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Determination of plasma cholesterol sulfate by LC–APCI-MS/MS in the context of pediatric autism

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ABSTRACT

Cholesterol sulfate (CS) has various biological functions. Previously, plasma CS was measured primarily as a means to diagnose X-linked ichthyosis; however, a recent hypothesis suggests that CS deficiency might be related to autism. As such, an assay capable of measuring both very high (in the case of X-linked ichthyosis) and very low (in the case of autism) plasma CS levels is required. Here we describe a novel LC–APCI-MS/MS method for the determination of CS in human plasma, and we propose normal CS ranges for children, based on studies of a local population of normal Chinese children between the ages of 2 and 10. In addition, we have used this method to measure plasma CS in autistic children. CS was isolated by solid-phase extraction, and quantified by isotope-dilution LC–APCI-MS/MS in negative ion mode monitoring 465.3 > 97.1 *m/z* (CS) and 472.3 > 97.1 *m/z* (CS-*d*7). Mean recovery of the assay ranged from 88.1 to 112.7%; within- and between-run imprecisions have CVs less than 7.2 and 8.1%, respectively. The assay was linear up to at least 100 μmol L^{-1}. The reference interval of plasma CS in males (range: 1.16–4.23 μmol L^{-1}) was found to be higher than in females (range: 0.86–3.20 μmol L^{-1}). Comparison of normal and autistic children showed no statistically significant difference in the plasma CS level. In conclusion, a robust LC–APCI-MS/MS method for plasma CS was developed, and a pediatric reference interval was derived from applying the method to normal and autistic children.

1. Introduction

Cholesterol sulfate (CS) is present in a variety of human body fluids, tissues and cells, such as erythrocytes, platelets, skin, hair, adrenal glands, lung and brain [1,2]. Its biological functions include the following: membrane stabilization [3], sperm capacitation [4], involvement in the activity of thrombin and plasmin [5], and regulation of the skin barrier [6]; it also serves as a precursor for steroid hormones [7]. Among them, the most investigated physiological roles for CS have been keratinocyte differentiation and development of the epidermal barrier [8,9].

CS represents only 0.1% of the total cholesterol in plasma but it can be significantly elevated in certain pathological conditions, such as liver cirrhosis [10], hypercholesterolemia [11] and hypothyroidism [12]; in X-linked ichthyosis, the plasma CS level is strikingly elevated [13–16]. Previously the measurement of plasma CS mainly helped in the differential diagnosis of ichthyosis. Recently, however, it has been suggested that CS deficiency might be related to autism. Since the rapid growth of infant brains places an exceptionally high demand on the supply of nutrients from the diet, Seneff's group suggested that insufficient supply of CS to the fetus during gestation and the infant postnatally might be related to autism [17]. The brain houses 25% of the body's cholesterol which is essential in the central nervous system for both synaptic fusion and myelin membrane growth. It was suspected that defective synaptic transmission is associated with autism spectrum disorders (ASD). Seneff's group hypothesized that cholesterol sulfate, which can cross the placental barrier much more readily than cholesterol, is the main source of cholesterol from the mother to the fetus. Therefore, maternal sulfur deficiency might be a key contributor to ASD in the child.

Autism is a disorder of neural development characterized by impaired social interaction and communication, and by restricted and repetitive behavior. It is one of three recognized disorders in the ASD, the other two being Asperger's syndrome and pervasive developmental disorder (PDD-NOS). The 20-year-old gunman responsible for the recent school shooting in Connecticut was suspected of having Asperger's syndrome, which is a mild form of autism often characterized by social awkwardness. Unlike classic autism, Asperger's syndrome does not typically involve delays in mental development or speech. The prevalence of autism has been estimated to be 1–2 per 1000, and of ASD, close to 6 per 1000 in developed countries [18]. In Hong Kong, ASD is estimated to affect 1.68 per 1000 children under 15 years old [19]. Based on data from

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the Center for Disease Control and Prevention, USA, the prevalence of several developmental disorders has decreased over recent years. But autism is on the other end of that spectrum, far outpacing all other disabilities in children and young adults up to the age of 22. From 2000 to 2008, the number of autism cases rose by 260% [20]. We do not know whether autism is a growing epidemic, or whether the increasing number is only due to increased awareness of the disorder. Although the relationship between CS deficiency and autism is only a hypothesis, it is worth exploring. Setting up a new analytical method for plasma CS, with simple sample preparation and high analytical sensitivity, and using it to determine plasma CS in autistic and non-autistic children are the specific purposes of this study. In the broader context, our main goal is to offer some help for those suffering from autistic conditions.

