Sensitive, Rapid and Easy Analysis of Three Catecholamine Metabolites in Human Urine and Serum by Liquid Chromatography Tandem Mass Spectrometry

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A sensitive and easy analytical method for catecholamine metabolites including 4-hydroxy-3-methoxyphenylglycol sulfate (HMPG sulfate), vanillylmandelic acid (VMA) and homovanillic acid (HVA) determination was developed based on liquid chromatography–tandem mass spectrometry in a negative multiple reaction monitoring mode. The analytes were rapidly separated on a reversed-phase Waters XBridge C18 column (150 × 2.1 mm i.d.) with the mobile phase of 15% (v/v) acetonitrile containing 2 mM ammonium formate and 85% (v/v) formic acid solution (0.05%, v/v). Mass spectrometric conditions, such as characteristic fragmentations and quantification ion transitions, both with chromatographic conditions including separation column type and mobile phase composition, were systematically investigated to get optimal sensitivity and specificity. The limits of detection were in the range of 0.03–0.7 ng/mL for the targets. Recovery rates of spiked urine samples with three different concentration levels (low, middle and high) were above 85% with precisions less than 5.7%. For serum analysis, acetonitrile chosen both as protein precipitation reagent and extraction solvent facilitates to reduce matrix effects. Recovery rates of spiked serum sample were in the range of 90.6% to 111.1% for three targets. The intra-day and inter-day precisions were satisfactory less than 8.7%. This proposed method was successfully applied to determine HMPG sulfate, HVA and VMA present in human urine and serum.

Introduction

Catecholamines (CAs), named as neurotransmitters or hormones, are biogenic amines that play an important role in the nervous system. The precursor and metabolites of some catecholamines have been used as tumor markers of pheochromocytoma, neuroblastoma and paraganglioma (1). 4-Hydroxy-3-methoxyphenylglycol (HMPG) is produced from both epinephrine and norepinephrine by sequential action of monoamine oxidase (MAO) and catechol-0-methyl-transferase (COMT) and is then further metabolised to vanillylmandelic acid (VMA) by alcohol dehydrogenase (2). Homovanillic acid (HVA) is another metabolite of dopamine by the two enzymes. Three metabolites are excreted in urine (3). Patients suffering neurological disorders produce an excess of catecholamines and their metabolites compared with healthy people. Analysis of CA metabolites in human urine is essential for diagnosis of neurological disorders. In addition, some researchers have confirmed that the determination of MHPG and VMA plasma levels could be an important tool in the study of mechanisms underlying drug addictions and in the development of new strategies for their control and prevention (2). To our best knowledge, the determination of serum levels of CA metabolites is rarely reported. However, Tang et al. (4) determined serum MHPG, VMA and HVA levels using high-performance liquid chromatography (HPLC) with electrochemical detection for the diagnosis of neuroblastoma in the early stages.

Low concentration levels of CA metabolites in biological samples pose a challenge to the detection sensitivity and pretreatment methods. Until now, the analysis of CA metabolites has primarily been based on HPLC with electrochemical detection (ECD) (5–8) and fluorescence detection (FLD) (9–11) because of their inherent high sensitivity. However, the reliability of HPLC–ECD is compromised because of coeluting interference and electrode fouling and HPLC–FLD generally requires derivatization steps, which is laborious and time-consuming. Alternatively, rapid measurement of some CA metabolites can be achieved by immunoassays. However, cross-reactivity and non-specific binding may lead to false-positive results. Given the limitations of existing analytical techniques, the need for a precise, easy and sensitive method of CA metabolites analysis is undoubtedly clear.

