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Research Article

# Tandem mass spectrometry of serum cholestanoic ($C_{27}$) acids – Typical concentration ranges and application to the study of peroxisomal biogenesis disorders

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ABSTRACT

*Background:* Primary bile acid synthesis is impaired in peroxisomal disorders, leading to the accumulation of long-chain bile acids, specifically dihydroxycholestanoic and trihydroxycholestanoic acids. Quantification of the diastereoisomers of these $C_{27}$ bile acids is essential for the differential diagnosis of these disorders.

*Methods:* High-performance liquid chromatography electrospray ionization-tandem mass spectrometry with stable-isotope dilution was used to quantify all eight diastereoisomers of cholestanoic acids in serum. Clinical ranges were established for patients with and without cholestatic liver disease, as well as for those with peroxisomal disorders.

*Results:* The assay was linear over the range of 20–2,500 ng/mL, and intra- and inter-assay imprecision was <20 % CV. The mean (±SEM) serum concentration of total $C_{27}$ bile acids in 20 adult controls was low (0.007 ± 0.004 μmol/L). In non-cholestatic, moderately cholestatic, and severely cholestatic patients, total $C_{27}$ bile acids measured 0.015 ± 0.011, 0.129 ± 0.034, and 0.986 ± 0.249 μmol/L, respectively. In contrast, patients with confirmed peroxisomal disorders (n = 49) exhibited concentrations >10-fold higher (14.06 ± 2.59 μmol/L). Patients with heterozygous mutations in PEX genes had low concentrations of serum $C_{27}$ bile acids. In all groups, the (25S)- and (25R)-diastereomers were present in a ratio of 0.3. In cases of 2-methylacyl-CoA racemase deficiency, serum total $C_{27}$ bile acids were markedly elevated (10.61 ± 0.92 μmol/L) and comprised exclusively the (25R)-diastereoisomer.

*Conclusions:* This tandem mass spectrometric assay quantifies all diastereoisomers of the $C_{27}$ cholestanoic acids in serum and was used to establish typical clinical concentration ranges. The method is applicable to the diagnosis of peroxisomal disorders and differentiates 2-methylacyl-CoA racemase deficiency from other peroxisomal biogenesis disorders.

*Abbreviations:* AMACR deficiency, 2-methylacyl-CoA racemase deficiency; CA, cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; CDCA, chenodeoxycholic acid, 3 α,7α-dihydroxy-5β-cholanoic acid; DCA, deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; DHCA, 3α,7α-dihydroxy-5β-dihydroxycholestanoic acid; GCA, Glycocholic acid; GCDCA, Glycochenodeoxycholic acid; GDCA, Glycodeoxycholic acid; GLCA, Glycolithocholic acid; GUDCA, Glycoursodeoxycholic acid; LCA, lithocholic acid, 3α-hydroxy-5β-cholanoic acid; PBD, Peroxisome biogenesis disorder; Tauro-DHCA, tauro conjugate of dihydroxycholestanoic acid; Tauro-THCA, tauro conjugate of trihydroxycholestanoic acid; D3-(25R)- and (25S)-DHCA, racemic mixture of (25R)- and (25S)-[26,26,26-2H3]3α,7α-dihydroxy-5β-cholestanoic acid; D3-(25R+25S)-THCA, racemic mixture of of (25R)- and (25S)-[26,26,26-2H3]3α,7α,12α-trihydroxy-5β-trihydroxycholestanoic acid; D4-Tauro-(25R)- and (25S)-DHCA, racemic mixture of [2H4]taurine conjugate of 3α,7α-dihydroxy-5β-cholestanoic acid; D4-Tauro-(25R)- and (25S)-THCA, racemic mixture of [2H4] taurine conjugate of 3α,7α,12α-trihydroxy-5β-trihydroxycholestanoic acid; TCA, Taurocholic acid; TCDCA, Taurochenodeoxycholic acid; TDCA, Taurodeoxycholic acid; THCA, 3α,7α,12α-trihydroxy trihydroxy-5β-cholestanoic acid; TLCA, Taurolithocholic acid; TUDCA, Tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; ZSD, Zellweger spectrum disorder.

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## Introduction

Peroxisomal biogenesis disorders (PBD), also referred to as Zellweger spectrum disorders (ZSD), are characterized by abnormal peroxisome biosynthesis, assembly and function. These autosomal recessive disorders are caused by mutations in various PEX genes or by single enzyme deficiencies [1–4]. There are at least 13 PEX genes and mutations have been reported causing a broad spectrum of disease in the Zellweger spectrum disorders. Single enzyme deficiencies in peroxisome function include, 2-alpha-methylacyl-CoA racemase (AMACR) deficiency [5,6], di-bifunctional protein deficiency (DBP) [7], sterol carrier protein X (SCPx) [8] and peroxisomal acyl-Coenzyme-A oxidase (SCOX) [9]. The differential diagnosis of a peroxisomopathy can be challenging, in part because peroxisomal biogenesis disorders present as a broad and overlapping phenotype that may include neurological disease, severe developmental delay, mild degenerative disease, ataxia, hearing loss, visual impairment, adrenal insufficiency and liver disease [4].

Peroxisomes play a key role in bile acid synthesis, being critical to side-chain oxidation of $C_{27}$ cholestanoic acid intermediates in the biosynthetic cascade from cholesterol to the primary $C_{24}$ bile acids, cholic (CA) and chenodeoxycholic (CDCA) acids [10,11]. DHCA (3α,7α-dihydroxy-5β-cholestanoic acid) and THCA (3α,7α,12α-trihydroxy-5β-cholestanoic acid) are the key intermediates in this complex pathway (Fig. 1) and these long chain cholestanoic acids accumulate in most, but not all, patients with peroxisomal disorders [2,12,13].

Identifying peroxisomal disorders requires a battery of biochemical tests, including the measurement of branched-chain fatty acids, very long-chain fatty acids, and hepatotoxic atypical $C_{27}$ cholestanoic acids. Urine analysis by fast atom bombardment ionization-mass spectrometry (FAB-MS) has proven a powerful tool in the discovery and diagnosis of genetic defects in bile acid synthesis [14], including peroxisomopathies [14,15], but, in the latter case, urinary $C_{27}$ bile acids are elevated only when there is significant liver dysfunction. However, an elevation in serum $C_{27}$ cholestanoic acids is a consistent feature of impaired side-chain oxidation, independent of whether there is liver dysfunction, and provides a highly sensitive indicator of a peroxisomal disorder [16]. Classically, taurine conjugates of THCA and DHCA have been measured in serum by gas chromatography (GC), with or without a mass spectrometer. This approach, however, requires time-intensive hydrolysis of the taurine conjugate, derivatization of the C-27 carboxylic acid and derivatization of the steroid ring hydroxyls to form a volatile and thermally-stable derivative [16–19]. More recently, liquid chromatography tandem mass spectrometry (LC-MS) methods have been described, but few afford separation of all of the diastereoisomers of DHCA, THCA and their conjugates, and in most cases, stable-labeled internal standards were not used for quantification [20–22].