CS has a relatively simple structure but one that is not easy to determine accurately. Because it represents only 0.1% of the total cholesterol in plasma, it is vulnerable to contamination from nonsulfated cholesterol which can result in significant ion suppression for mass spectrometry (MS)-based methods. Therefore it must first be separated from the free cholesterol and cholesterol esters. For accurate determination, an efficient sample clean up procedure and/or chromatographic separation is/are crucial.

Previously, gas chromatography (GC) has been used for the determination of CS [21] owing to its advantages in terms of specificity and sensitivity. However, a notable disadvantage of the GC method is the requirement to remove the conjugate sulfate moiety prior to GC analysis, because of the inherently high polarity and thermal instability of the intact CS. Study has indicated that a gas chromatography–mass spectrometry (GC/MS) method will overestimate plasma CS level [22]. Although a few papers have reported the measurement of plasma CS, for example, by using fast atom bombardment mass spectrometry [22] or LC–MS [23], they all have the pitfalls of large sample volume requirement, complicated sample preparation procedure and narrow dynamic range. In this paper we describe a novel liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (LC–APCI-MS/MS) method for the determination of CS in human plasma, and we use it to establish normal CS ranges for Chinese children between the ages of 2 and 10. In addition, we have used this method to measure plasma CS in normal and autistic children.

2. Experimental

2.1. Materials and reagents

Cholesterol sulfate, ammonium formate and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid of ULC/MS grade was obtained from LGC Promochem GmbH (Wesel, Germany). Cholesterol-*d*7 sulfate was purchased from C/D/N Isotopes Inc. (Quebec, Canada). HPLC-grade methanol was purchased from J.T. Baker (Philipsburg, NJ, USA). Bond Elut C18 (100 mg) solid phase extraction (SPE) cartridges were purchased from Agilent Technologies (Palo Alto, CA, USA). All other chemicals were of the highest purity available from commercial sources. Water used in the study was produced by a Millipore Milli-RO/Milli-Q Gradient system (Millipore, Bedford, MA, USA).

2.2. Standard preparation

Stock standard solution of CS was prepared at 10 mmol L^{-1} in methanol. Stock standard of cholesterol sulfate-*d*7 (CS-*d*7), as internal standard (IS), was prepared at 1 mmol L^{-1} in methanol. Both stock standards were aliquoted and stored at −70 °C. The CS stock standard was further diluted in methanol to obtain working standards which were made fresh on the day of analysis. The CS-*d*7 stock standard was diluted with methanol to obtain a working IS at 50 μmol L^{-1}.

2.3. Calibrator preparation

Three sets of calibrators were prepared: set 1 consisted of dilutions of CS and IS in methanol with final CS concentrations of 0, 0.08, 0.5, 1.0, 5.0, 10, 20, 50 and 100 μmol L^{-1}; and IS of 5 μmol L^{-1}. Set 2 consisted of extracts of plasma samples spiked with CS and IS after SPE procedure (post SPE spike) with concentrations same as set 1. Set 3 consisted of plasma samples spiked with CS and IS prior to SPE procedure (pre SPE spike). These 3 sets of calibrators were run 6 times, each using a different source of plasma. Assay linearity, recovery and matrix effects data were obtained from these calibrators [24,25].

2.4. Biological samples

102 (49 males, 53 females) blood samples from apparently healthy subjects aged from 2 to 10 years were analyzed for reference interval set up. 102 samples from autistic children (49 males, 53 females) aged 2–9 years were collected. The diagnosis of autism was made by developmental pediatricians based on the diagnostic criteria in The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) [26]. Lithium heparin whole blood was centrifuged at 3500*g* for 20 min at 4 °C. Plasma samples obtained were stored at −20 °C until analysis. For long term storage, the samples were kept at −70 °C.