HPLC coupled with mass spectroscopy (HPLC–MS) is a powerful technique that offers excellent specificity and sensitivity to the target. Many studies have been performed using high performance liquid chromatography–tandem mass spectrometry (HPLC–MS-MS) for the analysis of several compounds in the CA family in varied biological fluids (12–16). They used solid-phase extraction (SPE) or online SPE coupled with the HPLC–MS-MS approach provides a sensitive and specific alternative for the quantification of low nanomolar concentrations of free metanephrine and normetanephrine in plasma (13–14). Gu et al. divided 13 CAs and their metabolites into two groups, then developed two rapid and specific methods for their determination in adrenal gland by HPLC–MS-MS (12). However, interfering peaks may occur in the low-m/z region when biological samples are analyzed by MS. Cai et al established pre-column derivatized HPLC–MS-MS methods for the simultaneous determination of CA and their metabolites in human plasma (15) and human urine (16). All the previously mentioned papers used complex pretreatment procedures such as SPE or precolumn derivatization. The limits of detection were at 0.5–70 ng/mL levels (3, 12, 16). Obviously, the sensitivity needs further improvement to get more reliable and precise results because of the ultra-trace levels of CA concentration in biological fluids. Otherwise, the previous papers detected a single biological fluid. In the present work, we
describe a very easy, sensitive and rapid HPLC–MS-MS method for the simultaneous determination of HMPG sulfate, a marker of central norepinephrine metabolite (17), HVA and VMA, both in human urine and serum. The collaborative study of urinary and serum levels of CA metabolites are more reliable and effective for the diagnosis of neurological disorders than that of urinary or serum level alone. After a direct dilution of urine and a protein-preservation pretreatment with serum sample, respectively, the easy and rapid detection goal was realized.

**Experimental**

**Reagents and chemicals**

D.L-4-hydroxy-3-methoxymandelic acid (VMA), HVA and HMPG sulfate potassium salt were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and formic acid were purchased from Merck KGaA (Germany). Ammonium formate of HPLC grade was from Tedia Co.(Fairfield, OH). Two-deionized water was prepared by a Milli-Q system (Millipore, Bedford, MA). Urine samples were supplied by a healthy female volunteer and serum samples of healthy people were obtained from Ningbo First Hospital (China).

**Stock solutions and calibration curve standards**

The stock solutions of VMA, HVA and HMPG sulfate were prepared by dissolving their accurately weighted amounts in two-deionized water. They were stored in darkness at −30°C and brought to room temperature before use. Calibration curve standards were obtained by diluting corresponding stock solutions with two-deionized water, covering concentration ranges for VMA and HVA from 1 to 1,000 ng/mL and HMPG sulfate from 0.2 to 200 ng/mL.

**Pretreatment of urine and serum samples**

Urine and spiked urine samples were diluted 50 times with two-deionized water, then filtered through a 0.20-μm membrane. As-prepared urine samples were directly injected into the LC column for LC–MS-MS analysis.

For serum analysis, 500 μL serum and spiked serum samples were placed in 2.0-mL plastic centrifugation tubes. After adding 200 μL H2O and 1 mL acetonitrile, the resulting mixture was vortex-mixed for 10 min, then left to settle for a while and centrifuged for 3 min at 12,000 × g. The upper phase was transferred, then evaporated to dryness under N2 flow and precipitation was discarded. Finally, the residue was reconstituted with 500 μL two-deionized water and filtered through a 0.20-μm membrane into a clean sample vial before LC–MS-MS analysis.

**Liquid chromatography– mass spectrometry**

All samples were analyzed using a Shimadzu ultra fast liquid chromatography (UFLC) XR liquid chromatography system (Shimadzu Corporation, Japan) coupled with an AB SCIEX Triple QuadTM 5500 mass spectrometer (Applied Biosystems Instrument Corp.).

VMA, HVA and HMPG sulfate were separated on a reversed-phase Waters Xbridge C18 column (150 × 2.1 mm i.d.) with a mobile phase of 15% (v/v) acetonitrile containing 2 mM ammonium formate and 85% (v/v) formic acid solution (0.05%, v/v). The flow rate of the mobile phase was maintained at 0.4 mL/min. The injection volume was 10 μL and the temperature of the column was set at 30°C. Chromatographic separation was followed by negative ion electrospray ionization (ESI) tandem mass spectrometry in the multiple reaction monitoring (MRM) mode. The conditions of the mass spectrometer were as follows: ion-spray voltage, −2.0 kV; curtain gas, 40 psi; collision gas, 7 psi; source temperature, 550°C; ion source gas 1, 50 psi; ion source gas 2, 50 psi; entrance potential (EP), −10V; collision cell exit potential (CXP), −10V.

**Results and Discussion**

**Optimization of mass spectrometric conditions**

The structures and molecular weights of HMPG sulfate, VMA and HVA are shown in Figure 1. These three CA metabolites can be deprotonated in the negative mode and protonated in the positive mode. However, [M-H]− ions showed more intense abundance than [M+H]+ ions in their corresponding mode. The LC–MS–MS method excels in specificity and sensitivity for qualitative and quantitative analysis compared with LC–MS because it monitors ion transitions using MRM. Therefore, we chose negative ESI–LC–MS–MS in MRM mode to detect our targets.