We describe a stable-isotope dilution assay for the accurate quantification of the diastereoisomers of the unconjugated and taurine conjugated (25R)- and (25S)- cholestanoic acids in a single analysis, therefore permitting definitive diagnosis of an AMACR deficiency [5,6] from other peroxisomal biogenesis disorders. By application of this method we also report normal ranges for serum individual and total $C_{27}$ cholestanoic acids in adults, cholestatic infants and children, and a large cohort of patients with peroxisomal biogenesis disorders. These data are critical to establishing threshold levels for diagnosis of these disorders and for monitoring response to bile acid therapies [23–27].

## Materials and methods

### *Chemicals and reagents*

The reference compounds, DHCA and THCA, and the respective taurine conjugates and the corresponding stable-isotope labelled internal standards, (25S)- and (25R)-[$^2H_4$]Tauro-THCA, (25S)- and (25R)-[$^2H_4$]Tauro-DHCA, (25S) and (25R)-[$^2H_3$]THCA, and (25S)- and (25R)-[$^2H_3$]DHCA were custom synthesized by IRBM Science S.p.A. (Pomezia, Italy). All other solvents and chemicals were of analytical grade purity and obtained from Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA).

### *Human subjects*

This study was performed on de-identified, to be discarded, sera obtained after clinical investigations were completed. As only fully anonymized patient samples were used that were not obtained specifically for use in this study through an interaction or intervention with living individuals, neither informed consent nor IRB review were required. The only information provided with the deidentified sera was the confirmed diagnosis, and the patient age and sex in order to obtain normalized clincal ranges for the analytes in peroxisomal disorders. Sera were obtained from patients with Zellweger Spectrum Disorders (ZSD, 49 cases) and from adults not being investigated for liver disease, which served as controls (n = 20; age range, 18–62 yr). The cases had genetically confirmed peroxisomal biogenesis disorders and single enzyme defects (age range 1 week−18.9 yr). Of these, seven were confirmed to have AMACR deficiency. Heterozygous mutations in PEX genes were

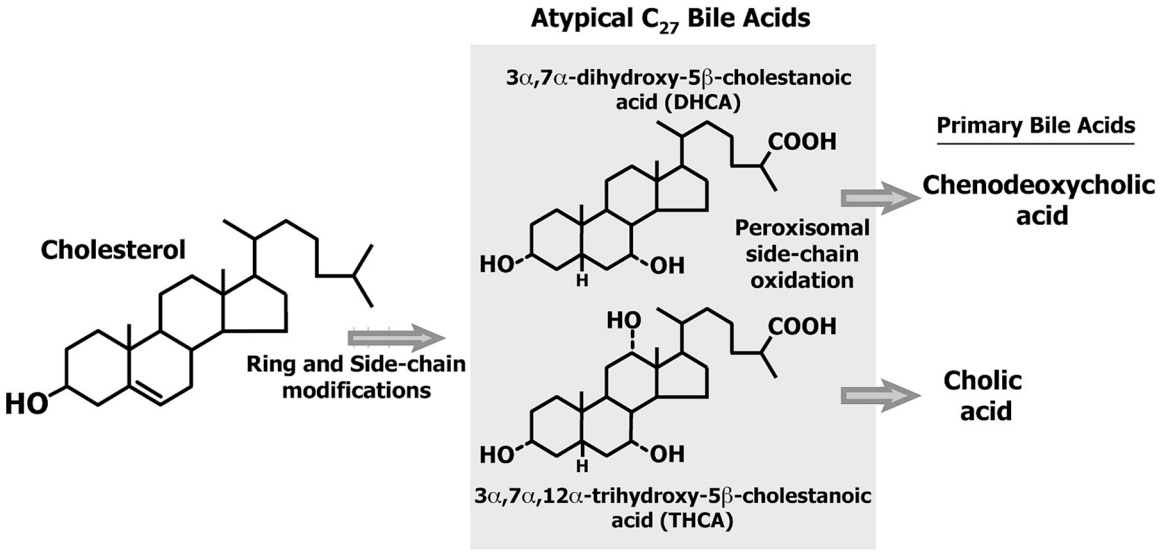


**Fig. 1.** The chemical structures of the key intermediate cholestanoic acids in the pathway for primary bile acid synthesis from cholesterol.

reported in 17 patients (age range 0.2–16.0 yr). None of the patients were on ursodeoxycholic acid or cholic acid therapy. Additionally, samples of discarded sera were obtained from de-identified pediatric patients being evaluated for underlying liver disease that were non-cholestatic and had serum total $C_{24}$ bile acid concentrations (sTBA) of <8 μmol/L (n = 8, ages 4.4 mo−18.8 yr), patients with mild to moderate cholestasis and sTBA concentrations 10–100 μmol/L (n = 10, ages 0.4 mo-17.7 yr), and patients with severe cholestasis and sTBA concentrations >100 μmol/L (n = 9, ages 0.4 mo-14.4 yr).

*Preparation of standards and samples for LC-MS/MS analysis*

Calibrators and Quality Control (QC) samples were prepared by spiking the reference compounds to charcoal stripped human serum (Cone Bioproducts, Seguin, TX). Calibrators over the concentration range 20–2500 ng/mL and four QC samples at concentrations of 40, 160, 800, 2000 ng/mL were prepared. Specifically, a series of standard mixture solutions at different concentration levels were prepared by serial dilution from stock solutions (1 mg/mL in 100 % MeOH), then spiked into matrix. Calibrators and QCs were prepared by spiking 50 μL of the standard solution into 950 μL stripped serum. All calibrators and QC samples were stored at −20 °C. The internal standard, a mixture of six compounds (50 μL of a 1 ng/μL methanolic solution) was added to serum (100 μL) from healthy subjects and patients with established peroxisomal disorders, and also to the calibrators and QC samples. The samples were vortexed for 10 secs and acetonitrile (1 mL) was added. The sample was centrifuged at 13,400 g at 4 °C for 10 min, and the supernant transferred to a glass tube and dried under nitrogen gas at 60 °C. The extract was redissolved in 200 μL of 70 % methanol/$H_2O$ and 10 μL injected on column.

