Simultaneous Determination of All Forms of Biopterin and Neopterin in Cerebrospinal Fluid

Pierre Guibal, Nathalie Lévêque, Diane Doummar, Nicolas Giraud, Emmanuel Roze, Diana Rodriguez, Rémy Couderc, Thierry Billette De Villemeur, and Fathi Moussa

ABSTRACT: In humans, genetic defects of the synthesis or regeneration of tetrahydrobiopterin (BH4), an essential cofactor in hydroxylation reactions, are associated with severe neurological disorders. The diagnosis of these conditions relies on the determination of BH4, dihydrobiopterin (BH2), and dihydroneopterin (NH2) in cerebrospinal fluid (CSF). As MS/MS is less sensitive than fluorescence detection (FD) for this purpose, the most widely used method since 1980 involves two HPLC runs including two differential off-line chemical oxidation procedures aiming to transform the reduced pterins into their fully oxidized fluorescent counterparts, biopterin (B) and neopterin (N). However, this tedious and time-consuming two-step indirect method underestimates BH4, BH2, and NH2 concentrations. Direct quantification of BH4 is essential for studying its metabolism and for monitoring the efficacy of BH4 supplementation in patients with genetic defects. Here we describe a single step method to simultaneously measure BH4, BH2, B, NH2, and N in CSF by HPLC coupled to FD after postcolumn coulometric oxidation. All target pterins were quantified in CSF with a small volume (100 μL), and a single filtration step for sample preparation and analysis. As compared to the most widely used method in more than 100 CSF samples, this new assay is the easiest route for accurately determining in a single run BH4, BH2, and NH2 in CSF in deficit situations as well as for monitoring the efficacy of the treatment.

KEYWORDS: Biopterin, neopterin, hydroxylases, cerebrospinal fluid, metabolism, neurologic disorders

In mammals, tetrahydrobiopterin (BH4) (Figure 1) is an essential cofactor for aromatic amino acid hydroxylases, glyceryl-ether mono-oxygenase, and three nitric oxide synthases. Thus, BH4 plays an important role in monoamine neurotransmitters metabolism. In humans, defects in the metabolism or recycling of BH4 (Figure 1) leading to BH4 deficiency are associated with neurological deterioration including progressive mental and physical retardation, central hypotonia and peripheral spasticity, seizures, and microencephaly. Lack of BH4 synthesis or regeneration can be due to one of five known inborn errors of metabolism leading to the deficiency of five enzymes (Figure 1). The diagnosis of these conditions relies on the accurate determination of neurotransmitters and pterins, notably BH4, dihydrobiopterin (BH2), and dihydroneopterin (NH2) in the cerebrospinal fluid (CSF).

As oxidized pterins are fluorescent while their reduced counterparts are not, the most widely used method for pterin determination in CSF requires carrying out two HPLC (high performance liquid chromatography) runs coupled to fluorescence detection (FD) including two off-line iodine oxidation procedures for complete analysis. The first run involves oxidation under acidic conditions of both BH4 and BH2 into biopterin (B), and NH2 into neopterin (N) prior to injection into the chromatograph. The second run involves the oxidation under alkaline conditions of BH2 into B and BH4 into pterin (P). Although time-consuming, this indirect chemical oxidation (ICO) method has been widely used since 1980, notably because it has appropriate sensitivity. Total B can be also determined after MnO2 oxidation but this method does not allow distinguishing BH4 from BH2.
Direct quantification of BH4, BH2, and NH2 is essential for studying BH4 and neurotransmitter metabolism (Figure 1).

This area of research is very active as reflected in a recent description of an unknown genetic disorder of neurotransmitter translocation into synaptic vesicles.9 Besides, as BH4 is used as a therapeutic agent in patients with BH4 deficiency,7,10 a direct and accurate method of BH4 quantification in biological fluids and tissues is needed for pharmacokinetic studies and for determining the efficacy of the treatment. Notably, two recent papers raised doubts about the ability of BH4 at usual doses (20 or 40 mg/kg/day) to cross the blood brain barrier in patients with genetic BH4 recycling disorder.11,12 In such situations, only treatments with neurotransmitter precursors can induce a significant change of the CSF neurotransmitters profile.11,12

