Matthias Grimmler*, Tobias Frömel, Angelique Masetto, Holger Müller, Tina Leber and Christoph Peter

Performance evaluation of enzymatic total bile acid (TBA) routine assays: systematic comparison of five fifth-generation TBA cycling methods and their individual bile acid recovery from HPLC-MS/MS reference

https://doi.org/10.1515/cclm-2024-1029
Received September 3, 2024; accepted November 15, 2024;
published online November 29, 2024

Abstract

Objectives: Serum total bile acid (TBA) levels are frequently assessed in clinical routine for the early detection of hepatobiliary dysfunction. However, the comparability of current 5th-generation TBA cycle assays based on 3α-hydroxysteroid dehydrogenase (3α-HSD) and their ability to quantify individual bile acids has not been systematically addressed.

Methods: Patient serum samples (n=60) across the diagnostically relevant TBA range (1–200 µmol/L) were analyzed using five TBA routine assays from Abbott, DiaSys, Diazyme, Beijing Strong (BSBE) and Randox on the same analyzer (BioMajesty® JCA-BM6010/C). The assays were compared using Passing-Bablok regression and the recovery of 11 individual BAs was evaluated against RP-HPLC-MS/MS as non-enzymatic reference method.

Results: Despite excellent correlation (Spearman r ≥0.99), the assays showed proportional differences (slope) ranging from 0.99 (BSBE/Randox) to 1.24 (Abbott/DiaSys). The assays showed considerable deviation in the recovery of competitor's calibrators and controls, and large heterogeneity in the recovery of individual BAs, with mean deviations from reference value between 13 % (DiaSys) and 42 % (Abbott). CA and TCA were measured most accurately and consistently, whereas GCA, CDCA, DCA, UDCA, and conjugates were over- or undermeasured to varying degrees.

Conclusions: The linear relationship and constant proportional bias between all five routine assays enable the harmonization of TBA measurements up to 60 µmol/L. However, for patient samples with high TBA levels and disease-specific overrepresentation of individual BAs, harmonization will require: i) optimized reaction conditions to equalize substrate specificity, and ii) calibration to a common, commutable reference material with well-defined BA composition instead of internal standards spiked with different BAs.

Keywords: bile acids; enzymatic assay; hepatobiliary disease; liver disease; method comparison; tandem mass spectrometry

***Corresponding author: Matthias Grimmler**, PhD, Professor, Institute for Biomolecular Research, Hochschulen Fresenius gemeinnützige Trägergesellschaft mbH, University of Applied Sciences, Limburger Str. 2, 65510 Idstein, Germany; and DiaSys Diagnostic Systems GmbH, Holzheim, Germany, E-mail: matthias.grimmler@ext.hs-fresenius.de. https://orcid.org/0000-0003-2860-3391
Tobias Frömel, Institute for Analytical Research, Hochschulen Fresenius gemeinnützige Trägergesellschaft mbH, University of Applied Sciences, Idstein, Germany. https://orcid.org/0000-0003-4812-0127
Angelique Masetto, Institute for Biomolecular Research, Hochschulen Fresenius gemeinnützige Trägergesellschaft mbH, University of Applied Sciences, Idstein, Germany; and DiaSys Diagnostic Systems GmbH, Holzheim, Germany
Holger Müller and Tina Leber, DiaSys Diagnostic Systems GmbH, Holzheim, Germany
Christoph Peter, Institute of Molecular Medicine I, Heinrich Heine University Düsseldorf, Düsseldorf, Germany. https://orcid.org/0000-0002-0048-2279

Introduction

The concentration of total bile acids (TBA) in human blood is a widely used marker for the early assessment of hepatobiliary dysfunction, as it directly reflects pathological changes in bile acid synthesis, secretion, and intestinal reabsorption [1–4]. The fast, reliable, and cost-effective quantification of serum TBA plays an increasingly important role in the timely diagnosis and treatment of various hepatobiliary diseases such as liver cirrhosis, hepatitis, and intrahepatic cholestasis of pregnancy (ICP) [1–6].

Open Access. © 2024 the author(s), published by De Gruyter. This work is licensed under the Creative Commons Attribution 4.0 International License.