*Analysis of dihydroxy-cholestanoic and trihydroxy-cholestanoic species by LC-MS/MS*

Quantitative analysis of DHCA and THCA was performed by ultra-high performance liquid chromatography electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) using a Waters TQ-XS triple quadruple mass spectrometer interfaced with a Waters Acquity UHPLC system (Milford, MA). Nitrogen was the nebulizer gas and argon the collision gas. The source temperature was maintained at 150 °C, and the desolvation temperature was maintained at 500 °C. The drying gas ($N_2$) was maintained at approximately 600 L/h, and the cone flow gas was set at 150 L/h. The multiplier was set at an absolute value of 500 V. Optimized parameters, i.e., cone voltage and collision energy for $C_{27}$ bile acid intermediates were determined by prior direct infusion of the individual compounds interfaced with LC flow (Supplemental Table 1). For quantification, the ESI-MS/MS was operated under negative ion mode and with multiple reaction monitoring (MRM). Detection of the conjugated cholestanoic acids was performed by monitoring transitions of the individual deprotonated parent ions and the common daughter ion at *m/z* 79.8. The following transition ions were monitored; *m/z* 556.1 > 79.8 for taurine conjugated trihydroxycholestanoic acids (Tauro-THCA), *m/z* 540.1 > 79.8 for taurine conjugated dihydroxycholestanoic acids (Tauro-DHCA). The analogous MRM transitions monitored for the internal standards were *m/z* 560.1 > 79.8 for (25S)- and (25R)-[$^2H_4$]Tauro-THCA and *m/z* 544.1 > 79.8 for (25S)- and (25R)-[$^2H_4$]Tauro-DHCA. With no significant collision-induced fragmentation of the unconjugated cholestanoic acids, only the parent ions were monitored. These were *m/z* 449.2 > 449.2 for (25S)- and (25R)-THCA and *m/z* 433.2 > 433.2 for (25S)- and (25R)-DHCA. The corresponding ions monitored for the internal standards were *m/z* 452.2 > 452.2 for the (25S)- and (25R)-[$^2H_3$]THCA and *m/z* 436.2 > 436.2 for (25S)- and (25R)-[$^2H_3$]DHCA reflecting the shift in mass due to deuterium incorporation.

The individual bile acid species were separated by HPLC on a Thermo ODS HYPERSIL C18 (5 μm, 250 x 4.6 mm) column (Thermo, Waltham, MA) with gradient elution consisting of mobile phase A (5 mM ammonium formate in methanol/water (70:30, v/v), with 100 μL formic acid and adjusted to pH 5.0) and mobile phase B (0.2 % formic acid in 100 % acetonitrile). The gradient profile was held at 100 % mobile phase A for 15 min, increasing to 100 % mobile phase B in 10 min, and reverting back to 100 % mobile phase A in 0.1 min, and then held at 100 % for 5 min for re-equilibration of the column. The LC flow rate was 0.5 mL/min and the total chromatographic run time was 30 min.

*Analysis of individual $C_{24}$ bile acids species by LC-MS/MS*

Total and individual $C_{24}$ bile acids in serum were measured by a separate clinical assay using stable-isotope dilution analysis with LC-ESI-MS/MS. The assay was fully validated according to CAP/CLIA requirements and performed in a CAP/CLIA accredited laboratory (CAP number 1667801; CLIA Number 36D0656333). The clinically relevant primary and secondary bile acids and their conjugates were quantified and these included TCA, TUDCA, TCDCA, TDCA, TLCA, GCA, GUDCA, GCDCA, GDCA, GLCA, CA, UDCA, CDCA, DCA, and LCA. Total $C_{24}$ bile acids was determined from the sum of the concentrations of the 15 individual bile acid species.

LC-ESI-MS/MS was performed on a Waters TQ-XS triple quadruple mass spectrometer interfaced with a Waters Acquity UHPLC system (Milford, MA). Nitrogen was the nebulizer gas and argon the collision gas. The source temperature was maintained at 150 °C, and the desolvation temperature was maintained at 500 °C. The drying gas ($N_2$) was maintained at approximately 600 L/h, and the cone flow gas was set at 150 L/h. The multiplier was set at an absolute value of 500 V. Optimized parameters, i.e., cone voltage and collision energy for $C_{24}$ bile acid was determined by prior infusion of the individual compounds interfaced with LC flow. For quantification, the ESI-MS/MS was operated under negative ion mode and with MRM, with detection of the transitions of the individual deprotonated parent ions and their common daughter ion *m/z* 79.8 for taurine conjugated bile acids, individual deprotonated parent ions and their common daughter ion *m/z* 73.9 for glycine conjugated bile acids. *m/z* 407.1 > 407.1 for CA, *m/z* 391.1 > 391.1 for UDCA + CDCA + DCA, *m/z* 375.1 > 375.1 for LCA were used for monitoring these five unconjugated species. The individual bile acid species were separated by HPLC on a Kinetex C18 (2.6 μm, 100 x 3.0 mm) column (Phenomenex, Torrance, CA) with gradient elution consisting of mobile phase A (20 % Acetonitrile/Water with 10 mM $NH_4OAc$) and mobile phase B (80 % Acetonitrile/Water with 10 mM $NH_4OAc$). The total run time was 20 min. The internal standard, a cocktail of 15 deutrium-labeled standards (50 μL of a 1 ng/μL methanolic solution) was added to serum (20 μL) from healthy subjects and patients with a established peroxisomal disorders, and also to the Calibrators and QC serum samples. The samples were vortexed for 10 secs before addition of acetonitrile (200 μL). After vortex and centrifuge at 13,400 g at 4 °C for 10 min, the supernatant was poured into a glass tube. The supernatant was dried under nitrogen gas at 65 °C and then redissolved in 250 μL of the reconstitution solution consisting of 50 % methanol/$H_2O$. A 10 μL volume of the sample extract was injected on column.

## Results and discussion

*Development and optimization of the LC-ESI-MS/MS assay for cholestanoic acids*

The ESI negative ion mass spectra of the taurine conjugated $C_{27}$ bile acids revealed an intense singly-charged deprotonated ion $[M-H]^-$, which for Tauro-THCA was at *m/z* 556.1 and Tauro-DHCA was *m/z* 540.1 (Fig. 2). The corresponding internal standards [$^2H_4$]Tauro-THCA and (25R)-[$^2H_4$]Tauro-DHCA also gave singly-charged deprotonated ions with mass shifts to *m/z* 560.1 and 544.1, respectively. With collision-induced dissociation (CID) of the parent ions, all yielded a

