic elution (5 % methanol in 25 mmol l^{-1} Na_2HPO_4, pH 6.0 ± 0.1). Stationary phase was an analytical column Discovery® HS C18, 250 × 4 mm i.d., 5 μm. The flow-rate was kept constant at 0.5 ml min^{-1} and separation ran at 30 the temperature of °C

Table II Precision of VMA and HVA for urine samples analysis

A) Precision (within-day)				
	VMA		HVA	
N	Mean ± *SD* μmol l^{-1}	*CV* %	Mean ± *SD* μmol l^{-1}	*CV* %
10	22.5 ± 0.9	4.0	25.3 ± 0.8	3.2
10	79.4 ± 2.9	3.7	97.3 ± 2.7	2.8
B) Precision (between-day)				
	VMA		HVA	
N	Mean ± *SD* μmol l^{-1}	*CV* %	Mean ± *SD* μmol l^{-1}	*CV* %
12	21.6 ± 1.7	7.8	24.4 ± 2.0	8.2
12	78.1 ± 5.8	7.4	93.1 ± 7.1	7.6

Table III Recovery of VMA and HVA for urine samples analysis. *Mean of triplicate assays is recorded

Recovery				
	VMA		HVM	
Added μmol l^{-1}	Observed μmol l^{-1*}	Recovery %	Observed μmol l^{-1*}	Recovery %
0	23.5 ± 0.9	-	27.1 ± 1.2	-
2	25.3 ± 1.1	90.0	28.9 ± 1.2	90.0
5	28.1 ± 1.2	92.0	31.7 ± 1.3	92.0
20	42.1 ± 1.9	93.0	45.9 ± 2.1	94.0
50	71.5 ± 2.9	96.0	76.4 ± 3.1	98.6
100	120.8 ± 4.7	97.3	129.5 ± 4.9	102.4
		Mean 93.7 ± 2.7		Mean 95.4 ± 4.5
		CV 2.8		*CV* 4.7

l^{-1} (19.0 fmol $inject^{-1}$), respectively.

VMA, iVMA and HVA were separated on a reverse-phase column using an isocratic system of methanol and Na_2HPO_4. The mobile phase was optimized in

order to obtain the best separation of the analytes in the shortest time. Standard solutions of VMA, iVMA and HVA as well urine were used for study of the mobile phase composition. Several mobile phases (namely different buffers containing methanol, ethanol and acetonitrile) were assayed and the best results were obtained for the conditions described in the experimental. The column temperature was varied from 25 to 45 °C. Optimal temperature interval was from 28 to 33 °C. The criteria were the resolution, electrode sensitivity, baseline stability and the analysis speed.

Determination of VMA and HVA in Human Urine

The levels of VMA and HVA in a group of donors are (19.7 ± 6.9) μmol d^{-1} and (23.3 ± 14.8) μmol d^{-1}, respectively. We found no significant differences in VMA and HVA concentration between women and men (18.2 ± 10.3 μmol d^{-1} versus 21.7 ± 9.1 μmol d^{-1}, p = 0,137 and 20.8 ± 17.1 μmol d^{-1} versus 24.7 ± 15.1 μmol d^{-1}, p = 0.226). We observed no significant correlation between VMA and HVA concentrations and age (R = 0.085, p = 0.443; R = 0.067, p = 0.489).

The aim of this study was to develop and validate LC method with coulometric electrochemical detection for the simultaneous determination of VMA and HVA in human urine. The major advantages of electrochemical detection are especially the simultaneous determination of VMA, HVA and 5-HIAA and a relatively rapid sample preparation without time-consuming pre-column derivatization. Coulometric electrochemical detection offers several advantages over the commonly used amperometric electrochemical detection [15]. First of all, in the amperometric mode about 5 % of the analyte is oxidized on the surface of the electrode; with a coulometric detector, close to 100 % of the analyte is oxidized in dual flow-through porous graphite electrodes. Within past 3 years we have carried out more than 1500 analyses. By virtue of careful clean-up of urine samples and a high sensitivity of flow-through porous graphite electrodes we can inject a small amount of enough diluted sample. Under these conditions, the lifetime of the analytical column is at least 1500 injects. The presented method is superior to similar methods in that it is inexpensive (about 2500$ per1000 analyses; Costs of guard cell, analytical cell, analytical column, mobile phase, SPE tubes, anion-exchange resin, nylon filters and chemicals for sample preparation costs) and suitable for clinical tests.

Conclusion

We developed a simple and very selective LC method with coulometric electrochemical detection for simultaneous determination of VMA and HVA in human urine. Careful pretreatment of human urine samples allow using of a simple

and inexpensive LC method. SPE procedure is relatively rapid and accurate.

Abbreviations used: VMA – vanillylmandelic acid; iVMA – isovanillylmandelic acid; HVA – homovanillic acid

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