2.5. Sample preparation

200 μL of plasma, calibrator or control samples was mixed with 20 μL of working IS. Protein precipitations took place by adding 2 mL methanol and vortexing for 1 min. After centrifugation, supernatant was transferred to a clean glass test tube and 1.8 mL water added. After mixing, samples were loaded to the C18 SPE column which had been pre-conditioned with 2 mL methanol and 2 mL water. After complete sample loading, SPE column was washed with 1 mL 25 mM ammonium acetate, 2 mL 25 mM ammonium acetate/methanol (6:4, v/v), 2 mL water, 2 mL hexane followed by 2 mL chloroform. CS was eluted with 1 mL chloroform/methanol (2:1, v/v) for 3 times, all eluates were pooled and evaporated to dryness. Sample was reconstituted with 200 μL methanol and ready for analysis.

2.6. LC–APCI-MS/MS

For LC, an Agilent 1200 series and a Waters X'Bridge C8 column (2.1 × 100 mm^2, 3.5 μm) was used, with a gradient of mobile phase A (100 μmol L^{-1} ammonium formate in 50% methanol with 0.1% formic acid) and mobile phase B (100% methanol). The flow rate was 200 μL min^{-1} using gradient elution: 0–1 min, 60% B; 1–2 min, 60–90% B; 2–10 min, 90% B; 10–12 min, 60% B. Total run time was 12 min, and injection volume was 10 μL.

Mass spectrometry was performed using triple quadrupole mass spectrometer (Applied Biosystems 3200 QTRAP) equipped with a TurboIon-Spray ionization source. To optimize mass spectrometer settings, compounds were infused with a syringe pump at 10 μL min^{-1} in 90% mobile phase B. Negative APCI ionization was performed with the following settings: ion spray voltage, −4500 V; curtain gas, 15 psi; collision gas, medium; ion source gas 1 and 2 at 45 psi; interface heater, on; needle current, −5.0 μA; and source temperature, 450 °C. The mass spectrometer was operated using Analyst software version 1.4. To optimize the compound-dependent mass spectrometer settings, CS and CS-*d*7 were infused

one by one with a syringe pump at 10 μL min^{-1} in methanol. CS and CS-*d*7 were monitored with multiple reaction monitoring (MRM). Declustering potential for CS at −90 V; mass transitions: *m/z* 465.3 > 97.1 and 465.3 > 80.1 with collision energy at −75 V and −130 V, respectively. CS-*d*7 with declustering potential at −85 V, mass transition: *m/z* 472.3 > 97.1 with collision energy at −80 V.

2.7. Method validation

The analytical method validation was performed according to the CDER guidelines [27] and the recommendations as described in the publications of Matuszewski et al. [24,25] for matrix effects and extraction recovery determination.

2.7.1. Linearity, recovery and matrix effect

These three parameters, linearity, recovery and matrix effect, can be accessed from results of the three sets of calibrators mentioned above. Slopes of standard lines were determined from the linear regression analysis of the peak area ratios of CS/IS versus CS concentrations. Calibration curves were considered linear for $R^2 > 0.999$ in this study.

Recoveries were determined by comparison of the peak areas of sets 2 and 3 after correcting for endogenous CS [25]. A recovery of 100 ± 15% was regarded as acceptable in this method.

For matrix effect, 6 calibration lines were obtained by spiking 6 different lots of plasma with eight different concentrations 0.08, 0.5, 1, 5, 10, 20, 50, 100 μmol L^{-1} of CS and subsequent calculation of slope and intercept. Absolute matrix-effect (AME) is defined as the difference in response between analyte spiked in sample matrix, in this case—plasma, and the response of analyte spiked in methanol; it can be calculated as the agreement between the slopes of calibration lines made in plasma and methanol [25].

The relative matrix effect (RME) is defined as the combination of the effect of matrix on both recovery of CS from different plasma lots and ion suppression or enhancement between different plasma lots. It was calculated as 100%−CV% of the slopes of the 6 plasma calibration lines. A RME of 100 ± 5% was considered acceptable [25].

Matrix effects were also studied by the direct infusion method. A CS standard solution was directly infused into the LC–MS/MS. At the same time, extracted normal plasma ($n=6$) was injected into the chromatographic system and the flows were merged using a polyetheretherketone (PEEK) tee before the source entrance, in a post-column infusion system.

