Title
Development of a simultaneous analytical method for five conjugated cholesterol metabolites in urine and investigation of their performance as diagnostic markers for Niemann–Pick disease type C

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Abbreviations: AUC, area under the curve; Cr, creatinine; GlcNAc, N-acetylglucosamine; HQC, high quality control; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LQC, low quality control; MQC, middle quality control; NPC, Niemann–Pick disease type C; NPC1, NPC intracellular cholesterol transporter 1; NPC2, NPC intracellular cholesterol transporter 2; Niemann–Pick disease type C; S7B-Δ⁵-CA, 3β-sulfooxy-7β-hydroxy-5-cholen-24-oic acid; S7O-Δ⁵-CA, 3β-sulfooxy-7-oxo-5-cholen-24-oic acid; SNAG-Δ⁵-CA, nonamidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ⁵-CG, glycine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholen-24-oic acid; SNAG-Δ⁵-CT, taurine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholen-24-oic acid; SRM, selected reaction monitoring; ROC, receiver operating characteristic.
Abstract

Niemann–Pick disease type C (NPC) is an autosomal recessive disorder characterized by progressive nervous degeneration. Because of the diversity of clinical symptoms and onset age, the diagnosis of this disease is difficult. Therefore, biomarker tests have attracted significant attention for earlier diagnostics. In this study, we developed a simultaneous analysis method for five urinary conjugated cholesterol metabolites, which are potential diagnostic biomarkers for a rapid, convenient, and noninvasive chemical diagnosis, using liquid chromatography/tandem mass spectrometry (LC/MS/MS). By the method, their urinary concentrations were quantified and the NPC diagnostic performances were evaluated. The developed LC/MS/MS method showed high accuracy and and satisfied all analytical method validation criteria. Analyzing the urine of healthy controls and patients with NPC, three of five urinary conjugated cholesterol metabolites concentrations corrected by urinary creatinine were significantly higher in the patients with NPC. As a result of receiver operating characteristics analysis, the urinary metabolites might have excellent diagnostic marker performance. 3β-sulfooxy-7β-hydroxy-5-cholenoic acid showed particularly excellent diagnostic
performance with both 100% clinical sensitivity and specificity, suggesting that it is a
useful NPC diagnostic marker. The urinary conjugated cholesterol metabolites exhibited
high NPC diagnostic marker performance and could be used for NPC diagnosis.
INTRODUCTION

Niemann–Pick disease type C (NPC) is a progressive and life-limiting autosomal recessive inherited disorder (1). The prevalence of this disease is approximately 1/100000 and is classified as a lysosomal disease. It is caused by mutations in the NPC intracellular cholesterol transporter 1 (NPC1) gene coding for membrane proteins or NPC intracellular cholesterol transporter 2 (NPC2) coding for secreted proteins (2,3). Lack of these functional proteins, that work cooperatively with lysosomal free cholesterol efflux, causes excessive accumulation of free cholesterol and sphingolipids (4). However, the relationship between the characteristic lipid abnormalities and pathology of the disease remains unclear, as patients with NPC present a wide variety of clinical symptoms (5). The onset age of NPC ranges from neonatal to adult, and the symptoms are diverse and include systemic, visceral, nervous, and psychiatric abnormalities. Because the prognosis of patients with this disease is poor, it is important to diagnose NPC early and apply the treatment to maintain the quality of life of the patient (5). However, few trained specialists are available and the process leading to the discovery and diagnosis of NPC is complex. As conventional
laboratory tests, the filipin test and genetic examination are considered to be the gold standards (5). However, both of these tests are complicated, so biomarker tests have attracted significant attention as a rapid screening method for NPC. Oxysterols are generated from the accumulated cholesterol in NPC cells, and is present in higher concentrations in the plasma of the affected patients (6). The concentration of lysosphingomyelin, which is metabolized from sphingomyelin, is also elevated in the plasma of patients with NPC (7). Lysosphingomyelin-509 is a blood biomarker that has been recently used, but its precise structure remains unknown (8).

