Original article

Simultaneous assay of urine sepiapterin and creatinine in patients with sepiapterin reductase deficiency

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1. Introduction

Sepiapterin reductase deficiency (SRD, MIM #612716) is a very rare inherited metabolic disease caused by biallelic pathogenic mutations in the *SPR* gene [1]. There are only a few dozen case reports in the world [2]. The *SPR* gene encodes sepiapterin reductase (SR, EC 1.1.1.153), which plays an important role in the biosynthesis of tetrahydrobiopterin (BH_4) [3]. BH_4 is the essential cofactor of aromatic amino acid hydroxylases, which include phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase. Therefore, decreased BH_4 results in the depletion of dopamine and serotonin. Patients with SRD have various central nervous system symptoms, such as global developmental delay, dystonia, oculogyric crisis, and diurnal variation of symptoms [4]. In infancy, patients often present nonspecific features and are therefore frequently misdiagnosed with cerebral palsy [2].

Since most SRD patients exhibit dramatic improvement in neurological symptoms with L-DOPA and 5-hydroxytryptophan supplementation [5,6], it would be ideal if this disorder could be screened for in patients with unexplained neurological symptoms. However, SRD does not cause hyperphenylalaninemia, as seen in many disorders involving BH_4 metabolism, and cannot be detected by newborn screening [7]. In the past, in order to diagnose SRD, the assessment of biogenic amines and pterins in the cerebrospinal fluid (CSF) or *SPR* gene sequencing was considered necessary. SRD patients have low concentrations of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), high concentrations of biopterin and sepiapterin (Sp) and normal concentrations of neopterin in the CSF [8]. However, lumbar puncture is invasive and cannot be performed for every patient with neurological symptoms, and genetic testing for the *SPR* gene is costly and time-consuming as a screening test. A urine sample can be non-invasively collected and is ideal for investigating disease biomarkers. There are conflicting reports regarding urine Sp in SRD. Although urine Sp in SRD patients was initially reported as not elevated by Zorzi et al. [9], a recent study demonstrated the elevation of urine Sp in SRD [10].

In this study, we developed a fast method to measure Sp and creatinine using high-performance liquid chromatography (HPLC) with ultraviolet and fluorescence detection. We analyzed urine and CSF samples obtained from SRD patients, their family members, and non-SRD controls to confirm the elevation of Sp in urine and CSF samples in SRD patients. Our method enables the rapid and non-invasive biochemical diagnosis of SRD.

2. Subjects and Methods

2.1. Subjects

We contacted pediatric neurologists who were following SRD patients in Japan using mailing lists

and personal communications. Subsequently, we asked these physicians to recruit SRD patients and their family members for this study. As controls, we also included patients who were considered to not have SRD based on their clinical presentation between July 2014 and July 2021.

2.2. Sample collection

We collected urine samples from SRD patients, their family members, and controls. CSF samples were also collected if there was an opportunity to have a lumbar puncture during the diagnostic work-up. All collected samples were protected from light and frozen below -70°C as soon as possible, because Sp is sensitive to light and oxygen. When the samples were collected outside Okayama University Hospital, they were shipped on dry ice to our laboratory and kept frozen at -80°C until analysis.

We also obtained clinical information, including age, sex, symptoms, CSF monoamines, genotypes, and treatment, from attending physicians.

This study was approved by the ethics committee of Okayama University Hospital (Approval number 1604-009). Written informed consent was obtained from the patients and their parents or guardians before the procedure.

2.3. Measurement of Sp and creatinine

We obtained Sp from Schircks Laboratories (Jona, Switzerland), xanthopterin from Santa Cruz Biotechnology, Inc. (Shanghai, China), and creatinine, ascorbic acid, disodium hydrogen phosphate, sodium dihydrogen phosphate, and 0.02 mol/L hydrochloric acid from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetonitrile was from Nacalai Tesque, Inc. (Kyoto, Japan). Ultrapure water was prepared using a Direct-Q UV3 system (Merck Millipore, Burlington, USA).