Two primary bile acids (PBAs) are synthesized from cholesterol in the liver: cholic acid (CA; 3α-, 7α-, and 12α-OH) and chenodeoxycholic acid (CDCA; 3α- and 7α-OH) (Figure 1A and B) The C-terminal carboxy group is amidated with either glycine (G) or taurine (T) before secretion into the bile, resulting in the amphipathic and more water-soluble T/GCA and T/GCDCA [1, 4]. Conjugated PBAs are secreted into the duodenum in response to dietary fat, where they act as emulsifiers of lipophilic molecules [1, 4]. In addition, BAs play a key role in endocrine signaling and the regulation of lipid and glucose metabolism [1, 2, 4, 5]. Dysregulated BA metabolism or signaling is a common feature of several chronic diseases such as obesity, type 2 diabetes, and Alzheimer's disease [1–8].

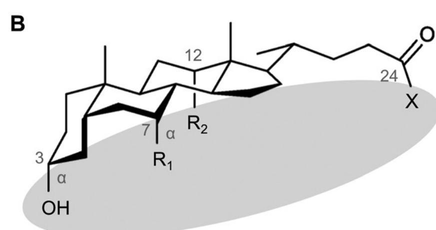
Secondary bile acids (SBAs) such as deoxycholic acid (DCA; 3α- and 12α-OH) and lithocholic acid (LCA; 3α-OH) are directly derived from PBAs by bacterial enzymes in the intestine [1, 4]. Since ursodeoxycholic acid (UDCA; 3α- and 7β-OH) is generated from CDCA in two steps, it is sometimes referred to as "tertiary" bile acid (Figure 1A and B). About 95 % of PBAs and SBAs are reabsorbed from the ileum and return to the liver via the portal vein, where the majority is re-secreted into the gallbladder together with newly synthesized PBAs (enterohepatic circulation) [1, 4, 9]. Less than 10 % escapes the hepatic extraction and reaches the systemic circulation, resulting in low fasting serum TBA levels (2–10 µmol/L) in healthy individuals [1, 4, 9]. Highly elevated serum TBA is hence an early marker of impaired hepatic clearance and various hepatobiliary diseases, including cholestasis, cholangitis, cirrhosis, hepatitis, nonalcoholic fatty liver disease (NAFLD), hepatocellular carcinoma (HCC), and cholangiocarcinoma (CCA) [1–3, 7–16]. Of note, CA, CDCA, DCA and their derived T/G-conjugates constitute >95 % of the BA pool in systemic circulation, with highest reference values for unconjugated and G-conjugated BAs such as GCDCA (≤5.1 µmol/L), CA (≤2.7 µmol/L), CDCA (≤2.3 µmol/L), and GUDCA (≤1.0 µmol/L), as compared to TCA (≤0.3 µmol/L) and LCA (≤0.09 µmol/L) [17–20]. Especially the T/G-conjugated PBAs seem to contribute to the highly elevated TBA levels in patients with liver cirrhosis [8, 14, 21], while an increased ratio of T- to G-conjugated BAs has been reported in patients with primary biliary cirrhosis [7, 18].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered the preferred method for the accurate quantification of TBA and discrimination of individual bile acids (IBA) [3, 6]. Luo et al. recently assessed the concentration of nine IBAs in a large study population by LC-MS/MS [7]. Serum TBA was 10-fold higher in patients with liver disease (51.3 ± 3.8 µmol/L) than in healthy subjects (4.8 ± 0.6 µmol/L). Again, the conjugated PBAs (TGC, GCA, TCDCA, and GCDCA) were disproportionately elevated in patients with liver disease [6, 7].

A

Primary bile acids		R_1	R_2	X
Cholic acid	CA	7α-OH	12α-OH	OH
Glycocholic acid	GCA	7α-OH	12α-OH	$NHCH_2COOH$
Tauroccholic acid	TCA	7α-OH	12α-OH	$NHCH_2CH_2SO_3H$
Chenodeoxycholic acid	CDCA	7α-OH	H	OH
Glycochenodeoxycholic acid	GCDCA	7α-OH	H	$NHCH_2COOH$
Taurochenodeoxycholic acid	TCDCA	7α-OH	H	$NHCH_2CH_2SO_3H$
Secondary bile acids		R_1	R_2	X
Ursodeoxycholic acid	UDCA	7β-OH	H	OH
Glycoursodeoxycholic acid	GUDCA	7β-OH	H	$NHCH_2COOH$
Deoxycholic acid	DCA	H	12α-OH	OH
Glycodeoxycholic acid	GDCA	H	12α-OH	$NHCH_2COOH$
Taurodeoxycholic acid	TDCA	H	12α-OH	$NHCH_2CH_2SO_3H$
Lithocholic acid	LCA	H	H	OH