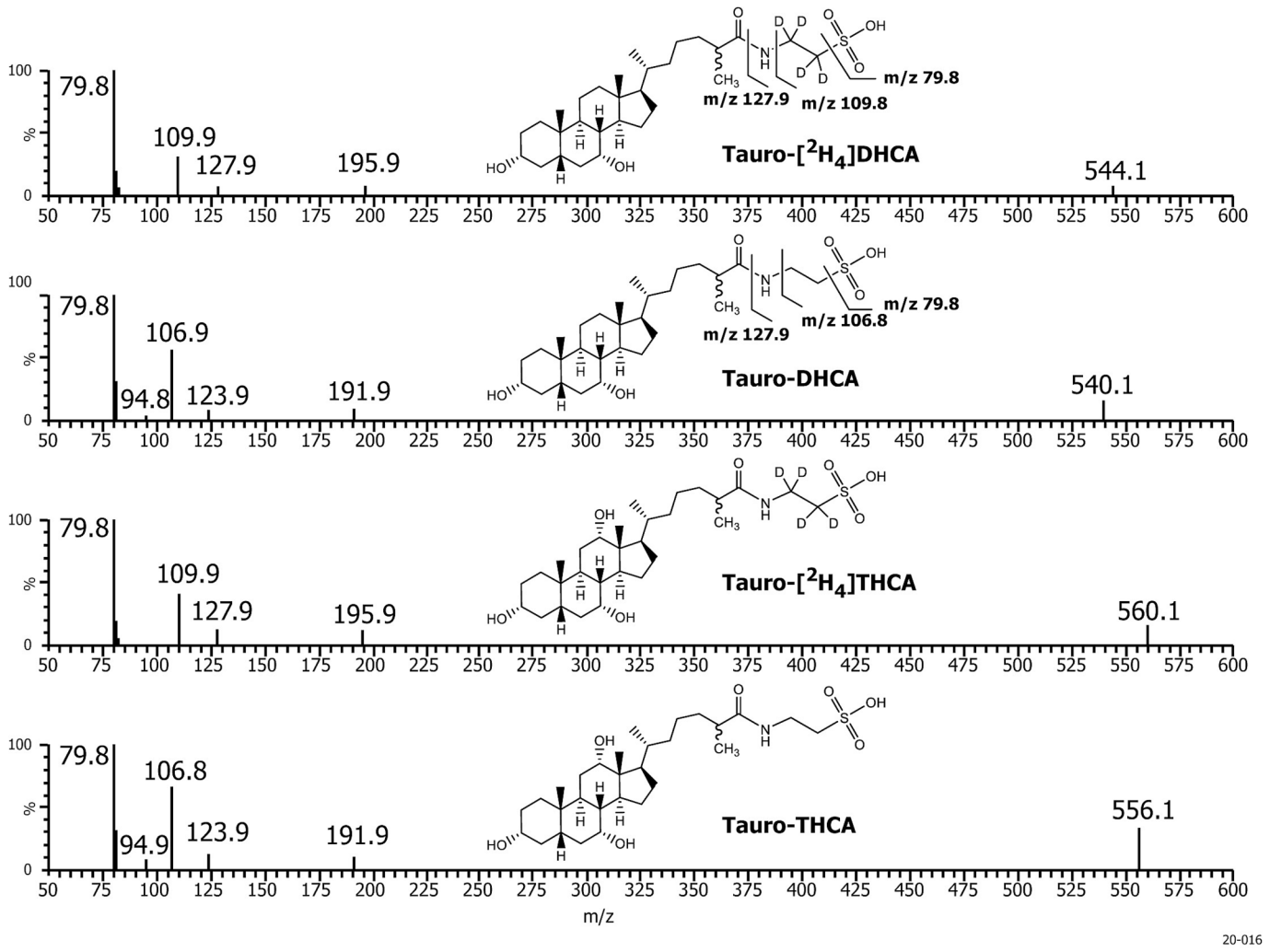


**Fig. 2.** Negative ion MS/MS mass spectra of the racemic forms of [$^2H_4$]Tauro-DHCA, Tauro-DHCA, [$^2H_4$]Tauro-THCA and Tauro-THCA. The chemical structures and fragmentation pattern is indicated along with the position of the deuterium ([$^2$H]) atoms, designated in the chemical structure with the letter 'D'.

common intense base peak at *m/z* 79.8, corresponding to [$HSO_3^-$] ion from the taurine conjugated side chain. Consequently, the deprotonated molecular ion and its [$HSO_3^-$] fragment were selected as the appropriate mass transition pairs for monitoring and quantification. Unconjugated THCA and DHCA also gave intense deprotonated molecular ions at *m/z* 449.2 and 433.2, respectively, but on CID yielded no significant fragments that could be usefully monitored and, therefore, an MRM transition was used of the same parent and daughter ions. The same applied to their deuterium labeled internal standards, [$^2H_3$]THCA and [$^2H_3$]DHCA, which yielded deprotonated molecular ions at *m/z* 452.3 and 436.2, respectively (data not shown).

The four pairs of $C_{27}$ diastereoisomers were separated with baseline resolution using a 250 mm Thermo C18 column with gradient elution. By using acetonitrile rather than methanol and increasing the proportion of formic acid to 0.2 % in mobile phase B, sharp peaks with complete resolution of the unconjugated DHCA and THCA diastereoisomers were achieved, as evidenced from mass chromatograms for a mixture of the eight internal standards spiked to serum (Fig. 3).

*Assay performance*

A full validation of the assay including linearity, within- and between-batch precision and accuracy, freeze–thaw cycle, dilution integrity, short- and long-term stability was performed, and the assay was found to be robust and reproducible. Calibration curves for all $C_{27}$ bile acids were linear up to 2500 ng/mL. The limits of detection (LOD), defined by a signal/noise ratio >5 was 1.5 pg on column for the taurine conjugates of (25S)-THCA, (25R)-THCA, (25S)-DHCA and (25R)-DHCA, and 16 pg for the corresponding unconjugated species. The lowest limit of quantification (LLOQ) defined as the lowest concentration measurable in serum with a coefficient of variation (CV) of <20 % was 40 ng/mL. The analytical recoveries of $C_{27}$ bile acids spiked into stripped human serum ranged from 83.0 – 92.7 % for concentrations of 160, 800, and 2000 ng/mL. Four different concentrations (40, 160, 800, 2000 ng/mL) of QC samples of human serum were measured to evaluate precision (CV%) and accuracy (bias%) for the eight analytes (detailed data in Table 1). The matrix effect was evaluted by comparing the response of post-spiked standards into a blank matrix extract to that of pure standards. The matrix effect factor was determined to be in the range of 0.9–1.1 for all $C_{27}$ bile acids.

The stability of the $C_{27}$ bile acids was evaluated by analyzing stripped human serum spiked with the reference standards at concentrations of 160 ng/mL and 2000 ng/mL, respectively, for the QC-low and QC-high samples, which were subjected to multiple freeze–thaw cycles according to the FDA bioanalytical assay validation guidance. After three freeze–thaw cycles of the QC-Low and QC-High samples the % differences ranged from −2.71 to 1.79 % and −1.22 to 0.93 %, respectively, at −20 °C. The $C_{27}$ bile acids were also stable for 6 h at ambient temperature, for 24 h in the autosampler, and when stored for 1, 3 and 6 months at −20 °C (data not shown).