When giving a moderate, proper collision energy in specific MS conditions, [HVA-H]- lose CO2, giving m/z 137 fragment. In the case of [VMA-H]-, the loss of CO2 is followed by the loss of methane (18). For MHPG, the simultaneous losses of SO3 and H2O result in an elimination of 98Da from [MHPG sulfate]. Table I clearly shows the transitions from precursor ions to product ions of the mixture under the MRM mode for quantification of the targets. In the case of HVA, the transition of 180.9/122.0 was used for quantification instead of the abundant transition 180.9/137.0 because the adopted mobile phase shows much background noise at the transition of 180.9/137.0. The signal-to-noise (S/N) value of the 180.9/122.0 transition was more sensitive than that of the 180.9/137.0 transition under the specific chromatographic conditions.

**Optimization of chromatographic conditions**

A short reversed-phase Waters Xbridge C18 column was chosen for the rapid separation of targeted CA metabolites. The Shimadzu UFLC XR liquid chromatography system can provide much higher pressure, up to 9,300 psi, to shorten analysis time and achieve high separation performance. Because acetonitrile offers lower noise and formic acid is compatible with ESI, both were chosen as mobile phase components.

Three targets, except HMPG sulfate, have carboxylic groups and can be easily deprotonated in neutral or basic environment. To achieve better interaction with the column and reduce interfering peaks, an acidic mobile phase; that is, 0.05% formic acid solution, was chosen. We studied the effect of 0.05% formic acid solution on the retention time and peak intensity, ranging the percentage in the mobile phase from 20 to 95%. As a result, the retention times of HMPG sulfate, HVA and VMA were shorter than 1.3 min and the peaks were overlapped to some extent at small formic acid content (<30%). It may be difficult to distinguish the targets from interfering peaks in
complicated matrix such as urine and serum. Retention time and separating degree of the targets was increased by improving the content of formic acid solution, but the peak intensity of HVA decreased extensively. When the formic acid content exceeded 90%, the latest-eluted peak (HVA) got broader and smaller and the peak intensities of other analytes also changed. Finally, 85% formic acid solution in the mobile phase was chosen to achieve better separation performance and sensitivity.

Besides addition of ammonium formate to the organic mobile phase, acetonitrile was examined to investigate how the intensities and retention times of the targets change. It was found that the HVA peak got sharper and nicer and the retention times of the targets remained unchanged when the molarity of ammonium formate was 2 mM. To increase HVA sensitivity, 15% (v/v) acetonitrile containing 2 mM ammonium formate was used as organic mobile phase, although the signals of HMPG sulfate and VMA decreased slightly under these conditions. Representative extract ion chromatograms of standard mixture containing HMPG sulfate, VMA and HVA (in order of elution) in the negative ESI MRM mode under the optimized conditions are shown in Figure 2.

Table I

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Transitions</th>
<th>DP (V)</th>
<th>CE (V)</th>
<th>Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPG sulfate</td>
<td>263.0 → 165.0</td>
<td>-140</td>
<td>-25, -35, -20</td>
<td>165.0, 150.0, 183.0</td>
</tr>
<tr>
<td>HVA</td>
<td>180.9 → 122.0</td>
<td>-90</td>
<td>-10, -20</td>
<td>137.0, 122.0</td>
</tr>
<tr>
<td>VMA</td>
<td>197.0 → 137.0</td>
<td>-50</td>
<td>-30, -16</td>
<td>137.0, 138.0</td>
</tr>
</tbody>
</table>

*DP, declustering potential; CE, collision energy.

Figure 1. Structures and molecular weights of the targets.

Linearity and limits of detection

Quantification was performed using the external standard method. Calibration curves were constructed based on relative peak intensities of signal to noise. Linearity was excellent over calibration range from 1.0 to 1,000 ng/mL with correlation coefficient ($R^2 > 0.999$) for VMA and HVA, and 0.2 to 200.0 ng/mL for HMPG sulfate.

The limit of detection (LOD) was defined as the injected amount that produced a signal 3-fold higher than the noise. We analyzed a series of gradually diluted standard mixtures until the S/N value of each analyte approached 3 (12). The LODs reflect instrument sensitivity rather than method sensitivity. Matrix effects in real samples and the loss of analytes during sample preparation were not considered. A matrix effect study and recovery test of our method were also performed. The results suggested that recovery rates were acceptable both in urine and serum, and matrix effects can be taken as negligible except for VMA in serum. Considering the negligible matrix effects, we take LODs of standard solutions as the LODs in the real samples. From this opinion, the LODs in urine and serum were estimated to be 0.2 ng/mL for VMA, 0.03 ng/mL for HMPG sulfate and 0.7 ng/mL for HVA. The linearity equations, calibration ranges, correlation coefficients and LODs for all targets are presented in Table II. The present LC–MS-MS method was more sensitive than those in other published articles (3, 12, 16).