Some methods of direct quantification through FD after chemical or photochemical postcolumn oxidation have been implemented.13−17 However, the sensitivities of these methods were not satisfactory as compared to those of ICO methods.5−8

More recently, LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) was used to determine B concentrations in plasma by applying an oxidation conversion ratio.18 An enzymatic approach was also developed to distinguish BH4 from B with a two-step procedure.19 These indirect methods requiring two run procedures are still tedious and time-consuming. LC-MS/MS was also used for the simultaneous detection of BH4, BH2, and B in urine samples, cell extracts, and rat brains.20−22 However, according to the authors, MS/MS detection is less sensitive than FD for pterin quantification.22

In the mid-1980s, sequential electrochemical detection (ECD) and FD were proposed for the simultaneous quantification of BH4 and BH2 in CSF.23,24 BH4 was detected electrochemically, while BH2 was detected by FD after postcolumn coulometric oxidation (PCCO-FD) into B. Due to the large number of electroactive compounds in CSF, detecting specifically BH4 by ECD is a challenge. Actually, ECD is less robust than FD for routine analyses,8 besides this method does not allow the detection of N and NH2.

Figure 1. Metabolism pathway of tetrahydrobiopterin (BH4). N (neopterin); B (bioperin); BH2 (dihydrobiopterin); NH2 (dihydroneopterin). GTP (guanosine triphosphate); GTPCH (guanosine triphosphate cyclohydrolase EC 3.5.4.16); PTPS (6-pyruvoyl-tetrahydropterin synthase EC 4.2.3.12); SR (sepiapterin reductase EC 1.1.1.153); PCD (pterin-4-carbinolamine dehydratase EC 3.5.4.16); DHPR (dihydropteridin reductase EC 1.5.1.34). (Dashed arrows: nonenzymatic.)

Figure 2. Pterin separation as a function of stationary phase and pH. (a) Atlantis dC18 (NBH2 = 19 230); (b) Eclipse XDB C18 (NBH2 = 11 510); (c) XTerra (NBH2 = 2080); (d) Zic-HILIC (N_{NH2} = 16 250). N_{BH2} and N_{NH2} = theoretical plate number for BH2 or NH2, respectively. Mobile phase for panels (a)−(c): pH 7.4, 0.05 M sodium citrate/methanol (97/3, v/v). Mobile phase for panel (d): pH 7.4, 0.2 M ammonium formiate/acetoniitrile (20/80, v/v). Flow rate 0.6 mL/min at 30 °C and UV detection at 260 nm for all columns (AU = absorbance units).
Here we focus on the effects of pH on the separation of the target pterins as a function of the stationary phase and on their electro-oxidation to develop a single method capable of simultaneous determination of all forms of target pterins present in CSF samples by HPLC coupled to PCCO-FD with a single filtration step for sample preparation and analysis.

For comparison, the proposed method was applied in parallel with the most widely used ICO method to analyze CSF samples collected from infants, children, and young patients aged 2 months to 20 years (median 5.8 years, \(n = 94\)) and adults aged from 20 to 41 years (median 30.8 years, \(n = 5\)) with a normal metabolic profile. We also included samples from patients aged 2 months to 38 years (median 15.6, \(n = 9\)) with defects in pterin metabolism, including a known sepiapterin reductase deficiency (SR), an amino acid decarboxylase deficiency (AADC), a tyrosine hydroxylase deficiency (TH), a secondary biopterin deficiency, and five cases of neopterin increase of different origins.

**RESULTS AND DISCUSSION**

**Characterization of Standard Pterins.** As most standards are reduced species, which are prone to oxidation, we systematically checked their identities by high resolution mass spectrometry (HRMS). We also checked the structure of BH4, the least stable standard, by NMR.

MS experiments confirmed the \(m/z\) ratios of all standards. The most abundant protonated ions in the spectra of BH4, BH2, and B were at \(m/z\) 242.1244, 240.1094, and 238.0942, respectively. As for NH2 and N, the most abundant protonated ions were at \(m/z\) 256.1037 and 254.0885, respectively.