Following the previous report regarding urinary metabolites in patients with NPC by Alvelius et al. (9), we developed a noninvasive diagnostic method using urine analysis. First, we developed an analytical method for three multi-conjugated cholesterol metabolites, 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholen-24-oic acid as well as its glycine and taurine conjugates (SNAG-Δ⁵-CA, SNAG-Δ⁵-CG and SNAG-Δ⁵-CT, respectively) using liquid chromatography/tandem mass spectrometry (LC/MS/MS) (10). It was observed that the metabolites in the urine of two patients with NPC were much higher than those of the controls without NPC. Subsequently, we collected over
20 urine samples and preliminarily investigated their diagnostic performance, assuming that they may be useful for NPC screening (11). However, several patients with NPC had extremely low concentrations of the relevant metabolites and false-negatives. Thus, a comprehensive analysis method was used to search for other biomarker candidates (12), which yielded two strongly detected metabolite peaks in urine of patients with NPC, 3β-sulfooxy-7β-hydroxy-5-cholenoic acid (S7B-Δ5-CA) and 3β-sulfooxy-7-oxo-5-cholenoic acid (S7O-Δ5-CA) (13). In this study, we evaluated the NPC diagnostic marker performance of five urinary conjugated cholesterol metabolites. To evaluate their diagnostic performances, it is necessary to accurately determine the concentration of all metabolites for every case. Therefore, we developed an LC/MS/MS method that could accurately and simultaneously analyze the urinary concentrations of the five conjugated cholesterol metabolites for each sample. The urinary conjugated cholesterol metabolites in all samples were quantified by the developed method, and their utility as NPC diagnostic markers were evaluated.

MATERIALS AND METHODS
Chemicals and reagents

SNAG-Δ⁵-CA, SNAG-Δ⁵-CG, SNAG-Δ⁵-CT, S7B-Δ⁵-CA, S7O-Δ⁵-CA, and 3β-sulfooxy-7β-hydroxy-23-nor-5-cholenoic acid (as an internal standard (IS)) were synthesized as described in previous reports (the structures are shown in the Fig. 1) (13-15). Ultrapure water was prepared with a PURELAB ultra apparatus (Organo Co., Tokyo, Japan). All reagents (HPLC grade) were purchased from FUJIFILM Wako Pure Chemical Co. Ltd. (Osaka, Japan). Urine samples were collected after obtaining informed consent from untreated patients diagnosed with NPC and healthy volunteers. The urine samples were collected in the morning, stored at -80 °C, and analyzed within 1 month. All experiments were performed according to the protocol approved by the Ethics Committee of the Graduate School of Medicine in Tohoku University (Approval number, 2013-1-293).

LC/MS/MS analysis

A Prominence model high performance liquid chromatograph system (Shimadzu Co., Kyoto, Japan) was connected to a triple quadrupole tandem mass
spectrometer API 5000 equipped with an electrospray ionization probe (SCIEX, Framingham, MA, USA). MS/MS was acquired in selective reaction monitoring (SRM) mode with negative ion detection. Ion spray voltage, turbo spray temperature, curtain gas, nebulizer gas, turbo gas, and collision gas were set at -4500 V, 700 °C, 20 psi, 50 psi, 50 psi, and 6 units, respectively. SRM conditions were set as listed in Supplemental Table 1. The dwell and pause times were set to 160 and 5 msec. Data acquisition was performed using analyst version 1.5.0 (SCIEX) and SCIEX OS-Q software (SCIEX) for data integration. With respect to the LC, a column switching system was used (10-13, 16). After injection of the sample aliquot, 20 mM ammonium acetate buffer (pH 5.5)/methanol (9:1, v/v) mixture was loaded on OASIS HLB column (2.1 mm i.d. × 20 mm, 5 μm, Waters, Milford, MA). Pretreatment of the sample was performed at a flow rate of 1.0 mL/min for 3 min. After washing and concentrating the analytes, the sample eluent was loaded on a Capcell pak C18 BB-H column (2.1 mm i.d. × 150 mm, 3 μm, Osaka Soda, Osaka) by switching the valve used for changing the flow path. Mobile phase A (20 mM ammonium acetate buffer (pH 5.5)) and mobile phase B (methanol) were gradually changed from A:B=65:35 to A:B=45:55 over 50 min.
Preparation of the stock and working solutions