Recovery and precision

Spiked urine samples at three different concentration levels (low, middle and high) were prepared by adding appropriate standard mixtures to urine. The amount of each analyte thus calculated by the linear regression equation, minus the background value in the matrix, was compared to the former fortified amount, and the recovery rate of the method was obtained. Table III shows the detailed recovery rates and precision results. The recovery rates of all the targets are
satisfactory. The results also reflect that matrix effects of urine are negligible after 50-times dilution. Slight ionic enhancement to HMPG sulfate and small ionic suppression to VMA and HVA are observed, which does not affect the determination of the targets. For HVA, the choice of 180.9/122.0 transition as quantitation pair achieved a recovery rate of 89% better than that of 180.9/137.0. In addition, precision of the method was determined by analyzing six parallel urine and spiked urine samples after pretreatment as mentioned. As a result, the precisions (relative standard deviations; RSDs) were lower than 5.7% for all the compounds.

To serum, recovery rates were estimated by measuring the peak areas of processed serum spiked with a known concentration of targets against those of corresponding concentration of targets added in the processed serum. After pretreatment as mentioned. As a result, the precisions (relative standard deviations; RSDs) were lower than 5.7% for all the compounds.

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Matrix effect

The extent of matrix effect can be assessed by standard addition, sample clean-up, post-column infusion, matrix-matched standards and internal standards (19). Standard addition method was applied to investigate the matrix effects of urine and serum. In the analysis of urine, matrix effects on all the targets are negligible after 50-times dilution. It has also been reported that dilution of matrix extract help to reduce matrix interferences (20–21). With regard to serum, matrix effects were investigated by comparing signals of standard substances added to the blank serum extract with the signals of standard solution at the same concentration of 10.0 ng/mL described in six parallel experiments were lower than 4.9%. Additionally, the intra-day and inter-day precisions of the urine and serum analysis were satisfactory less than 8.7% (N = 6).

Table II
Analytical Performance of the Developed Method*

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear regression equation</th>
<th>Calibration range (ng/mL)</th>
<th>Number of calibration points</th>
<th>Correlation coefficient</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVA</td>
<td>$y = 1.5 \times 104x + 6.59 \times 103$</td>
<td>1.0–1,000</td>
<td>8</td>
<td>0.9999</td>
<td>0.7</td>
</tr>
<tr>
<td>VMA</td>
<td>$y = 1.94 \times 105x + 1.27 \times 104$</td>
<td>1.0–1,000</td>
<td>8</td>
<td>0.9993</td>
<td>0.2</td>
</tr>
<tr>
<td>HMPG sulfate</td>
<td>$y = 5.45 \times 104x - 0.2$</td>
<td>2.0–200</td>
<td>7</td>
<td>0.9996</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*x = the concentration of analyte; y = the peak area of analyte.

Table III
Recoveries and Precision Results of Spiked Urine Sample

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Background value (ng/mL)</th>
<th>Fortified value (ng/mL)</th>
<th>Average recovery rate (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPG sulfate</td>
<td>121.4</td>
<td>4</td>
<td>105%</td>
<td>3.8</td>
</tr>
<tr>
<td>VMA</td>
<td>255.2</td>
<td>20</td>
<td>86%</td>
<td>4.3</td>
</tr>
<tr>
<td>HVA</td>
<td>433.3</td>
<td>50</td>
<td>89%</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*x = the concentration of analyte; y = the peak area of analyte.

Figure 2. Representative extract ion chromatograms of standard mixture in the negative ion electrospray MRM mode. Concentrations of HVA, VMA and HMPG sulfate were 100, 100 and 20 ng/mL. Separation conditions: separation column, reversed-phase Waters Xbridge C18 column (150 x 2.1mm i.d.) with mobile phase of 15% (v/v) acetonitrile containing 2 mM ammonium formate and 85% (v/v) formic acid solution (0.05%, v/v); flow rate, 0.4 mL/min.
the article (22); a kind of standard addition method. However,
because HMPG sulfate, HVA and VMA are endogenous sub-
stances, a real blank extract is impossible to get. Therefore, the
signals of standards added to the blank serum extract are
obtained by subtraction of native background signals from total
responses of spiked serum. The matrix effects of serum were
shown in Figure 3. The figure shows that matrix effects to
three targets except VMA are ignorable after the pretreatment
process. Almost 60% of the VMA signal was lost by the ionic
suppression.