\(^1H\) NMR, and \(^1H–^{13}C\) heteronuclear correlation NMR confirmed the structure of BH4 \(\{^1H\) NMR (400 MHz, D2O) \(\delta\) ppm: \(1.06 (d, J = 4.3 Hz, 3H, CH_3), 3.32 (dd, J = 13.7 Hz, \(J' = 9.7 Hz, 1H, CH_2^{N'}), 3.51 (dd, J = 13.8 Hz, \(J' = 4.0 Hz, 1H, CH_2^{N'}), 3.57 (m, 2H, CH_3CH_2), 3.58 (m, 1H, CH_3)\}; and \(^{13}C\) NMR (400 MHz, D2O), \(^{13}C\) edited HSQC and \(^1H–^{1}H\) COSY 2D spectra \(\delta\) ppm: 19.5 (1C, C\(_3\)H), 36.8 (1C, C\(_2\)H\(_2\)), 53.5 (1C, C\(_4\)H), 68.0 (1C, C\(_4\)H\(_3\)). Typical correlation patterns were observed on \(^1H–^{13}C\) edited HSQC and \(^1H–^{1}H\) COSY 2D spectra, indicating the presence of a fragment \(H_2C–C_6H–C_6H–C_6H–C_6H=\) that can be unambiguously assigned to the structure of BH4 (Figure 1).

**Method Development.** Figure 2 shows UV–visible chromatographic profiles of a standard solution of the target pterins obtained on different stationary phases. To evaluate the efficiency of each column, we determined the \(N\) value (number of theoretical plates) for the last eluting compound in each chromatogram. \(N\) is then used to calculate \(h\) [reduced plate height \(h = H/d_p\)], where \(H\) is the theoretical plate height (column length/\(N\)) and \(d_p\) is the particle diameter both expressed in the same length unit], with \(h\) comparing efficiencies of different columns with different geometries filled with different particle sizes.

As the target pterins are basic compounds with low hydrophobicity, we used a polar embedded C\(_{18}\) column (Atlantis) to separate them without using an ion pairing reagent. At pH 7.4, the Atlantis column showed better efficiency \(h_{BH2/4} = 3.6\) (Figure 2a) than the Eclipse XDB column designed for reverse-phase separation of basic compounds \(h_{BH2/4} = 4.5\) (Figure 2b).

To investigate the effects of pH on pterin separation and detection between pH 2 and 10, we used the X Terra column filled with hybrid particles having a widened useable pH range.

As expected in HILIC (hydrophilic interaction liquid chromatography) separations, the ZIC-HILIC column showed different selectivity as compared to RP-columns (Figure 2d). While this column showed high efficiency for the standard mixture \(h_{BH2} = 2.65\) due to solvent-mobile phase incompatibility (data not shown). Hence, further studies were carried out on the Atlantis column (Figure 2b).

Figure 3 shows the hydrodynamic voltammograms and the oxidation yields as a function of pH of the target pterins as determined by HPLC-PCCO-FD. While high oxidation yields (\(>50\%\)) of BH2 and NH2 can be obtained irrespective of the pH range between pH 3 and pH 8, a sufficient yield...
In agreement with previous observations, attributed to this oxidized form because it was not detected after PCCO (Figure 4c). The X2 unidentified peak eluting at 13.5 min corresponding to the retention time of B cannot be attributed to this oxidized form because it was not detected without oxidation (Figure 4b1, dashed line). As this interfering peak was detected in all analyzed samples after PCCO-FD, we investigated its electrochemical and fluorescence behavior.

Figure 3a shows that while the PCCO product of X2 was fluorescent at an electrode potential lower than 500 mV, further oxidation at higher potentials results in decreased fluorescence intensity. At an electrode potential of +600 mV, this interference was no longer detected under our experimental conditions (Figure 4d). Thus, to avoid this interference, we decided to oxidize the effluent at 600 mV for pterin analyses. At this potential, the chromatographic profile of the same pooled CSF sample clearly shows that the three circulating forms of pterins are well separated and detected without interfering peaks (Figure 4d).