Sp primary stock solution (0.01%, w/v) was prepared by dissolving Sp ≈3 mg into a 0.02 mol/L NaOH aqueous solution to achieve the concentration of 1 mg/mL (=0.1%, w/v), then diluting it ten-fold by 0.02 mol/L HCl with ascorbic acid (1%, w/v). Sp secondary stock solution (0.001%, w/v) was prepared by diluting the primary stock solution (0.01%, w/v) by 0.02 mol/L HCl with ascorbic acid (1%, w/v). Xanthopterin stock solutions (0.01%, 0.001%, w/v) were similarly prepared using 0.02 mol/L HCl as diluent. These stock solutions were frozen at -80°C. The secondary stock solutions of Sp and xanthopterin were mixed and diluted with ultrapure water to make a calibrator (Sp 2 μmol/L, xanthopterin 2 μmol/L). We also prepared a 2 mmol/L calibrator of creatinine in ultrapure water. We prepared two types of quality control (QC) samples. QC sample 1 (QC1) was prepared by diluting calibrators with ultrapure water to achieve final concentrations of 200 nmol/L

Sp, 200 nmol/L xanthopterin, and 0.4 mmol/L creatinine. QC sample 2 (QC2) of urine was a heathy control sample spiked with 100 nmol/L of Sp. The QC2 of CSF was the non-SRD pooled sample spiked with 100 nmol/L of Sp.

Calibrators, QC1, CSF samples, and CSF QC2 were filtered through 0.45-μm Millex LH filters (Merck Millipore). Thawed urine samples (50 μL) and urine QC2 were added to 200 μL of 0.125% (w/v) ascorbic acid in mobile phase A (described later) and ultrafiltered using a Nanosep centrifugal device with Omega membrane 10k (Pall Corporation, Port Washington, USA) at 14000 × *g* for 10 mins at 25°C.

The HPLC system consisted of a Waters Alliance 2695 module with a tandemly connected Waters 2489 ultraviolet/visible detector and a Waters 2475 multi λ fluorescence detector (Waters Japan, Tokyo, Japan). The mobile phase A was 60 mmol/L sodium phosphate buffer at an approximate pH of 6.2. (10.2 mmol disodium hydrogen phosphate and 49.8 mmol sodium dihydrogen phosphate in 1 L of ultrapure water) and the mobile phase B was 100% acetonitrile. A gradient setting shown in Table 2 was applied. The samples were cooled at 4°C in the autosampler and the injection volume was 5 μL for urine and 30 μL for CSF. The chromatographic separation was performed at a flow rate of 1.0 mL/min through a reverse-phase column (Atlantis T3, 3 μm, 3.0 mm × 50 mm, Waters Japan) at 38°C with a guard column (Atlantis T3 VanGuard Cartridge, 3 μm, 2.1 mm × 5 mm, Waters Japan). Sp and xanthopterin were detected using excitation at 425 nm and emission at 530 nm. Creatinine was detected using absorbance at 234 nm. The entire analysis time was 10 min. Concentrations were calculated using peak heights with a single-point calibrator (Sp 2 μmol/L, creatinine 2 mmol/L). When creatinine concentration of a urine sample turned out to be above 2 mmol/L (= 10 mmol/L in the original sample), the sample was further diluted to achieve a creatinine concentration below 2 mmol/L and re-analyzed.

2.4. Statistical analysis

Spearman's correlation tests were performed using R Ver 4.1.2 software (https://www.r-project.org/). The results were considered statistically significant when *p*-values were below 0.05.

3. Results

3.1. Subject characteristics

There were three patients with SRD who were diagnosed by genetic analysis in advance. Table 1 shows a summary of the patients. Case 1 was previously reported [11]. All patients had had oculogyric crisis (OGC) during infancy or childhood, but in Case 3, OGC spontaneously resolved by 5 years of age without treatment. Cases 2 and 3 are siblings, and all three had the same mutation in

the *SPR* gene. There were two SRD carriers (the parents of Case 2 and 3: age 612 and 696 months, respectively) and four patients' siblings (SRD siblings: mean age: 208.5 months, range: 129–264 months). They had no clinical symptoms. It is unclear whether the siblings are SRD carriers, because they did not undergo genetic testing. There were 102 control patients without SRD (mean age: 12.5 months; range: 0–377 months). Control patients had symptoms, including epilepsy, developmental delay, autism, etc., but no symptoms commonly observed in neurotransmitter diseases, such as involuntary movements.

3.2. Validity of Sp and creatinine assay using HPLC

The retention time of Sp and creatinine was 5.2 min and 0.6 min, respectively (Fig. 2). Xanthopterin, which has fluorescence properties similar to Sp, was well separated from Sp and did not interfere with the measurement of Sp at all. There was a good linear relationship over the concentration range of 2.5 to 2000 nmol/L for Sp (R^2 = 0.9999) and 5 to 2000 μmol/L for creatinine (R^2 > 0.9999). Because the intercept of the calibration lines for Sp and creatinine was not significantly different from zero, we chose to use single-point calibrators at 2000 nmol/L (Sp) and 2000 μmol/L (creatinine). The limit of detection (LOD, signal-to-noise [S/N] ratio≥3) was approximately 2 nmol/L for Sp and 5 μmol/L for creatinine. The limit of quantification (LOQ, S/N ratio≥10) was approximately 5 nmol/L for Sp and 10 μmol/L for creatinine. The intra-day coefficients of variation (CV, n = 10) were 2.4% (Sp) and 1.1% (creatinine) for QC1 and 5.2% (Sp) and 0.5% (creatinine) for QC2. The inter-day CVs (n = 10) were 2.3% (Sp) and 0.8% (creatinine) for QC 1 and 8.6% (Sp) and 1.3% (creatinine) for QC2.