The analytes and IS were adjusted to a concentration of 100 μg/mL using water/ethanol (1:1, v/v, as stock solution). IS was diluted with water/ethanol (1:1, v/v) to 33 ng/mL and used as the IS solution. The analytes were mixed and diluted with water/ethanol (1:1, v/v) to 0.3, 1, 3, 10, 30, 100, 300 and 1000 ng/mL (working solutions for the calibration curve). For quality control (QC), mixed solutions of 2, 50, and 800 ng/mL were set as the low quality control (LQC), middle quality control (MQC), and high quality control (HQC) (working solution for QC), respectively.

Calibration curve

A total of 50 μL of water was used as a surrogate matrix and 50 μL of IS solution, 50 μL of working solution for the calibration curve, and 350 μL of water were added and mixed. The mixture was then centrifuged at 15,000×g at 4 °C for 3 min and 200 μL of the supernatant was injected for LC/MS/MS analysis. The peak area ratio of each analyte to IS was plotted against the standard concentration and the calibration
curves were prepared using the least squares method with $1/x^2$ weighting.

**Matrix effects**

To determine matrix effects, 50 μL of the IS solution, 50 μL of water/ethanol (1:1, v/v) or QCM solution, and 350 μL of water were added to 50 μL of urine from a healthy control or water. After mixing and centrifugation, the supernatant was injected into the LC/MS/MS system. The matrix factor (MF) for each analyte was calculated using the following formula and the ratio considering the MF of IS was calculated as the IS normalized MF (7).

\[
MF(\%) = \frac{(\text{Peak area of spiked urine}) - (\text{peak area of blank urine})}{(\text{Peak area of standard solution})} \times 100
\]

IS normalized MF (\%) = \frac{(\text{Matrix factor of each analytes})}{(\text{Matrix factor of IS})} \times 100

**Intra-assay and inter-assay reproducibility**

To determine intra- and inter-assay reproducibility, 50 μL of QC solution (blank, LQC, MQC, HQC), 50 μL of IS solution, and 350 μL of water were added to 50 μL of urine from a healthy control, and the specimens were analyzed using the
procedure described above. Every three days, urine samples were prepared and analyzed
for every blank, LQC, MQC, and HQC (N = 6). Generally, the recovery (%) was
calculated by relative error (R.E. (%)). However, since the analytes in this study are
endogenous, it was calculated by adding the concentration contained in the healthy
control urine (Blank).

\[
R.E. (\%) = \frac{(\text{Calculated concentration}) - ((\text{Added concentration})+(\text{Blank concentration}))}{(\text{Added concentration})+(\text{Blank concentration})} \times 100
\]

Precision (%) was calculated by relative standard deviation (R.S.D. (%)).

\[
R.S.D. (\%) = \frac{(\text{Standard deviation})}{(\text{Mean concentration})} \times 100
\]

**Stability test**

For the stability test, 50 μL of QC solution (blank, LQC, HQC) was dried under
a nitrogen gas stream, and the urine of healthy control was added and stored under
various conditions including: 6 months at -80 °C, 24 h at 4 °C, 12 h at 25 °C as room
temperature, 3 times repeated freeze-thaw cycles, and 48 h in an autosampler.

Afterwards, analysis was performed using the same pretreatment as described above,
and the ratio between the data immediately after preparation and the quantitative value
was calculated as Recovery (%).