B



C

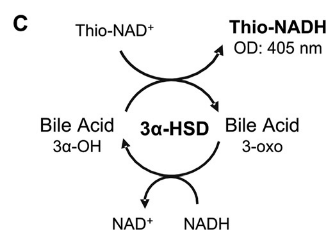


Figure 1: Bile acids and TBA cycling assay principle. (A) Functional groups of individual bile acids used in this study; (B) Structure of bile acids including position and orientation of hydroxylation (R1/R2), and amidation (X) with taurine (T) or glycine (G). Hydrophilic face (grey); (C) Principle of 5th-generation TBA cycling assays. Amplified generation of Thio-NADH by 3α-hydroxysteroid dehydrogenase (3α-HSD) is detected as a linear increase in OD at 405 nm.

Over the years, several enzymatic TBA assays have become commercially available, which are more suitable for clinical laboratory routine due to lower costs and shorter turnaround time. These assays are based on 3α-hydroxysteroid dehydrogenase (3α-HSD; BRENDA:EC1.1.1.50), which catalyzes the reversible oxidation of 3α-hydroxylated steroids using nicotinamide adenine dinucleotide (NADH) as coenzyme [3, 21, 22]. Previous 3rd-generation endpoint assays used an additional reaction step to generate proportional amounts of formazan (OD: 570 nm) from NADH [22–24]. Modern 5th-generation cycling assays rely on the amplified linear generation of a modified coenzyme Thio-NADH (OD: 405 nm) from Thio-NAD^+, in the excess of NADH (Figure 1C) [3, 22, 23]. This method enables higher detection sensitivity, shows lower interference for hemolytic and lipemic serum samples, and requires smaller sample volumes [22].

Only three studies have evaluated the performance of routine TBA assays using chromatography-mass spectrometry as reference method. Ducroq et al. [25] compared two 3rd-generation assays and one 5th-generation assay using isotope dilution gas chromatography-mass spectrometry (ID-GCMS) as reference [25]. Danese et al. [26] evaluated three 5th-generation assays against LC-MS/MS, whereas Žížalová et al. [27] used one 3rd-generation TBA assay to conclude that enzymatic methods may generally underestimate serum TBA levels [26, 27].

Here we systematically evaluated the performance of five 5th-generation routine TBA cycling assays from Asian, European, and US-American manufacturers on the same clinical chemistry platform, by measuring 60 patient serum samples across the diagnostically relevant TBA range (1–200 µmol/L), as well as 11 individual BAs with the highest abundance in human serum. Due to the low solubility of unconjugated and deoxygenated BAs in aqueous matrices, we determined the final concentration of each bile acid standard solution using reverse-phase high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (RP-HPLC-ESI-MS/MS) as a non-enzymatic reference method.

Materials and methods

Bile acid solutions and reagents

Bile acid salts for the preparation of aqueous standard solutions (≥95 % purity) and bile acid solutions in methanol for HPLC-MS/MS calibration (≥98 % purity) were purchased from AppliChem (Darmstadt, Germany), Merck (Darmstadt, Germany), and Cambridge Isotope Laboratories (Tewksbury, USA), respectively. See Supplementary Tables S1 and S2 for details. Aqueous stock solutions (200 µmol/L) for 12 individual bile acids (CA, CDCA, DCA, GCA, GCDCA, GDCA, GUDCA, LCA, TCA, TCDCA, TDCA, and UDCA) were prepared and stored at RT until use. Complete dissolution in deionized water was achieved by sonification for 10–30 min and pH adjustment with 4 M NaOH as required. Three aliquots of each BA standard solution (50 µmol/L) were analyzed in separate Eppendorf tubes in a single determination by five TBA cycling assays. Due to the low water solubility of hydrophobic BAs, three additional aliquots were analyzed by HPLC-MS/MS to determine the final concentration of each BA standard solution. HPLC-grade methanol and acetonitrile (ACN) were purchased from Carl Roth (Karlsruhe, Germany).