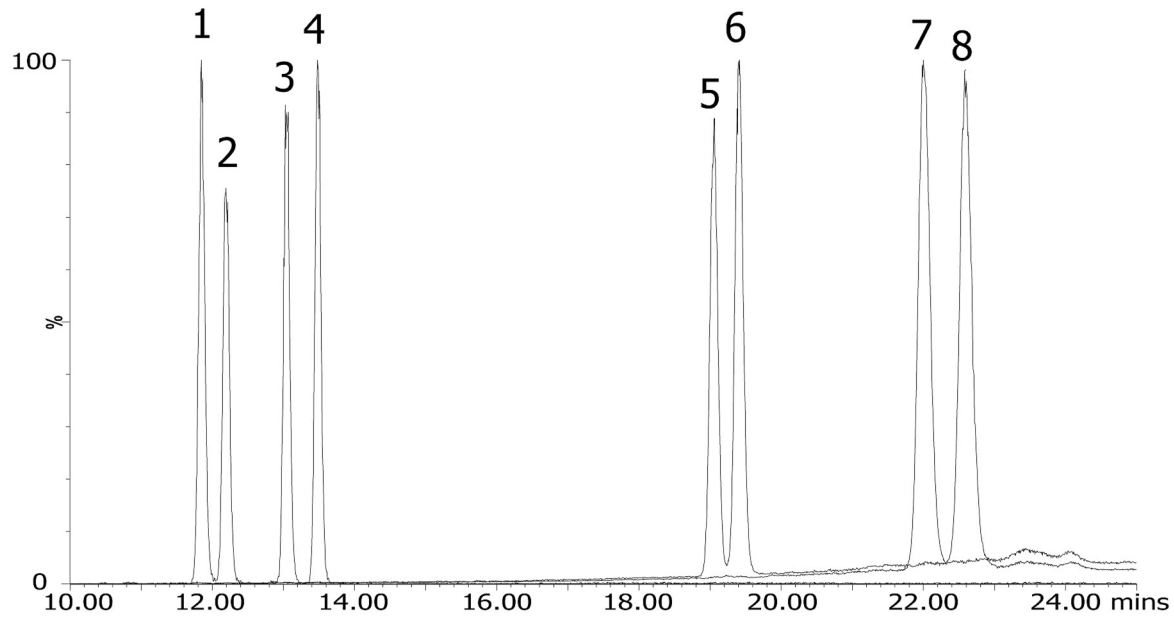


**Fig. 3.** Combined MRM mass chromatograms for the internal standards that were added to a sample of human serum to produce at concentration of 250 ng/mL and separated by HPLC. The following C27 bile acids are indicated numerically as: 1 and 2, (25S)- and (25R)-[$^2H_4$]Tauro-THCA, *m/z* 560.1 > 79.8; 3 and 4, (25S)- and (25R)-[$^2H_4$]Tauro-DHCA, *m/z* 544.1 > 79.8; 5 and 6, (25S)- and (25R)-[$^2H_3$]DHCA, *m/z* 436.2 > 436.2; 7 and 8, (25S)- and (25R)-[$^2H_3$]THCA *m/z* 452.2 > 452.2.

**Table 1**
Between-batch and within-batch imprecision (CV%) and accuracy (Bias%) of four levels of quanlity control samples. Data from six replicates of each level of QC within-batch and five separate batches (n = 6 x 5 = 30 measurements) for between-batch validation experiments.

| C27 Bile acid species | Between-batch CV% (n = 6 × 5 = 30) | | | | Between-batch bias% (n = 6 × 5 = 30) | | | |
|---|---|---|---|---|---|---|---|---|
| QC | 40 ng/mL | 160 ng/mL | 800 ng/mL | 2000 ng/mL | 40 ng/mL | 160 ng/mL | 800 ng/mL | 2000 ng/mL |
| 25S-Tauro-THCA | 5.4 | 2.9 | 4.0 | 2.9 | −2.0 | 0.8 | −1.5 | −4.8 |
| 25R-Tauro-THCA | 5.9 | 2.7 | 2.6 | 2.1 | 2.8 | 1.0 | −1.0 | −5.3 |
| 25S-Tauro-DHCA | 4.9 | 2.8 | 2.7 | 2.5 | 3.0 | 0.5 | −1.2 | −5.6 |
| 25R-Tauro-DHCA | 4.5 | 3.0 | 4.5 | 3.5 | −0.7 | −1.9 | −4.5 | −7.5 |
| 25S-THCA | 8.1 | 1.7 | 1.8 | 1.4 | 3.2 | 9.4 | 6.9 | −1.2 |
| 25R-THCA | 9.1 | 2.4 | 2.3 | 1.9 | 0.4 | 8.8 | 6.8 | −1.2 |
| 25S-DHCA | 5.8 | 1.7 | 2.0 | 1.7 | 7.7 | 13.0 | 8.5 | 0.8 |
| 25R-DHCA | 9.9 | 2.7 | 2.0 | 1.6 | 1.0 | 8.9 | 8.8 | 1.2 |
| | | | | | | | | |
| C27 Bile acid species | Within-batch CV% (n = 6) | | | | Within-batch bias% (n = 6) | | | |
| 25S-Tauro-THCA | 0.0 | 2.6 | 3.1 | 1.0 | −4.3 | −0.3 | −2.9 | −5.2 |
| 25R-Tauro-THCA | 7.0 | 1.8 | 2.1 | 2.6 | 4.3 | 2.0 | 0.3 | −4.6 |
| 25S-Tauro-DHCA | 0.0 | 2.5 | 1.6 | 2.7 | 5.2 | −0.8 | −3.5 | −5.8 |
| 25R-Tauro-DHCA | 0.0 | 1.9 | 1.1 | 1.3 | −2.3 | −3.4 | −5.6 | −6.8 |
| 25S-THCA | 5.0 | 1.1 | 1.9 | 1.2 | −2.1 | 8.4 | 5.7 | −0.6 |
| 25R-THCA | 6.2 | 1.9 | 1.2 | 0.8 | −11.4 | 5.8 | 5.7 | −0.9 |
| 25S-DHCA | 5.2 | 1.9 | 1.5 | 1.0 | 11.9 | 12.7 | 7.2 | 1.0 |
| 25R-DHCA | 5.5 | 2.9 | 1.5 | 0.9 | 3.5 | 10.4 | 7.9 | 2.1 |

*Clinical application and utility of LC-ESI-MS method*

Dihydroxy- and trihydroxy-cholestanoic acid diastereoisomers are the major $C_{27}$ bile acid intermediates in the pathway for primary bile acid synthesis (Fig. 1). The final steps in this pathway involve side-chain shortening, with the loss of three carbons and the formation of a C-24 carboxylic acid that is then conjugated to taurine and glycine. These reactions are catalyzed by enzymes residing within the peroxisome [1,10–12]. Thus, disorders of peroxisomal assembly, structure or function generally lead to impaired synthesis of primary bile acids and the accumulation of long-chain cholestanoic acids in serum and urine. The diagnosis of a peroxisomal disorder requires a battery of biochemical tests complemented by molecular diagnosis to identify specific gene mutations in PEX genes. Biochemical confirmation of a peroxisomopathy includes the measurement of the $C_{27}$ bile acids, THCA and DHCA. The earliest methods for the determination of THCA and DHCA, described decades ago, utilized GC or GC–MS analysis, but stable-labeled internal standards were either not available, or of limited availability [16–19]. Furthermore, the requirement for hydrolysis, derivatization and extensive sample work-up made this approach time-consuming and not ideal for screening. A further drawback of the early methods was the inability to chromatographically separate the diastereoisomers of THCA and DHCA [6], which becomes critical in making a differential diagnosis of a AMACR deficiency from other peroxisomal biogenesis disorders [5,6]. AMACR deficiency is characterized by the exclusive production of only the (25R)-diastereoisomer of THCA and DHCA [5,6] in contrast to other peroxisomal disorders where both diastereoisomers are present in serum and urine [19], and likewise in controls or patients with liver disease (Fig. 4).