2.7.2. Lower limit of quantification and lower limit of detection

As clinical samples with extremely low CS concentrations were not available, CS-*d*7, which is absent in patient samples, was used to determine the lower limit of quantification (LLOQ). Plasma was spiked with different concentrations of CS-*d*7 and measured 20 times. The lowest concentration of CS-*d*7 where the imprecision was less than 20% and where the signal to noise (*S*/*N*) ratio was at least 10 was regarded as the LLOQ. The lower limit of detection (LLOD) was defined as three times the baseline noise (*S*/*N*≥3).

2.7.3. Accuracy and precision

For accuracy study, since commercial quality control (QC) sample for CS was not available, in-house prepared QCs were used. The accuracy of the method was determined at four levels (QC 1: low normal level; QC 2: high normal level; QC 3: pathological level; QC 4: high pathological level) of CS by using pooled plasma sample spiked with CS at different concentrations. Accuracy of the method was expressed as [(mean observed concentration−endogenous CS)/(spiked concentration−endogenous CS)] × 100. The mean value within 15% of the actual value was considered acceptable [25].

For precision, four different CS levels were used. Un-spiked pooled plasma with CS concentration 1.8 μmol L^{-1}, denoted as CS-L. Spiked plasma with CS at concentrations of 6.0, 73 and 120 μmol L^{-1} denoted as CS-M, CS-H and CS-VH respectively. To determine within-run precision, samples were run 20 times in a single run. For between-run precision, samples were run once a day for 20 days. Within-run and between-run precisions were expressed as coefficients of variation (CV, %). The CDER's acceptance criteria for precision (CV < 15%) was applied [27].

For "QC 4" and "CS-VH", since the expected concentrations were above the upper limit of the standard curve, they were diluted with water (1:1, v/v) before SPE and results multiply by 2 to obtain the reported values. This helps to show the ability to dilute samples originally above the upper limit of standard curve.

2.7.4. Stability

For stability studies, 6 plasma samples were collected and split into aliquots. They were stored at room temperature (RT), 4, −20 and −70 °C and analyzed at intervals over 3 months of storage. The stability of the CS stock standard was also determined under the same conditions. Since concentration of the CS stock standard was very high, it was diluted to 100 μmol L^{-1} before analysis. Freeze/thaw stability of plasma CS was investigated by up to three cycles of freezing at −20 °C and unassisted thawing. Stability of CS after sample preparation was evaluated by keeping the freshly prepared and measured samples in the auto-sampler (on board) at 20 °C for 24 h. All results obtained from these stability experiments were compared with the results of the samples freshly measured.

3. Results and discussion

For the determination of CS in plasma by MS based methods, only a few articles were found. They used GC [21], fast atom bombardment MS [22] or LC/MS [23]. All articles were published more than 20 years ago with complicated sample preparation procedures, large sample volume requirements, and narrow dynamic range. With advancement in technology and higher standards in assay performance required by clinician, a new method for plasma CS determination is appropriate and timely.

Although CS is an ionic compound that easily to be ionized by an ESI source in negative mode, we encountered severe ion suppression when using this mode. As a result, an APCI source in negative mode was used and ion suppression was reduced.

CS and CS-*d*7 were monitored by MRM with mass transitions: *m/z* 465.3 > 97.1 and 465.3 > 80.1 for CS and 472.3 > 97.1 for CS-*d*7. For CS, *m/z* 465.3 > 97.1 gave a better *S*/*N* ratio and was used as quantifier; *m/z* 465.3 > 80.1 was used as qualifier. A typical chromatogram is shown in Fig. 1

3.1. Linearity, recovery, matrix effect, LLOQ and LLOD

The methanol calibrators and plasma-based calibrators were subjected to linear regression analysis of peak area ratios of CS/IS versus CS concentrations, all calibration curves showed correlation with R^2 greater than 0.999 ($y=0.2083x+0$) and were considered linear, and the assay linearity was up to at least 100 μmol L^{-1}.

CS was fortified at different concentrations ranging from 0.08 to 100 μmol L^{-1} to plasma samples. After trying various organic solvents to improve assay recovery, we found that SPE elution with chloroform/methanol (2:1, v/v) gave the highest recovery and was selected. The mean recovery for CS was 99.4% with a range of 85.7–112.7% with CV less than 15% (Table 1).