Total bile acid cycling assays

Five TBA cycling assays were adapted for the use on the fully-automated clinical chemistry analyzer BioMajesty® JCA-BM6010/C (DiaSys Diagnostic Systems, Holzheim, Germany) according to the manufacturer protocols: Total Bile Acids (Abbott Diagnostics, Abbott Park, USA), Total bile acid Assay Kit (Beijing Strong Biotechnologies/BSBE, Beijing, China), Total bile acid 21 F S (DiaSys Diagnostic Systems, Holzheim, Germany), Total Bile Acids Assay (Diazyme Laboratories, Poway, USA), and Bile Acids reagent (Randox Laboratories, Crumlin, UK). All TBA assays were calibrated following the manufacturer's instructions. See Supplementary Tables S3 and S4 for further details. Patient samples, calibrators, controls, and blank (0.9 % NaCl) were analyzed in duplicate. All controls were within the manufacturer's reference range. Thio-NADH formation was quantified by time interval measurement (TIM) using the change in optical density (OD) at 410 nm (primary wavelength) and 596 nm (secondary wavelength), to reduce interference from serum components such as lipids, hemoglobin, and bilirubin. A consistent volume of reagent R1 (90 µL) and R2 (30 µL) was used for all TBA assays. Time intervals and reaction temperature were kept constant. Sample volume and detection cycles were adjusted as follows: BSBE (1.2 µL; cycles 25/35), Randox (1.2 µL; cycles 25/35), Abbott (1.2 µL; cycles 25/32), DiaSys (1.3 µL; cycles 25/32), Diazyme (1.5 µL; cycles 26/31). For reaction kinetics and linearity within measuring range see Supplementary Figure S4. For method comparison, anonymous patient serum samples (n=60; TBA range: 1–200 µmol/L; Median: 11 µmol/L, IQR: 6–26) were analyzed in duplicate with all five TBA assays on the same analyzer.

HPLC-MS/MS method

A 3200 QTRAP® LC-MS/MS System (AB Sciex Pte. Ltd., Framingham, USA) was used with two Series 200 Micro Pumps and a Series 200 Autosampler (PerkinElmer, Waltham, USA). Multiple reaction monitoring (MRM) parameters were optimized from individual BA solutions (1–3 µg/mL) in ultra-pure water 1 (UPW1):methanol (50:50; v/v) by infusion at flow rates (10–20 µL/min) using automatic 'compound optimization'. All measurements were carried out in negative ion mode. The precursor ion was selected manually for MRM transitions, as the ion with the highest abundance in a full scan (Supplementary Table S5). RP-HPLC-ESI-MS/MS was performed using reversed-phase chromatography on a CORTECS T3 Column (120 Å, 2.7 µm, 2.1 mm × 50 mm) and CORTECS T3 VanGuard Cartridge (120 Å, 2.7 µm, 2.1 mm × 5 mm; Waters Corporation, Milford, USA). Eluent A was UPW1:ACN (95:5; v/v) and eluent B was UPW1:ACN (5:95; v/v), both containing 5 mM ammonium formate (pH 3.0). The injection volume was 10 µL, mobile phase flow rate was 300 L/min, and ESI source gas temperature was set to 600 °C. The gradient was held constant at 70 % A for 1.5 min, linearly decreased to 20 % A until 8.0 min, held at 20 % A until 9.0 min, and increased again to 70 % A until 9.1 min. Finally, the column was re-equilibrated from 9.1 to 14.0 min at 70 % A. The final methanol proportion of calibrators and standard solutions was 30 %. Five calibration points between 0 and 3 µmol/L were measured in triplicate for each calibrator solution (Supplementary Table S2), with UPW1:methanol (70:30; v:v) as blank. Three aliquots were measured in separate Eppendorf tubes in a single determination to determine the actual concentration of each aqueous BA standard solution (Supplementary Table S1).

Statistical analysis and software

Passing-Bablok regression, Bland-Altman, and statistical analysis were performed using MedCalc® (Version 18.10.2, MedCalc Software Ltd, Belgium) and Microsoft Excel 2021. LC-MS/MS data was evaluated using Analyst software (Version 1.5.1, build 5218) and MultiQuant™ software (Version 3.0.2, build 8664.0), respectively (Sciex, Framingham, USA).

Results

Recovery of calibrators and controls

All TBA assays were calibrated using the calibration standard provided by the manufacturer, and all calibrators and recommended controls were measured in duplicate with all TBA assays. While the proprietary calibrator was measured with high accuracy, all assays showed significant deviation from the target value of competitor's calibrators (Figure 2A) and controls (Figure 2B). For example, Diazyme recovered both DiaSys controls too high (+38 % and +29 % deviation), whereas DiaSys measured Randox controls too low (−13 % and −24 % deviation) (Figure 2B). Of note, the BSBE results were very similar to those of the Randox assay, suggesting a similar composition of calibrators and other assay components. Since no certified reference material is available, two independent controls with normal (12 µmol/L) and pathological TBA concentration (54 µmol/L) were analyzed in duplicate with all TBA assays (Figure 2C). Abbott recovered both independent controls with the highest accuracy (+7 % mean deviation), while BSBE and Randox had the highest mean deviation from the target values (−15 % and −16 %), again with very similar recovery rates (Figure 2C).