Direct analysis of bile acids in urine by FAB-MS was key to the discovery of the first six genetic defects in bile acid synthesis [14,15,28,29]. This soft ionization technique yields mass spectra highlighted by intense

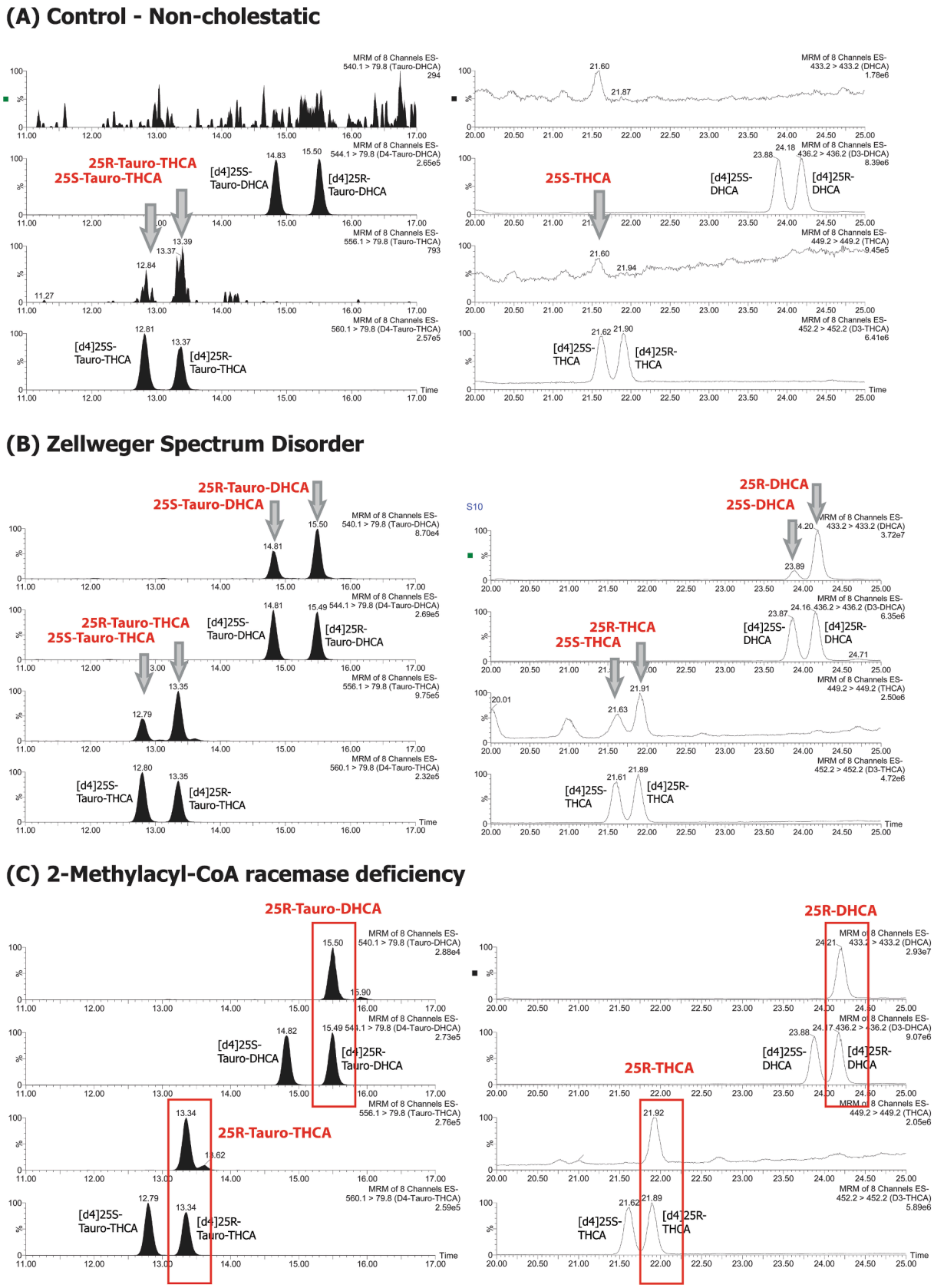


**Fig. 4.** Typical mass chromatograms for the MRM transitions of DHCA, THCA and their taurine conjugates and internal standards are shown for sera from a control subject with no evidence for liver disease, a patient with a confirmed peroxisomal disorder of Zellweger, and a patient with a confirmed 2-methylacyl-CoA racemase deficiency.

deprotonated molecular ions and little to no fragmentation and the spectra generated are definitive for the diagnosis of a bile acid synthesis disorder [14]. While FAB-MS remains a clinically relevant diagnostic tool, it provides only semi-quantitative information [30]. Electrospray ionization (ESI-MS) with direct flow injection and no chromatography yields similar, but not identical, mass spectra to that of FAB-MS, but again suffers from the drawback of not being able to distinguish positional or steroisomeric forms of bile acids [20,31]. From our experience, the diagnosis of a peroxisomal biogenesis disorder is possible by FAB-MS when there is significant underlying liver dysfunction, which is not uncommon in peroxisomal disorders [32]. The ability to accurately quantify concentrations of these bile acid intermediates is, however, crucial not only to diagnosis, but also in the evaluation of response to oral primary bile acid therapy, in which efficacy is contigent upon suppressing endogenous bile acid synthesis to effect a reduction in the synthesis and urinary excretion of these atypical bile acids [23–27].

Very few methods to quantify DHCA and THCA by LC-MS have been described [20–22] and the accuracy of reported values for these $C_{27}$ bile acids is unclear given the lack of availability of all the reference compounds and especially the stable-labeled internal standards. Until recently, only unconjugated THCA and its taurine conjugate were available as reference compounds and there were no isotopically-labeled analogs commerically available for use as internal standards. The LC-ESI-MS/MS methodology described here enables the direct quantification of all conjugated and unconjugated $C_{27}$ bile acids, including the (25S)- and (25R)-diastereoisomers without the need for prior hydrolytic steps. It is the first stable-isotope dilution mass spectrometric assay to utilize all eight reference compounds and to incorporate the corresponding isotopically-labeled standards.

There is a paucity of data on the typical concentrations of $C_{27}$ bile acids in peroxisomal disorders, and in patients with liver disease. By application of this method we report for the first time a comparison of the concentrations of $C_{27}$ bile acids in normal adult and pediatric controls, in patients with cholestasis, and in patients with a variety of peroxisomal disorders. $C_{27}$ bile acids were present in negligible amounts, or in traces, in patients in the absence of liver disease. The mean (±SEM) serum concentration for total $C_{27}$ bile acids in 20 adults (age range 18–62 yr) was 0.007 ± 0.004 μmol/L (Fig. 5). The levels of $C_{27}$ bile acids in patients that were non-cholestatic and had a normal serum total $C_{24}$ bile acid concentration (<8 μmol/L), in patients that were moderately cholestatic with total serum $C_{24}$ bile acids levels between 10 and 100 μmol/L, and in severe cholestatic patients with total $C_{24}$ bile acids levels of >100 μmol/L were 0.02 ± 0.01, 0.13 ± 0.03, and 0.99 ± 0.25, respectively (Table 2). Thus, even in the presence of cholestasis, patients with liver disease have extremely low serum concentrations of $C_{27}$ bile acids. For peroxisomal disorders there was a wide variation in serum total $C_{24}$ bile acids measured by a routine clinical assay (Fig. 5), which presumably reflects the variable extent of liver dysfunction. In patients with genetically confirmed peroxisomal disorders the mean (±SEM) total serum $C_{27}$ bile acid concentration was markedly elevated at 14.06 ± 2.59 μmol/L (Fig. 5), being about 10-fold higher than in patients with severe cholestasis, and consistent with impaired peroxisomal side-chain oxidation of these cholestanoic acids. In all these subjects both (25S)- and (25R)- diastereoisomers were present in serum with the ratio of (25S)-/(25R)- being on average 0.3 (Table 2).