Dilution test

A mixture of standard solutions was added to 1.5 mL of healthy human urine to a final standard solution concentration of 645 ng/mL (Dilute 1). Dilute 1 was further diluted 20-fold with water (Dilute 2) and Dilute 1 and 2 were analyzed as described above. Dilution factor (%) was calculated as follows.

\[
\text{Dilution factor (\%)} = \left( \frac{\text{Concentration of Dilute 2} \times 20}{\text{Concentration of Dilute 1}} \right) \times 100
\]

Urine analysis

For analysis of the urine samples, 50 μL of urine from healthy subjects (N = 38) and patients with NPC (N = 28) were subjected to analysis. The data was processed using JMP Pro version 13.2.1 software (SAS Institute Inc., NC, USA). Wilcoxon's t-test and receiver operating characteristic (ROC) analysis were used for intergroup analysis and diagnostic performance tests. Urinary creatinine was analyzed with enzymatic creatinine analysis kit (Serotec, Sapporo, Japan). The urinary concentrations of five
metabolites were corrected with the urinary creatinine concentration.

**RESULTS AND DISCUSSION**

**Detection and separation of analytes with column switching LC/ESI-MS/MS**

The analytes and IS, which are sulfate conjugates (Fig. 1), were detected with high sensitivity in negative ion mode \((10-13)\). As a result of optimization, SRM condition was set as listed in Supplemental Table 1. A column switching LC system, which was capable of large volume injection and online solid phase extraction, was used for the analysis \((10-13,16)\). Under this LC condition, the separation of all analytes and IS was achieved with sharp peak shapes (Fig. 2A). In addition, the peaks were separated from urinary contaminant peaks, which were detected constantly at the SRM transitions of \(m/z\ 469>97\) and \(467>97\) (Fig. 2B).

**Calibration curves and matrix effects**

In general bioanalysis, working solution spiked sample matrices are used for preparing calibration curves. Because the analytes in this study are endogenous in urine,
it is necessary to use a surrogate matrix. Therefore, we investigated the matrix effects for quantification of analytes. Procedure of sample preparation for calibration curves, QC samples and urine samples were summarized in Supplementary Table 2, respectively. We prepared calibration curves using water as a surrogate matrix, and the all calibration curves showed high linearity over wide range from 0.3 to 1000 ng/mL (Supplemental Table 3A). Next, the matrix effects were investigated. The matrix effect is usually calculated by the ratio of peak intensity of the standard solution spiked in a pretreated matrix to that of the neat standard solution (17). However, the analytical system used herein features an online solid phase extraction, so we could not evaluate the typical method (17). Therefore, it was evaluated using MF which is the parameter combining the pretreatment extraction efficiency and matrix effects from biological contaminants (7). As a result, the MFs of all analytes and IS was 101–105% (Supplemental Table 3B). The IS normalized MFs of all analytes were nearly 100% and it was found that the analytes could be quantified without considering the matrix effect.

Reproducibility test
The method reproducibility was investigated using QC samples. Accuracy was evaluated by subtracting the concentration in the healthy control urine as Blank. The accuracy of the inter- and intra-day assays were within 100% ± 10% for all QC samples and their precision (%) were within 10% (Table 1).

**Stability test**

The QC solution spiked urine samples were stored under various conditions and the analytes were subsequently quantified. All analytes could be stably stored under all conditions tested and could be quantified even for the long-term preserved specimens (Table 1).

**Dilution test**

When the upper limit of the calibration curve was exceeded, it became necessary to dilute with the matrix and re-measure the sample using general bioanalytical techniques. Because endogenous analytes of this study are included in urine, water was used as a surrogate matrix. The influence on the quantitative value was
investigated and it was found that 20-fold dilution of the urine sample by water did not affect the quantitative results (Table 1B).