Applications
The present LC–MS-MS method was applied to detect CA
metabolites, VMA, HVA and HMPG sulfate in human urine and
serum. Quantifications were performed using standard curve,
except serum VMA.

To urine sample, 50-times diluted samples were directly
injected for analysis without complex manipulation. According
to Commission Decision 2002/657/EC, a minimum of four
identification points are required for confirmation of com-
pound identity in LC–MS methods (22). In this study, four
identification points for each target were obtained as 1.0 point
for one precursor ion and 3.0 points for two daughter ions.
Along with retention time and standard addition method, char-
acteristicfragmentations were used together to identify the
targets. The details of these detected compounds are clearly
shown in Figure 4. In the case of HVA, interfering peaks oc-
curred in the low-m/z region of urine matrix when using MRM
mode. However, two characteristic fragmentations of HVA (m/
2137.0, m/z 122.0) appeared at exactly the retention time of
the standard. The prolonged retention time of HVA facilitated
the selectivity from urine matrix disturbance. Under the opti-
mized conditions, three targets were selectively detected
without disturbance. The concentrations of VMA, HVA and
HMPG sulfate detected in the urine sample were calculated to
be 2.55, 4.33 and 1.21 μg/mL, respectively. The results were
consistent with published papers (3, 17, 23).

To serum, samples were processed as mentioned previously.
Considering the significant matrix effect to VMA, the concen-
trations of detected VMA present in serum were calculated
based on the ratio of response generated from serum sample to
fortified serum sample of a close concentration. The typical

Figure 3. Matrix effect of serum. Concentrations of three targets were 10 ng/mL.

Figure 4. Extract ion chromatograms of detected compounds in 50-times diluted urine sample under optimal conditions (the same as in Figure 2).
extract ion chromatograms of detected CA metabolites in serum and fortified serum are shown in Figure 5. Serum matrix exhibited some interference peaks toward HMPG and VMA, but HMPG sulfate can be identified by the fragmentations ($m/z$ 165.0, $m/z$ 150.0), and VMA can be identified by the typical retention time, both using the standard addition method. Excellent selectivity is a benefit of using the HPLC–MS-MS method. Concentrations of VMA, HVA and HMPG sulfate detected in the serum were calculated as 3.3, 10.9 and 3.1 ng/mL, respectively. Tang et al. (4) found that serum concentrations of VMA, HVA and HMPG sulfate of healthy people were 3.14 ± 2.63, 12.58 ± 6.91 and <28.16 (detection limit) ng/mL, respectively. Our results were in good agreement with their findings. Furthermore, the sensitivity of this developed method exceeded that of Tang’s method.

Conclusions

We proposed an ultrasensitive and easy analytical method for determination of three CA metabolites (VMA, HVA and HMPG sulfate) in both human urine and serum using UFLC–MS-MS technique in negative MRM mode. Determination of urine levels of CA metabolites has been accepted as a general method for the diagnosis of some neurological disorders, but urinary CA metabolite levels are affected by kidney function. When patients suffer from renal inadequacy, CA metabolites accumulate in the serum (4), so the diagnosis of neuroblastoma is more reliable based on simultaneous determination of urine and serum VMA, HVA and HMPG levels. The present method provides higher sensitivity, easier manipulation and shorter analysis time than other existing methods. For example, our method greatly exceeds in the detection sensitivities of three targets compared to others, because their LODs were in the range of 0.5–70 ng/mL (3, 12, 16). In addition, our method does not require an on-line or off-line SPE process in serum analysis, unlike other methods described in the literature (2, 13), but does not sacrifice precision and feasibility. In the case of HVA, the 180.9/122.0 transition was creatively chosen for quantitation instead of the than normal quantitative 180.9/137.0 transition to reduce background interference. In summary, the proposed LC–MS-MS method is sensitive, easy, rapid and efficient for the analysis of three targeted CA metabolites in human urine and serum. The cooperative urinary and serum levels of CA metabolites obtained by this general method are more helpful and effective for the diagnosis of neurological disorders than urinary or serum levels alone.

Acknowledgments

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References