There were wide inter-individual variations in total serum $C_{27}$ bile acids for patients with peroxisomal disorders (0.05–86.76 μmol/L). This was particularly the case for some of the patients with homozygous PEX mutations (Fig. 5). This variability likely reflects the broad phenotypic presentation of peroxisomal biogenesis disorders and may also be influenced by age of the patient and the degree of liver dysfunction. The

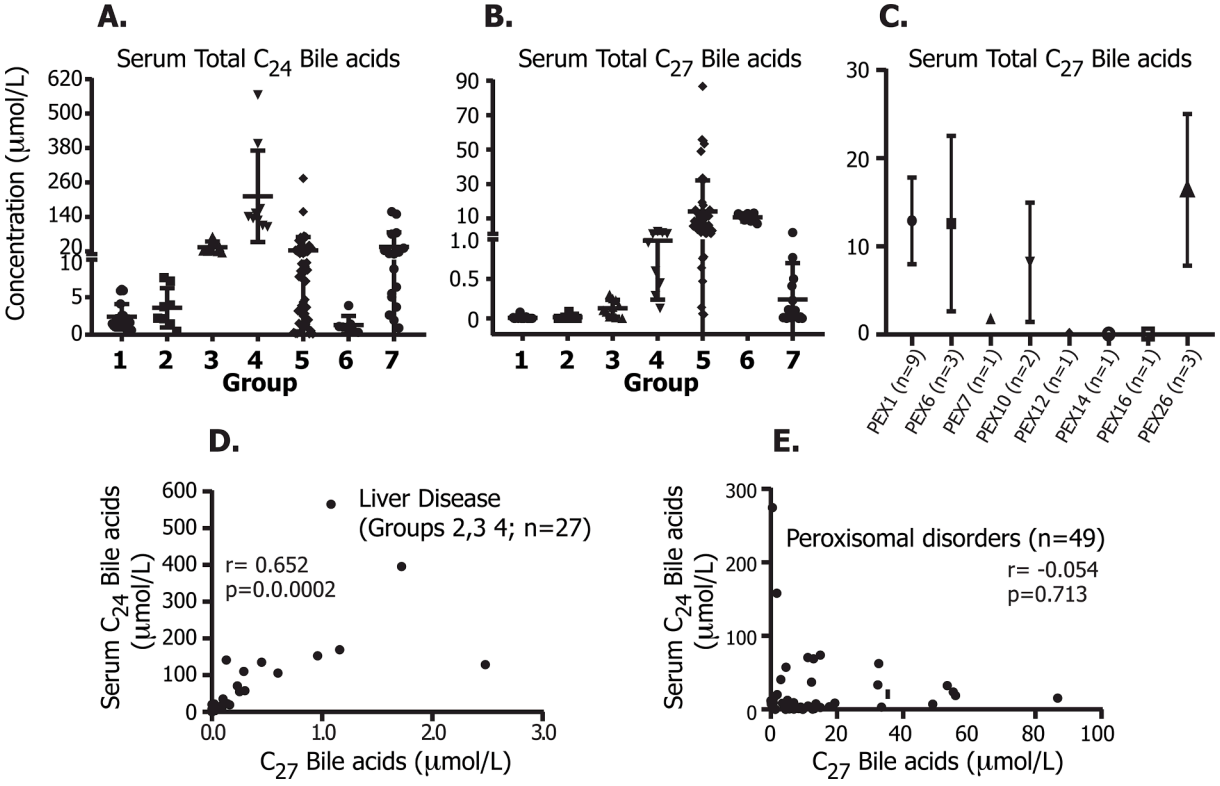


**Fig. 5.** The serum concentrations of (A) total $C_{24}$ bile acids and (B) total $C_{27}$ bile acids for the following groups; (1) normal adults n = 20, (2) non-cholestatic infants and children n = 8, (3) infants with moderate cholestasis n = 10, (4) severely cholestatic infants and children n = 9, (5) patients with genetically confirmed peroxisomal disorders n = 49, (6) patients with 2-methylacyl-CoA racemase deficiency n = 7, and (7) patients with confirmed heterozygous mutations in PEX genes n = 17. The serum total $C_{27}$ bile acids concentrations are shown (C) for patients with mutations in the different PEX genes. Below are the correlations between $C_{24}$ bile acids and $C_{27}$ bile acids for patients with liver disease (D) and patients with Peroxisomal disorders (E).

**Table 2**
Individual DHCA and THCA and total $C_{27}$ Bile Acid data in patients.

| | Concentration (μM, Mean ± SD) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (25S)-Tauro-THCA | (25R)-Tauro-THCA | (25S)-Tauro-DHCA | (25R)-Tauro-DHCA | (25S)-THCA | (25R)-THCA | (25S)-DHCA | (25R)-DHCA | Total C27 Bile Acid | Total 25S | Total 25R | S/R ratio |
| Adults w/o liver disease (n = 20) | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.a |
| (Min-Max) | 0–0.02 | 0–0.06 | n.d | n.d | 0–0.02 | n.d | n.d | n.d | 0–0.08 | 0–0.03 | 0–0.06 | n.a |
| Non-cholestatic (n = 8) | n.d | 0.01 | n.d | n.d | n.d | n.d | n.d | n.d | 0.02 ± 0.03 | n.d | 0.01 ± 0.02 | n.a |
| (Min-Max) | 0–0.03 | 0–0.06 | n.d | n.d | n.d | n.d | n.d | 0–0.02 | 0–0.09 | 0–0.03 | 0–0.06 | n.a |
| Moderately cholestatic (n = 10) | 0.02 ± 0.02 | 0.10 ± 0.08 | n.d | n.d | 0.01 ± 0.01 | n.d | n.d | n.d | 0.13 ± 0.11 | 0.03 ± 0.03 | 0.10 ± 0.08 | 0.22 ± 0.14 |
| (Min-Max) | 0–0.05 | 0–0.21 | n.d | n.d | 0–0.04 | 0–0.01 | n.d | n.d | 0–0.30 | 0–0.08 | 0–0.22 | 0–0.40 |
| Severely cholestatic (n = 9) | 0.20 ± 0.17 | 0.73 ± 0.56 | n.d | 0.02 ± 0.03 | 0.03 ± 0.04 | n.d | n.d | n.d | 0.99 ± 0.75 | 0.23 ± 0.18 | 0.75 ± 0.57 | 0.32 ± 0.08 |
| (Min-Max) | 0.02–0.58 | 0.10–1.86 | n.d | 0–0.08 | 0–0.11 | 0–0.01 | n.d | n.d | 0.13–2.48 | 0.03–0.59 | 0.10–1.89 | 0.19–0.50 |
| Peroxisomal disorder (n = 56)* | 1.40 ± 1.89 | 4.02 ± 5.77 | 0.11 ± 0.15 | 0.24 ± 0.30 | 0.95 ± 1.79 | 3.78 ± 7.8 | 0.96 ± 1.15 | 5.72 ± 8.02 | 17.17 ± 20.52 | 3.41 ± 3.97 | 13.76 ± 16.90 | 0.30 ± 0.15 |
| (Min-Max) | 0.01–8.51 | 0.03–26.02 | 0–0.64 | 0–1.31 | 0–9.45 | 0–31.51 | 0–5.06 | 0–39.60 | 0.05–86.76 | 0.01–15.88 | 0.03–70.88 | 0.01–8.51 |
| PD with Het PEX mutation (n = 19) | 0.06 ± 0.09 | 0.23 ± 0.43 | n.d | 0.01 ± 0.02 | n.d | n.d | n.d | n.d | 0.30 ± 0.53 | 0.06 ± 0.09 | 0.24 ± 0.44 | n.a |
| (Min-Max) | 0–0.29 | 0–1.50 | 0–0.01 | 0–0.06 | 0–0.04 | n.d | 0–0.01 | 0–0.02 | 0–1.81 | 0–0.29 | 0–1.56 | 0.01–8.51 |
| AMACR deficiency (n = 7) | n.d | 4.68 ± 2.57 | n.d | 0.52 ± 0.62 | n.d | 0.42 ± 0.61 | n.d | 5.00 ± 3.34 | 10.61 ± 2.44 | n.d | 10.61 ± 2.44 | n.a |
| (Min-Max) | n.d | 1.81–9.29 | n.d | 0.09–1.82 | n.d | 0–1.72 | n.d | 0.28–9.40 | 6.96–13.06 | n.d | 6.96–13.06 | n.a |