Analysis of five urinary cholesterol metabolites in healthy controls and patients with NPC

Subsequently, all urine samples from the healthy controls and patients with NPC were analyzed. A total of 66 specimens were collected from every patients with NPC and healthy controls, and their demographics are listed in Supplemental Table 4. The age of each groups did not differ between healthy controls (0.33–47 years) and patients with NPC (0.0274–48 years; \( P=0.1739 \)), but a larger proportion of females were recruited in the NPC patient group (\( P=0.0179 \)). The typical SRM chromatogram of patient with NPC was shown in Fig. 2C. The data are summarized in both creatinine-corrected concentrations, which are often used for biochemical examinations (Fig. 3 and Supplemental Table 5), and uncorrected concentrations (Supplemental Fig. 1 and Supplemental Table 6). All metabolites were significantly higher in patients with NPC in terms of creatinine-corrected concentrations and uncorrected concentrations other
than SNAG-Δ⁵-CT (Fig. 3 and Supplemental Fig. 1). The correlations between each of
the metabolites were investigated and observed to generally correlate (Supplemental
Fig. 2). In other, the correlation for S7B-Δ⁵-CA and other metabolites was slightly lower
than other combinations. Similar to the reports of Mazzacuva et al. and Jiang et al. (18,
19), we speculate that the analytes in this study were produced via oxysterols. It was
also assumed that S7O-Δ⁵-CA is metabolized from 7-ketocholesterol and SNAG-Δ⁵-
CA, SNAG-Δ⁵-CG, SNAG-Δ⁵-CT, and S7B-Δ⁵-CA are produced from 7β-
hydroxycholesterol. The sequence of cleavage of the side chain, conjugation with
sulfuric acid, amino acid, and GlcNAc remains unknown. Because SNAG-Δ⁵-CA,
SNAG-Δ⁵-CG, and SNAG-Δ⁵-CT showed high correlations, it is expected that they are
produced via similar metabolic pathways. In contrast, S7B-Δ⁵-CA and S7O-Δ⁵-CA may
pass through a slightly different route. In addition, S7B-Δ⁵-CA did not overlap at all
between the samples from the patients with NPC and healthy controls in any cases
tested. In our previous studies (11) and the report by Mazzacuva et al. (18), several
cases where metabolites bearing the 7β-GlcNAc group were present in extremely low
concentration were observed due to mutation of the UGT3A1 gene, which codes for
UDP glucosyltransferase 3A1 as a GlcNAc conjugation enzyme (20). In this study, the concentrations of the metabolites of SNAG-Δ⁵-CA, SNAG-Δ⁵-CG, and SNAG-Δ⁵-CT were very low in the urine of patients with NPC Nos. 10 and 17. Conversely, the concentration of S7B-Δ⁵-CA, which does not contain a GlcNAc group, in NPC samples was higher than those of healthy controls, and it is likely that the discrimination between patients with NPC from other subjects by urinary S7B-Δ⁵-CA concentration may be possible. Similarly, S7O-Δ⁵-CA does not contain a GlcNAc group, but some overlap was observed between the concentrations present in the urine samples of the patients with NPC and healthy subjects. The results suggested that analysis of urinary S7B-Δ⁵-CA may prevent overlooking of patients with NPC with false negative results based on abnormally low concentrations due to the UGT3A1 mutation (18, 20).

Because the concentrations of urinary cholesterol metabolites were generally higher than plasma oxysterols (Fig. 3, Supplemental Table 7 and (6)), these metabolites act as an excretion pathway of excessive accumulated cholesterol due to metabolic abnormalities similar to other cholesterol metabolic disorder diseases (22-26).
Finally, the NPC diagnostic performance of each urinary cholesterol metabolites was evaluated using ROC analysis (Fig. 4). This study investigated the biomarkers for a rare lysosomal disease NPC, and we experienced difficulty collecting urine specimens and collected a total of 66 specimens. This limited sample size is not ideal, but the sample number in this study exceeded the threshold which could yield significant differences as result of power analysis (data not shown). Accordingly, the analytical results were subjected to statistical analysis and the AUC value exceeded 0.92 for each metabolite. In particular, because S7B-Δ⁵-CA exhibited no overlap between NPC and control patients, the AUC value of the metabolite was 1.0. The cut-off concentration was set to the concentration with the highest value of sensitivity-(1-specificity) which is representative of the highest true positive rate and lowest false positive rate. The sensitivity was 92.6–100% and specificity was 81.1–100%, but S7B-Δ⁵-CA showed 100% for both parameters. These results were nearly equivalent to the plasma oxysterols (6) and their metabolites (18, 19). Therefore, the metabolites investigated herein represent a series of metabolites produced from cholesterol.
accumulated by NPC pathology (18, 19). These results also suggest that urinary
to metabolites are a series of metabolites generated from cholesterol accumulation in an
NPC-dependent manner (6, 18, 19). In addition, some patients with other lysosomal
diseases and cholesterol metabolic disorders provided almost low concentrations
(Supplementary Table 5 and 6). Thus, it is suggested that these urinary metabolites can
serve as useful NPC diagnostic biomarkers, reflecting the pathology of NPC.