Sp recoveries from the urine samples spiked with 50 nmol/L and 200 nmol/L (n = 5) were 95.4 to 108.3% (mean, 101.3%) and 98.6 to 103.2% (mean, 99.7%), respectively. Creatinine recoveries from the urine samples spiked with 0.1 mmol/L and 0.4 mmol/L (n = 5) were 94 to 135% (mean, 105.8%) and 90 to 109.3% (mean, 99.8%), respectively.

When the injection volume was increased to 30 μL for Sp assay in the CSF, there was a good linear relationship over the concentration range of 0.25 to 2000 nmol/L. Because the intercept of the calibration line was not significantly different from zero, we chose to use a single-point calibrator at 2000 nmol/L. LOD was 0.5 nmol/L and LOQ was 1.0 nmol/L. The intra-day coefficients of variation (CV, n = 10) were 1.6% (Sp) for QC1 and 2.7% (Sp) for QC2. The inter-day CVs (n = 10) were 3.8% (Sp) for QC1 and 3.4% (Sp) for QC2.

Sp recoveries from the CSF samples spiked with 50 nmol/L and 200 nmol/L (n = 5) were 100.8 to 106.9% (mean, 104.2%) and 99.1 to 104.9% (mean, 100.8%), respectively.

3.3. Sepiapterin concentrations

Figure 1 shows Sp concentrations in the urine of SRD patients, SRD carriers, SRD siblings, and controls. Sp concentrations in the controls ranged from 1.5 to 47.9 μmol/mol creatinine (mean ± SD: 15.1 ± 10.2), those in SRD carriers were 6.8 and 22.2 μmol/mol creatinine, and those in SRD siblings ranged from 3.4 to 17.2 μmol/mol creatinine. These values were consistent with the previously reported reference value of <101.7 μmol/mol creatinine [10]. In Cases 1 to 3, Sp concentrations in urine were 1086, 914, and 575 μmol/mol creatinine, respectively. Patients with SRD had obviously higher urine Sp concentrations than the other groups. Sp concentrations of SRD carriers and siblings were not different from those of the control group. Sp concentrations were not correlated with age (ρ = 0.039, p = 0.6836; Figure 3). CSF Sp concentrations in all controls (28 non-SRD patients) were below the detection limit (<0.5 nmol/L), which was consistent with the previously reported reference value of <0.5 nmol/L [9]. A CSF sample was available in Case 2 only, and its Sp concentration was 4.1 nmol/L.

4. Discussion

We established a fast method to measure Sp and creatinine concentrations in urine simultaneously, utilizing HPLC with ultraviolet and fluorescent detection. We administered this method for SRD patients diagnosed in Japan, and confirmed the increased excretion of Sp in their urine. The SRD carriers and controls did not demonstrate elevation of urine Sp. Although Case 3 had milder symptoms than Cases 1 and 2 despite the same *SPR* gene mutation, owing to the small number of cases, the relationship between Sp concentrations in urine and phenotypic severity is unclear. In addition to urine samples, we confirmed that this method could be used to measure Sp in CSF, as previously reported by Zorzi et al. [9]. Although the CSF sample from only one SRD patient was available, we succeeded in detecting Sp in the CSF. In contrast, CSF Sp concentrations in all controls were below the detection limit (<0.5 nmol/L).

The diagnosis of SRD can be challenging. It is necessary to consider this disease based on symptoms specific to neurotransmitter diseases such as dystonia, oculogyric crisis, and diurnal variation of symptoms in addition to developmental retardation from infancy. Otherwise, it can be misdiagnosed as atypical cerebral palsy of unknown cause, for example [2]. Patients with mild or atypical symptoms may also go underdiagnosed because neurotransmitter diseases are not considered as differential diagnoses initially. Because treatment with L-DOPA and 5-hydroxytryptophan can be effective in motor symptoms for most patients [5,6], early diagnosis and treatment is essential. Urine has a great advantage of non-invasive sample collection compared with blood and CSF, and it is suitable for disease screening, especially in young patients including neonates. The caveat is that it is

necessary to quickly protect urine from light and freezing as soon as possible, because Sp is sensitive to light and oxygen.