*Data of 7 AMACR patients were excluded from this group.
n.d: Not detected; n.a: Not available.

patient ages ranged from one week of birth to adulthood. For comparison among the groups (Fig. 5), only pediatric subjects defined by age < 18 yr were compared. It is possible that in the first month of life the immaturity in hepatic bile acid transporters that contribute to a physiological cholestais [33] accounts for higher levels of $C_{27}$ bile acids in the neonatal period. Serum $C_{24}$ bile acids were also measured in most samples (Fig. 5). In patients that did not have a peroxisomal disorder, an expected positive correlation was observed between the serum $C_{24}$ and $C_{27}$ bile acid concentrations for non-cholestatic and cholestatic patients ($r = 0.652$, $p = 0.0002$, $n = 27$; Fig. 5). By contrast there was a inverse negative correlation between $C_{24}$ and $C_{27}$ bile acid concentrations for patients with peroxisomal disorders, although this did not reach statistical significance (Fig. 5). Impaired side-chain oxidation generally results in elevated $C_{27}$ bile acids and a low serum $C_{24}$ bile acid concentration, and this was observed in this study.

Included in this report were 17 patients with heterozygous mutations in one of the PEX genes and these were found to have relatively low total serum $C_{27}$ bile acids (0.24 ± 0.11 μmol/L). Two patients with compound heterozygous mutations, one with PEX14/HSD17B4 and one with PEX12/PEX6 had negligible levels of DHCA and THCA in serum. It has been reported that heterozygotes for PEX6 mutations may have disease expression [34], but this was not found in this study. It is apparent that heterozygous mutations in PEX genes do not always result in a significant elevation in serum $C_{27}$ bile acid intermediates.

A major advantage of this assay is the ability to discriminate the (25R)- from (25S)- diastereoisomers of all the cholestanoic acids. Among the 49 cases of peroxisomal disorders, 7 patients from 3 families were genetically confirmed with a 2-methylacyl-CoA racemase deficiency. This enzyme is critical to the racemization of the (25R)- to the (25S)-forms of cholestanoic acids to then enable entry of these long-chain bile acids into the peroxisome for subsequent side-chain oxidation [35]. The enzyme is also essential to the metabolism of branched-chain fatty acids [35]. Biochemical diagnosis of this genetic defect, therefore, requires the separation of the diastereoisomers of THCA and DHCA [6,22]. In this single enzyme deficiency, concentrations of total serum total $C_{27}$ bile acids were markedly elevated (10.61 ± 0.92 μmol/L) independent of age, which ranged from 1.7−18.9 yr. In all cases, THCA and DHCA and the taurine conjugates were exclusively present as the (25R)-diastereoisomer, and there were no detectable (25S)- diastereoisomers, consistent with a definitive diagnosis of AMACR deficiency (Fig. 4). The serum $C_{24}$ bile acid concentrations were 0.3–3.9 μmol/L and within the normal range (<8 μmol/L). The AMACR deficiency has a phenotype of overlaping symptoms, and liver disease is not always a feature [5,6,36]. This may be because the low levels of primary bile acids synthesized maintain some bile flow. Interestingly, since the first discovery of AMACR deficiency several decades ago, and prior to writing this report there had been only 12 cases reported in the literature. Because this assay is capable of simultaneously quantifying both diastereoisomers of THCA and DHCA and the conjugates, we have identified 7 patients with AMACR deficiency during investigations of potential peroxisomal disorders in the last two years. It is possible that the frequency of AMACR deficiency may be underestimated in early life due to the relatively mild clinical features, because, interestingly, most of the reported cases have presented later in life with neurosensory symptoms [5]. One patient was reported to have oculocutaneous albinism Type 4 [36]. Early identification of patients with AMACR deficiency using the described method may allow early identification of affected patients and initiation of dietary restriction of phytanic acid leading to attenuation of neurologic symptoms.

## Conclusions

In summary, a novel assay for the quantification of individual $C_{27}$ cholestanoic acids in serum is described that is applicable to the study of

peroxisomal biogenesis disorders, and specifically to facilitate the differential diagnosis of AMACR defiency from general PBD. Normative data for total and individual serum $C_{27}$ cholestanoic acids in one of the largest cohorts of adult and pediatric patients are compared and reported for the first time. These data are invaluable for establishing threshold levels for diagnosis and, furthermore, since cholic acid has been approved to treat peroxisomal disorders [25,26], for monitoring biochemical responses to bile acid therapies.

## CRediT authorship contribution statement

**Wujuan Zhang:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Monica Narvaez Rivas:** Writing – review & editing, Methodology, Formal analysis. **Kenneth D.R. Setchell:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: KDRS discloses equity in Asklepion Pharmaceuticals LLC and Aliveris s.r.l, Italy, was a consultant to Retrophin (later Travere Therapeutics) at the time of this research study and is a consultant to Mirum Pharmaceuticals Inc, California. None of the other authors have anything to declare.

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### *Ethics Statement*

This study was not considered 'Human Subject Research' and required neither informed consent nor IRB review because it used fully anonymized patient samples that were not obtained specifically for use in this study through an interaction or intervention with living individuals.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2024.10.005.

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