**Proposed Method.** Considered together, these results led us to adopt the following chromatographic conditions: separations were performed on an Atlantis dC18 (4.6 × 150 mm, 3 μm) column with a mobile phase consisting of pH 7.4, 0.05 M sodium citrate/methanol (97/3, v/v) delivered at a flow rate of 0.6 mL/min at 30 °C. The fluorimetric detection was performed at λex 350 nm and λem 450 nm after coulometric oxidation at +600 mV. The final run time was 16 min.

**Method Evaluation.** The identification of pterins in authentic CSF samples was based on their retention factors, increases in peak areas after spiking with known amounts of each pterin, and on electrochemical and fluorescence properties. The stability of the samples at 10 °C in the dark in the injector was checked after several hours. We did not observe significant differences after 6 h. After 8 h, NH2 and BH4 concentrations decreased between 4% and 31% of their initial values (n = 10, data not shown). Thus, using this method, samples should be analyzed not more than 6 h.

We checked the stability of pterins in CSF samples (n = 40) frozen at −80 °C immediately after collection. As compared with the initial values determined at day 7 after collection,
we did not observe significant differences after 24 months of storage. Thus, we avoided adding a preservative agent to the samples facilitating the collection procedure.

The comparison of within-run data between artificial and authentic CSF samples, spiked under the same conditions with each pterin, showed no significant differences between the two matrices (Table S-1, Supporting Information). The recoveries were equal to or higher than 88% irrespective of pterin concentration (Table S-1). Thus, we used an artificial CSF rather than authentic pooled CSF samples for method calibration.

The method was linear for all five pterins, up to 100 nmol L\(^{-1}\), which allowed direct BH4 determination in pharmacokinetic studies. The LOQs (limits of quantification) enabled the determination of all pterins even in deficit situations, and the CV (coefficient of variation) of the method never exceeded 9.9% (Table S-1).

As the sample preparation consisted of a single dilution in the mobile phase before protein filtration, and as precision was equal to or better than 9.9% in all instances (Table S-1), we did not use an internal standard for method validation. However, in clinical practice, there is a need for internal standards, thus we included lumazine as an internal standard (IS).

## Table 1

<table>
<thead>
<tr>
<th>Pterin</th>
<th>Concentration (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH4</td>
<td>0.5</td>
</tr>
<tr>
<td>BH2</td>
<td>1.0</td>
</tr>
<tr>
<td>NH2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

## Figure 5

Chromatographic profiles of a standard mixture and a pooled CSF sample \((n = 5)\) under the proposed chromatographic conditions: column = Atlantis dC18 (4.6 × 150 mm, 3 μm); mobile phase: pH 7.4, 0.05 M sodium citrate/methanol (97/3, v/v); flow rate: 0.6 mL/min at 30 °C; postcolumn oxidation at +600 mV detection: λ\(_{ex}\) 350 nm, λ\(_{em}\) 450 nm (FU: fluorescence units (arbitrary units)). (a) Standard mixture; (b) pooled CSF without internal standard; (c) pooled CSF with lumazine as internal standard (IS).
Table 1. Pterin Concentrations (nmol/L) in Human CSF As Determined by the Proposed Method, Categorized by Age and Sex (M: male; F: female)

<table>
<thead>
<tr>
<th>age (years)</th>
<th>N</th>
<th>M</th>
<th>F</th>
<th>mean</th>
<th>SD</th>
<th>med</th>
<th>max</th>
<th>min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–0.33</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>61.3</td>
<td>19.2</td>
<td>61.3</td>
<td>74.9</td>
<td>47.7</td>
</tr>
<tr>
<td>0.34–0.66</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>39.7</td>
<td>18.2</td>
<td>37.5</td>
<td>64.0</td>
<td>19.8</td>
</tr>
<tr>
<td>0.67–1.00</td>
<td>6</td>
<td>5</td>
<td>1</td>
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<td>46.8</td>
<td>63.9</td>
<td>28.6</td>
</tr>
<tr>
<td>1.1–5.00</td>
<td>29</td>
<td>13</td>
<td>16</td>
<td>33.6</td>
<td>13.2</td>
<td>33.4</td>
<td>76.3</td>
<td>12.8</td>
</tr>
<tr>
<td>5.001–20</td>
<td>53</td>
<td>27</td>
<td>26</td>
<td>31.7</td>
<td>11.9</td>
<td>30.8</td>
<td>61.1</td>
<td>7.6</td>
</tr>
<tr>
<td>20–42</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>16.1</td>
<td>3.5</td>
<td>14.7</td>
<td>20.8</td>
<td>12.3</td>
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<tr>
<td>all</td>
<td>99</td>
<td>54</td>
<td>45</td>
<td>33.3</td>
<td>13.9</td>
<td>32.3</td>
<td>76.3</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Although the number of samples is not sufficient to establish definitive normative values according to age and sex (Figure 6a), the average concentrations were close to those obtained by Hyland et al.26 However, our results were systematically higher than those obtained by HPLC after either MnO2 or I2 ICO.25 When we compared the mean values, they were 38% and 67% higher than those reported by Komori et al.6 and Ormazabal et al., respectively. When we compared the median values, they were 98% higher than those observed by Verbeek et al.25