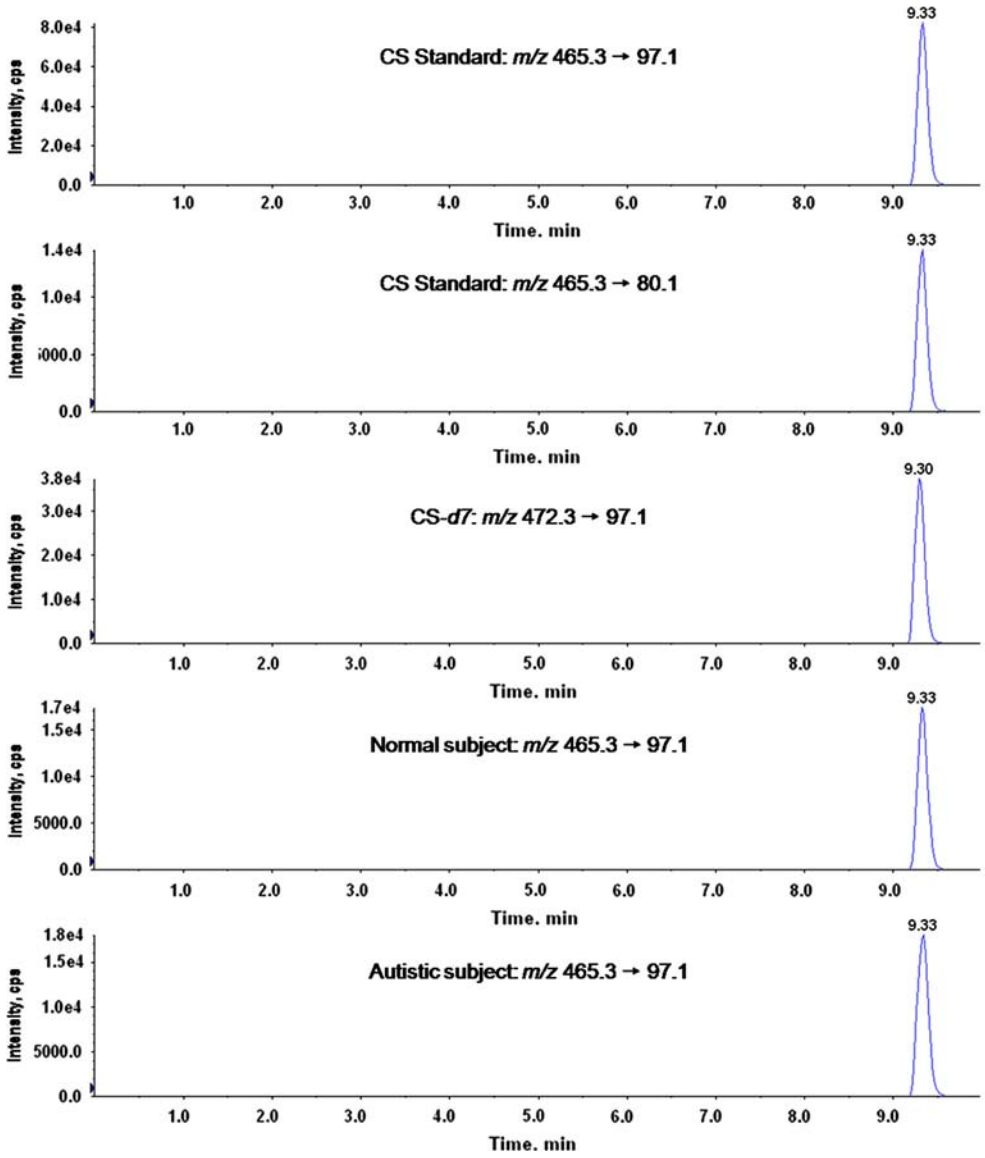

Fig. 1. Chromatogram of CS and CS-*d7*.CS-quantifier (pane 1), CS-qualifier (pane 2) both at 10 μmol L^{-1}, CS-*d7* (pane 3) at 5 μmol L^{-1}, normal child (pane 4) and autistic child (pane 5).