CONCLUSION

A simultaneous analytical method for five urinary conjugated cholesterol
metabolites identified from the urine of patients with NPC was developed using
LC/MS/MS. The performance of the five metabolites as NPC diagnostic biomarkers
was also evaluated. First, we developed a reliable analytical method using column
switching LC/MS/MS, then five NPC diagnostic biomarker candidates in urine were
quantified. All five metabolites were generally present in higher concentrations in the
urine of patients with NPC compared to those of healthy controls and showed excellent
diagnostic marker performance. It was observed that the conjugated cholesterol
metabolites are useful as diagnostic markers of NPC. In particular, S7B-Δ⁵-CA is a valuable biomarker, exhibiting both 100% sensitivity and specificity. In the future, it is expected that these five urinary cholesterol metabolites, and S7B-Δ⁵-CA in particular, will be used for a noninvasive diagnostic screening method for NPC.

Acknowledgements

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REFERENCES


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Fig. 1 Chemical structure of analytes and internal standard.

3β-Sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid (SNAG-Δ5-CA) (A), glycine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid (SNAG-Δ5-CG) (B), taurine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid (SNAG-Δ5-CT) (C), 3β-sulfooxy-7β-hydroxy-5-cholenoic acid (S7B-Δ5-CA) (D), 3β-sulfooxy-7-oxo-5-cholenoic acid (S7O-Δ5-CA) (E), 3β-sulfooxy-7-oxo-23-nor-5-cholenoic acid (Internal standard, IS) (F).

Fig. 2 SRM chromatograms of analytes and IS. 30 ng/mL standard mixture (A), an urine of a healthy control (B), an urine of patient with Niemann-Pick disease type C (C). All of analytes and IS were separated from each other and completely separated from the contaminant peaks. SRM, selected reaction monitoring; IS, internal standard.
Fig. 3 The creatinine-corrected concentrations of SNAG-Δ⁵-CA (A), SNAG-Δ⁵-CG (B), SNAG-Δ⁵-CT (C), S7B-Δ⁵-CA (D), S7O-Δ⁵-CA (E), and their total concentration (F) in the urine of healthy controls and patients with NPC. SNAG-Δ⁵-CA, S7B-Δ⁵-CA, and their total concentration in the urine of patients with NPC were significantly higher than those observed in healthy controls. NPC, Niemann–Pick disease type C; SNAG-Δ⁵-CA, 3β-Sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; SNAG-Δ⁵-CG, Glycine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; SNAG-Δ⁵-CT, Taurine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; S7B-Δ⁵-CA, 3β-Sulfooxy-7β-hydroxy-5-cholenoic acid; S7O-Δ⁵-CA, 3β-Sulfooxy-7-oxo-5-cholenoic acid.