The mechanism for increased urinary Sp in SRD patients is based on the following hypothesis [10]. In the absence of SR activity, there are two alternative pathways for BH_4 biosynthesis via Sp (salvage pathway 1) and via 2'-Oxo-1'-hydroxypropyltetrahydropterin (2'-oxo-TP) (salvage pathway 2) (Fig.3) [12,13]. The increase of Sp in urine suggests that salvage pathway 1 may be more dominant than salvage pathway 2 in the liver in SRD. This is also consistent with the result that 1'-Oxo-2'-hydroxypropyltetrahydropterin (1'-oxo-TP), which is a precursor of Sp, accumulates when SR is inhibited by human liver lysate *in vitro* [13]. This means that although dihydrofolate reductase is expressed in the liver and can produce enough BH_4 to not cause hyperphenylalaninemia, the liver of SRD patients may accumulate excessive amounts of 1'-Oxo-TP, Sp, and dihydrobiopterin (BH_2), which are not converted to BH_4, and these metabolites may leak into the urine.

This study is significantly limited by the small sample size. Future research must involve larger cohorts of patients with SRD and other neurological disorders, and healthy controls to check the sensitivity and specificity of urine Sp for the diagnosis of SRD. We hope that the application of this method to patients with unexplained neurological symptoms will help detect undiagnosed SRD patients and achieve early treatment that results in the improvement of their symptoms, development, and quality of life.

5. Conclusion

We developed the simultaneous assay of Sp and creatinine in urine and Sp in CSF and applied this method to SRD patients, their family members, and non-SRD controls. This method provides rapid and non-invasive diagnosis for SRD, and can potentially be used for disease screening.

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Conflict of Interest Disclosures

The authors have no conflicts of interest to disclose.

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Figure Legend

Figure 1

Sepiapterin (Sp) concentrations in the urine of sepiapterin reductase deficiency (SRD) patients, SRD carriers, SRD siblings, and controls.

A: Scatter plot showing the relationship between Sp concentrations and age in months. In the control group, there was no correlation between Sp concentrations and age. (Spearman's correlation test; $\rho = 0.039$, $p = 0.6836$ [>0.05])

B: Box-and-whisker plot showing the Sp concentrations for each group.

Figure 2

Chromatograms of a standard solution, urine, and cerebrospinal fluid (CSF) sample.

A) A standard solution of xanthopterin and sepiapterin at 2 µmol/L and a standard solution of creatinine at 2 mmol/L. B) A urine sample from an sepiapterin reductase deficiency (SRD) patient showing a clear peak of sepiapterin. C) A urine sample of controls in which a sepiapterin peak is unclear. D) A CSF sample from an SRD patient showing a peak of sepiapterin. E) A CSF sample of controls showing no peak of sepiapterin.

Figure 3

Biosynthesis of tetrahydrobiopterin.

AKR1B1: aldose reductase. AKR1C3: 3 α -hydroxysteroid dehydrogenase type 2. CR: carbonyl reductase. DHFR: dihydrofolate reductase. GTP: guanosine triphosphate. GTPCH: GTP cyclohydrolase. PTPS: 6-Pyruvoyl tetrahydropterin synthase. SR: sepiapterin reductase.

Table 1

Profile of SRD patients.

Patient number	Age	Sex	Age at onset	Symptoms	CSF monoamines	Genotype*	Sepiapterin/creatinine (μmol/mol creatinine)	Therapy（age）/Effectiveness
1	6y0m	Female	5m	DD, dystonia, OGC	HVA 132 nmol/L 5-HIAA 12 nmol/L	c.512G>A c.304+2_304+13del	1085.5	L-DOPA (10m)/excellent
2	15y10m	Female	4m	DD, OGC	HVA 48 nmol/L 5-HIAA 9 nmol/L	c.512G>A c.304+2_304+13del	914.2	L -DOPA (4y10m)/partial
3	24y	Male	infant?	OGC, mild ID	not performed	c.512G>A c.304+2_304+13del	575.2	L-DOPA+carbidopa(24y)/ effective for sleepiness

CSF: cerebrospinal fluid, DD: developmental delay, HVA: homovanillic acid, 5-HIAA: 5-hydroxyindoleacetic acid

ID: intellectual disability, m: months, OGC: oculogyric crisis, SRD: sepiapterin reductase deficiency, y: years

*: *SPR* gene (NM_003124.5)

Table 2

High-performance liquid chromatography gradient settings

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Gradient curve
Initial	1.0	100	0	0	
0.5	1.0	100	0	0	Linear
5.5	1.0	95	5	0	Linear
6.0	1.0	40	60	0	Step
6.5	1.0	100	0	0	Step
10.0	1.0	100	0	0	Step