For matrix effects, two different types can occur, AME and RME. Unlike the study by Shackleton and Reid [23], we found washing the SPE with hexane and chloroform were essential to reduce ion suppression (Table 2 and Fig. 2). Protein precipitation can be accomplished simply by mixing plasma samples with methanol and vortexing for 1 min. The mean AME of our CS assay was 101.4% (range: 98.3–104.7%) with CV of less than 2.8%, and RME of 100–2.8=97.2%. The LLOQ for the assay was 0.08 μmol L^{-1}; and the LLOD was 0.02 μmol L^{-1}.

Table 1
Recovery characteristics of LC–MS/MS for plasma CS, $n=6$.

μmol L^{-1}	Mean recovery (%)	CV (%)
0.08	85.7	14.83
0.5	88.1	9.47
1.0	92.7	6.25
5.0	102.8	4.13
10	96.5	3.60
20	109.6	1.78
50	106.4	2.79
100	112.7	3.09

3.2. Accuracy and precision

For accuracy, deviation between the mean measured value and the theoretical value at four different concentrations are given in Table 3. When CS deviated $<15\%$ from the mean it was considered acceptable [27]. Table 3 also shows the precision data with between-run CVs ranging from 1.95% to 8.06% and within-run CVs ranging from 1.71% to 7.15%.

3.3. Sample stability

Results showed minimal change (range from −7.1% to +2.4%) in CS concentrations under the studied conditions (Table 4); this suggested that CS was stable in human plasma for at least three months when stored below −20 °C.

Table 2
Degree of ion suppression by using different solvents during sample clean-up (CS spiked in plasma with a concentration of 10 μmol L^{-1}).

Solvent[a]	Degree of ion suppression (%)
Methanol	85
Ether	70
Ethyl acetate	87
Hexane	22
Chloroform	38
Hexane+chloroform	4.3

[a] 2 mL each.

3.4. Application to biological samples

Reference interval was established according to CLSI guidelines C28-A3c [28]. The distributions of CS in the control plasma were skewed toward the right. After log transformation, all results were normally distributed as assessed by the Anderson–Darling test ($p > 0.05$). Demographic data of normal and autistic children are presented in Table 5. The data from the autistic patient and normal subjects were compared using the Kruskal–Wallis test, as a nonparametric alternative to the ANOVA test. Differences were considered significant when the two-sided p values were less than