Fig. 4 ROC analysis results of the urinary concentration of SNAG-Δ⁵-CA (A), SNAG-Δ⁵-CG (B), SNAG-Δ⁵-CT (C), S7B-Δ⁵-CA (D), S7O-Δ⁵-CA (E), and their total concentration (F). AUC, cut-off concentration, sensitivity, and specificity are also shown. The AUC values ranged between 0.916 and 1.0. The sensitivities were 92.6% to 100% and the specificities were 81.1% to 100%. The cut-off concentrations ranged from
15 to 800 ng/mg Cr and S7B-Δ⁵-CA showed clear-cut diagnostic performance. SNAG-Δ⁵-CA, 3β-Sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; SNAG-Δ⁵-CG, Glycine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; SNAG-Δ⁵-CT, Taurine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; S7B-Δ⁵-CA, 3β-Sulfooxy-7β-hydroxy-5-cholenoic acid; S7O-Δ⁵-CA, 3β-Sulfooxy-7-oxo-5-cholenoic acid; ROC, receiver operating characteristic.
Table 1 Analytical validation data.

(A) Intra-day and inter-day assay

### Intra-day assay (N=6)

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<th>Accuracy (%)</th>
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### Inter-day assay (N=6)

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### (B) Stability and dilution test

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<table>
<thead>
<tr>
<th>LQC</th>
<th>HQC</th>
<th>LQC</th>
<th>HQC</th>
<th>10 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAG-Δ⁵-CA</td>
<td>95.5±3.23</td>
<td>95.8±2.16</td>
<td>92.2±2.80</td>
<td>93.7±0.797</td>
</tr>
<tr>
<td>SNAG-Δ⁵-CG</td>
<td>98.4±2.36</td>
<td>95.9±1.15</td>
<td>99.7±2.21</td>
<td>94.4±0.844</td>
</tr>
<tr>
<td>SNAG-Δ⁵-CT</td>
<td>95.6±2.17</td>
<td>94.6±0.399</td>
<td>94.7±2.38</td>
<td>96.3±2.18</td>
</tr>
<tr>
<td>S7B-Δ⁵-CA</td>
<td>94.4±7.45</td>
<td>93.8±2.30</td>
<td>101±3.09</td>
<td>106±1.09</td>
</tr>
<tr>
<td>S7O-Δ⁵-CA</td>
<td>95.3±2.92</td>
<td>96.9±3.36</td>
<td>103±1.43</td>
<td>109±1.40</td>
</tr>
</tbody>
</table>

LQC, low quality control (2 ng/mL); MQC, middle quality control (50 ng/mL); HQC, high quality control (800 ng/mL); SNAG-Δ⁵-CA, 3β-Sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; SNAG-Δ⁵-CG, Glycine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; SNAG-Δ⁵-CT, Taurine-amidated 3β-sulfooxy-7β-N-acetylglucosaminyl-5-cholenoic acid; S7B-Δ⁵-CA, 3β-Sulfooxy-7β-hydroxy-5-cholenoic acid; S7O-Δ⁵-CA, 3β-Sulfooxy-7-oxo-5-cholenoic acid.
Maekawa M. et al., Fig. 1
Maekawa M. et al., Fig. 2

(A) Maekawa M. et al., Fig. 2

(B) Maekawa M. et al., Fig. 2

(C) Maekawa M. et al., Fig. 2

Retention time (min)

Intensity (cps)

m/z 455→97
m/z 467→97
m/z 469→97
m/z 389→97
m/z 364→97
m/z 672→97
Maekawa M. et al., Fig. 4

(A) AUC 0.961
Cut-off 180 ng/mg Cr
Sensitivity 92.6%
Specificity 100%

(B) AUC 0.928
Cut-off 100 ng/mg Cr
Sensitivity 92.9%
Specificity 97.3%

(C) AUC 0.916
Cut-off 15.0 ng/mg Cr
Sensitivity 96.3%
Specificity 81.1%

(D) AUC 1.00
Cut-off 140 ng/mg Cr
Sensitivity 100%
Specificity 100%

(E) AUC 0.987
Cut-off 80 ng/mg Cr
Sensitivity 96.4%
Specificity 97.4%

(F) AUC 0.998
Cut-off 800 ng/mg Cr
Sensitivity 96.4%
Specificity 100%