However, ICO methods are known to underestimate amounts of reduced forms of pterins. The calculated oxidation yields were approximately 63% for BH4 and BH2 and only about 41% for NH2, suggesting that the oxidation mechanism of reduced forms of pterins leads to a more complex mixture than a simple oxidation into B and N. As no other peaks were detected by fluorescence at the usual emission and excitation wavelengths, we assume that BH2, BH4, and NH2 might also give rise to nonfluorescent byproducts after chemical oxidation. These results agree with those previously observed.16,22

Figure 6c shows that using reduced forms of pterins to calibrate B and N determinations can attenuate the differences with the proposed PCCO-FD method. Nevertheless, as already reported23 within-run CVs for ICO are high irrespective of pterin concentration, they ranged from 6.4% to 35% (n = 6) under our experimental conditions.

Finally, Figure 8 shows the feasibility of the translation of the proposed method for the diagnosis of pterin metabolic disorders. Figure 8c shows the chromatographic profiles of CSF samples from patients with defects of pterin metabolism. The first panel (Figure 7a) shows the chromatogram of a CSF sample from a patient with a TH deficiency exhibiting a normal pterin profile. Figure 7b and c shows chromatograms associated with an AADC deficiency. It is noteworthy that this profile with normal pterin levels exhibited two additional intense peaks corresponding to 3 OMD (3-ortho-methyl-dihydroxyphenylalanine) and 5 OHT (5-hydroxytryptophan) in the presence of severe HVA (Homovanillic acid) and 5 HIAA (5-hydroxy-indole-acetic acid) deficiencies, which makes it characteristic of AADC deficiency. Panel (d) shows the chromatographic profile of a known SR deficiency.11,28 This chromatogram clearly shows the absence of BH4 accompanied by an elevated level of BH2. The chromatographic profile of panel (e) shows secondary BH4 and BH2 deficiencies in an adult suffering from extrapyramidal symptoms of unknown causes. In this patient, the NH2 CSF level was normal while the dopaminergic and serotoninergic symptoms of unknown causes. In this patient, the NH2 CSF level was normal while the dopaminergic and serotoninergic syndromes of unknown causes. In this patient, the NH2 CSF level was normal while the dopaminergic and serotoninergic syndromes of unknown causes. In this patient, the NH2 CSF level was normal while the dopaminergic and serotoninergic syndromes of unknown causes. In this patient, the NH2 CSF level was normal while the dopaminergic and serotoninergic syndromes of unknown causes. In this patient, the NH2 CSF level was normal while the dopaminergic and serotoninergic syndromes of unknown causes.

Taken together, these examples highlight the applicability of the proposed method for the diagnosis of pterin metabolic disorders.

Finally, Figure 8 shows the feasibility of the translation of the proposed method to ultrahigh performance liquid chromatography (UHPLC). This technique has been recently proposed...
for the quantification of neurotransmitters in microdialysis fractions.\textsuperscript{30} Due to extra-column effects, the separation is less efficient (\(h_{\text{BH2}} = 12\)) than that obtained with the conventional HPLC system. Nevertheless, if we assume that a resolution of about 1.3 between the unidentified peak X\(_1\) and BH4 is acceptable, the run time is shortened to about 3.5 min, which allows analyzing more than 100 samples during the stability period (6 h). Additional optimization and validation of this UHPLC method are in progress.