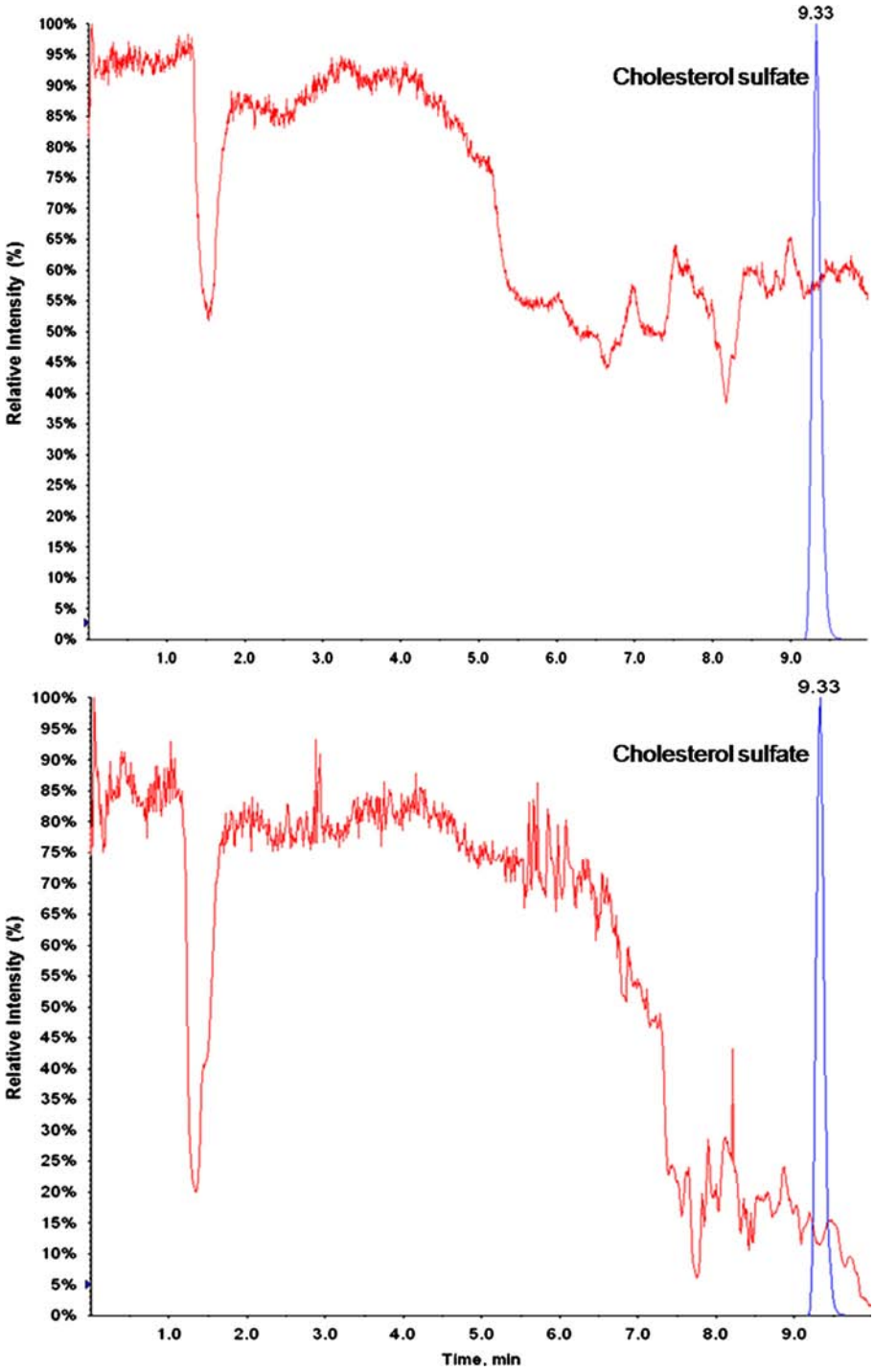

Fig. 2. A typical chromatogram showing the washing effect. Washing with hexane and chloroform (upper pane); without hexane and chloroform (lower pane).

Table 3
Accuracy and precision of LC–APCI-MS/MS of plasma CS.

	Theoretical value (μmol L^{-1})	Mean measured value (μmol L^{-1})	Accuracy (%)	CV (%)
Accuracy characteristic ($n=5$)				
QC 1	2.00	1.78	89.0	7.76
QC 2	10.0	9.15	91.5	5.84
QC 3	60.0	64.6	107.7	1.90
QC 4	150.0	143.7	95.6	2.13

	Target value (μmol L^{-1})	Within-run		Between-run	
		Mean measured value (μmol L^{-1})	CV (%)	Mean measured value (μmol L^{-1})	CV (%)
Precision characteristic ($n=20$)					
CS-L	1.80	1.83	7.15	1.94	8.06
CS-M	6.00	5.74	4.92	5.66	6.44
CS-H	73.0	76.3	1.71	77.2	1.95
CS-VH	120.0	125.1	1.98	124.7	2.04

Table 4
Plasma CS stability after storage under various conditions.

Storage condition	Mean difference[a] (%)	CV (%)
24-h RT	−5.6	5.4
1 week 4 °C	−2.1	3.1
2 weeks −20 °C	+1.4	4.1
1 month −20 °C	−3.6	4.8
2 months −20 °C	−4.3	5.5
3 months −20 °C	−3.4	2.7
2 weeks −70 °C	−1.9	4.3
1 month −70 °C	−2.7	3.7
2 months −70 °C	−2.2	2.8
3 months −70 °C	−3.7	2.4
1 × freeze/thaw	+2.4	3.1
2 × freeze/thaw	−4.9	3.8
3 × freeze/thaw	−7.1	4.3
On board	−4.8	6.3

[a] Difference of the CS results after storage under mentioned conditions compared with the CS results of the same samples freshly measured ($n=6$).

Table 5
Demographics of normal and autistic subjects.

	Males		Females	
	Normal	Autistic	Normal	Autistic
Number (n)	49	49	53	53
Min. age (y)	2.0	2.1	2.0	2.1
Max. age (y)	9.7	8.3	9.0	8.1
Mean age (y)	3.9	3.3	3.9	3.7
Median age (y)	3.0	2.9	3.2	2.9

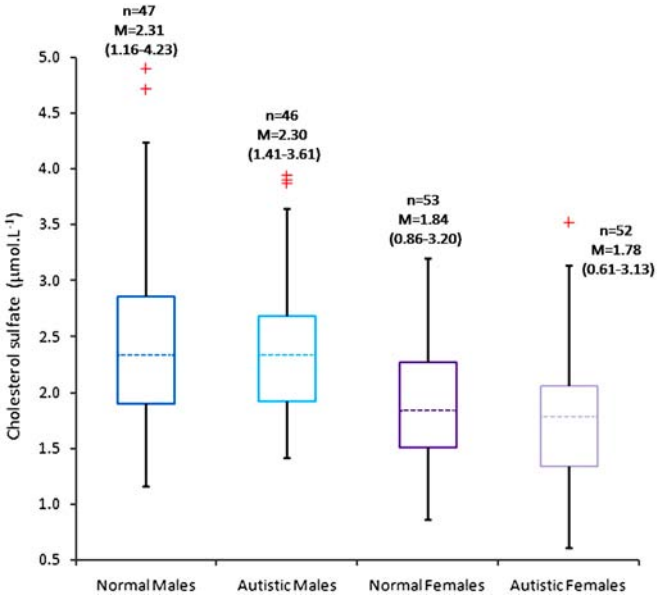

Fig. 3. Cholesterol sulfate concentrations for the 4 sample groups: normal males, autism males, normal females, and autism emales. Dash lines=medians; boxes=higher and lower quartiles; whiskers=higher and lower non-outlier values; and plus sign=outliers. For each group (excluding outliers): n=number of samples; M=median; minimum and maximum values are in brackets (μmol L^{-1}).

0.05. All data analysis was made using the Analyze-it for Microsoft Excel version 2.26 (Analyze-it Software, Ltd., Leeds, UK).

The subjects were divided into four groups according to gender and clinical status. The analysis of plasma specimens from normal males ($n=47$) and normal females ($n=53$) showed significant gender differences ($p<0.001$) in CS levels. Plasma CS levels were higher for normal males (median=2.31 μmol L^{-1}; range, 1.16–4.23 μmol L^{-1}) compared to normal females (median=1.84 μmol L^{-1}; range, 0.86–3.20 μmol L^{-1}) which was in agreement with previously published data [21]. But our data on reference intervals for both males and females were lower than those reported by Muskiet et al (male: 2.87–6.90 μmol L^{-1}, female: 2.51–4.59 μmol L^{-1}) [21]. This difference is probably due to more efficient sample clean-up in our method. When compared to another LC–MS method [23], our method used a simpler SPE procedure for all samples and showed a wider dynamic range. Shackleton and Reid [23] used different sample preparation procedures for plasma from normal subjects and from X-linked ichthyosis patient. Their method also required two sets of standards in order to cover both normal and high values.

Our data showed no significant differences in plasma CS levels for autistic males (median=2.30 μmol L^{-1}; range, 1.41–3.61 μmol L^{-1}) compared to normal males. Plasma CS levels were slightly lower for autistic females (median=1.78 μmol L^{-1}; range, 0.61–3.13 μmol L^{-1}) compared to normal females, and the two groups overlap with no significant difference ($p>0.05$) (Fig. 3).

The deviation of our study results from that of the hypothesis might be due to sampling time. Perhaps we need to measure CS levels in pregnant women. We should measure the plasma CS level of pregnant subjects especially during the stage of fetal brain development, such as (1) at week 3, when the fetal brain begin to

form, and (2) at week 29–40, the third trimester stage, when the brain is increasing rapidly in size. In addition, we should measure the children's plasma CS level early in their life, at the postnatal period. By measuring plasma CS at these times and monitoring the growth of children, we might be able to have a clearer picture of the relationship between plasma CS and autism.

4. Conclusion

In summary, we have developed and fully validated a high-throughput bioanalytical method for the determination of plasma CS by LC–APCI-MS/MS. This method requires less sample volume and simpler sample preparation and has a wider dynamic range when compared with methods reported by others [21–23]. We reported here the first set of data for pediatric reference of plasma CS in local Chinese children aged 2–10 years, and we found a gender difference with male levels higher than female.

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