\section*{CONCLUSIONS}

An HPLC method for the simultaneous determination of circulating forms of biopterin and neopterin in cerebrospinal fluid is presented. The use of an embedded-polar-group bonded phase (Atlantis) allows separation of the target pterins without using an ion pairing reagent. Setting the pH of the mobile phase at 7.4 allows the postcolumn coulometric oxidation of the reduced pterin forms enabling their simultaneous determination by fluorescence detection.

All target pterins were quantified in a small volume (100 \(\mu\)L) of CSF using a single filtration step for sample preparation and analysis.
As the samples are stable for a maximum of 6 h in an autosampler at 10 °C and as the run time is 16 min (20 min for AADC deficiency), the number of samples including calibrators and quality control that can be analyzed per run is 20. Vitamin C or dithiothreitol (DTT) are usually added in large excess to stabilize BH4 in biological media.22 While vitamin C had no effect on BH4 stability under our chromatographic conditions, DTT entailed a significant increase of BH4 stability up to 10 h. However, this lengthened the run time by 14 min because 30 min was necessary to completely elute DTT. The translation of the proposed method to UHPLC is one avenue to improve overall throughput of this method.

■ METHODS

Chemicals and Reagents. All reagents were purchased from Sigma (Saint-Quentin Fallavier, France) and were used without further purification.

Patient Samples. CSF samples were collected by lumbar puncture from patients suffering several neurological disorders with initially unknown etiology including movement disorders with or without encephalopathy, epileptic or neurodegenerative encephalopathy, and unknown etiology including movement disorders with or without encephalopathy, epileptic or neurodegenerative encephalopathy, and mening-encephalitis.

Lumbar punctures were performed at the Department of Pediatric Neurology of the Hôpital Trouseau (Paris, France) as part of normal clinical management with the written informed consent of parents or legal representatives of each patient. This study using only CSF sample residues was performed according to French Public health regulations (Code de la santé publique - Article L1121-3, modified by Law n°2011-2012, December 29 2011 - Article 5). Samples were collected in five fractions of 0.5 mL each. The first three fractions were processed for quantitative bacterial culture and routine biochemical tests, including amino acids, lactate, and pyruvate determinations. The two remaining fractions devoted to neurotransmitters, folate, and pterin metabolism were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. To avoid a possible effect of the ventriculo-spinal gradient on CSF parameters, all pterin investigations were performed on the fourth fraction.

Exclusion criteria were traumatic punctures, inadequate collection and preservation of the samples, and L-dopa and BH4 treatment. Determinations of 5-methyltetrahydrofolate by HPLC-FD25 and of RNA in the proposed method to UHPLC is one avenue to improve overall throughput of this method.

■ ASSOCIATED CONTENT

Supporting Information

Additional experimental data on method validation. This material is available free of charge via Internet at http://pubs.acs.org.

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Author Contributions

Pierre Guibal, Nathalie Lévêque, and Nicolas Giraud performed research and analyzed data. Diane Doummar, Emmanuel Roze, Diana Rodrigues, and Thierry Billette De Villemeur managed children and collected CSF samples. Remy Courdev provided material support. Fathi Moussa designed research and analyzed data. Pierre Guibal and Fathi Moussa wrote the paper. All authors discussed the results and commented on the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; NH2, dihydronicotinamide; CSF, cerebrospinal fluid; HPLC, high performance liquid chromatography; B, bipterin; N, neopterin; P, pterin; ICD, indirect chemical oxidation; FD, fluorescence detection; ECD, electrochemical detection; PCCO-FD, postcolumn coulometric oxidation-fluorescence detection; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; SR, sepiapterin reductase; AADC, alpha-dihydroxyphenylalanine; TH, tyrosine hydroxylase; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance; HILIC, hydrophilic interaction liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; CV, coefficient of variation; 3 OMD, 3 ortho-methyl dopa; 5 OHT, 5 hydroxy tryptophan; HVA, homovanillic acid; 5 HIAA, 5 hydroxy indole acetic acid; UHPLC, ultra high performance chromatography; QC, quality control; DTT, dithiothreitol.
REFERENCES