Method comparison

Patient serum samples (n=60) with TBA concentrations in the diagnostically relevant range (1–200 µmol/L) were measured in duplicate with all TBA assays on the same analyzer. Method agreement was determined using Spearman's rank correlation, Passing-Bablok regression, and Bland-Altman analysis (Figure 3A–D; Table 1; Supplementary Figure S1–3). Despite a strong overall correlation (Spearman r ≥0.996 for all assays), the TBA assays showed varying proportional differences (slope) ranging from 0.99 (BSBE/Randox) to 1.24 (Abbott/DiaSys) (Figure 3A–D, Table 1). [Place Table 1 near here] For some assay combinations, such as BSBE/Randox, BSBE/Abbott, and Abbott/DiaSys, the linear relationship and proportional bias were constant over the entire analytical measurement range (up to 200 µmol/L). For Diazyme/Abbott and DiaSys/Diazyme, the Cusum test indicated a significant deviation from linearity ($p<0.05$) (Table 1), which is most likely due to the non-linear dynamics of the Diazyme assay for samples >150 µmol/L (Supplementary Figure S4). Nevertheless, all five methods showed excellent correlation, strong linear relationship, and constant proportional bias around the clinically relevant 10 µmol/L cut-off (up to 60 µmol/L) (Supplementary Figure S2 and S3). The conversion factors provided in Table 1 may help to harmonize TBA measurements for patients with moderate (<60 µmol/L) and high TBA elevation (>60 µmol/L). It is reasonable to assume that assay-specific differences in substrate specificity may account for the lower agreement in high TBA samples

A

Calibrators	Diazyme	BSBE	Randox	Abbott	DiaSys	MD	Target value [µmol/L]
	Mean recovery from target value						
Diazyme Calibrator	102%	96%	94%	111%	104%	5%	50.0
DiaSys Calibrator	107%	93%	93%	107%	99%	6%	40.3
Randox Calibrator	108%	99%	101%	115%	78%	9%	45.2
Abbott Calibrator	97%	83%	84%	101%	58%	16%	51.6
Mean deviation (MD)	5%	7%	8%	9%	17%		
	min				max		

B

Controls		BSBE	Randox	DiaSys	Abbott	Diazyme	MD	Target value [µmol/L]
		Mean recovery from target value						
DiaSys (TruLab)	TruLab N	100%	99%	98%	114%	138%	11%	8.3
	TruLab P	88%	88%	98%	102%	129%	11%	32.4
Diazyme (TBA Control)	Level 2	86%	83%	97%	104%	102%	8%	31.2
	Level 3	87%	87%	98%	109%	94%	9%	109.8
Randox (Assayed Chemistry Premium Plus)	Level 2	98%	101%	87%	109%	106%	6%	25.5
	Level 3	97%	98%	76%	113%	107%	10%	48.0
Abbott (Technopath Multichem S Plus)	Level 1	94%	94%	101%	109%	123%	9%	11.9
	Level 3	93%	93%	116%	109%	131%	14%	57.2
Mean deviation (MD)		7%	7%	8%	9%	18%		
		min				max		

C

Independent control		Abbott	DiaSys	Diazyme	BSBE	Randox	MD	Target value [µmol/L]
		Mean recovery from target value						
Trinity Biotech Bile Acid Control	Normal	108%	110%	84%	87%	85%	12%	12.0
	Abnormal	105%	110%	97%	84%	83%	10%	54.0
Mean deviation (MD)		7%	10%	10%	15%	16%		
		min				max		

Figure 2: Recovery of calibrators and controls. (A) Cross-recovery of calibrators from other manufacturers; (B) Cross-recovery of competitor controls; (C) Recovery of independent control (Trinity Biotech) with normal and pathological TBA level. Assays are sorted by mean deviation (MD) from the target value from minimum (white) to maximum (green). Color scheme for mean recovery: <100 % (blue), 100 % (white), >100 % (red). Recovery of own calibrator and controls are outlined.

(>100 µmol/L), as highly elevated serum TBA levels are mainly observed in patients with severe hepatobiliary dysfunction, which is often accompanied by disease-specific overrepresentation of selected individual bile acids.

Recovery of individual bile acids

To compare the quantification of individual bile acids by all five TBA assays, the concentration of 11 aqueous BA standard solutions was determined in triplicate by HPLC-MS/MS as a

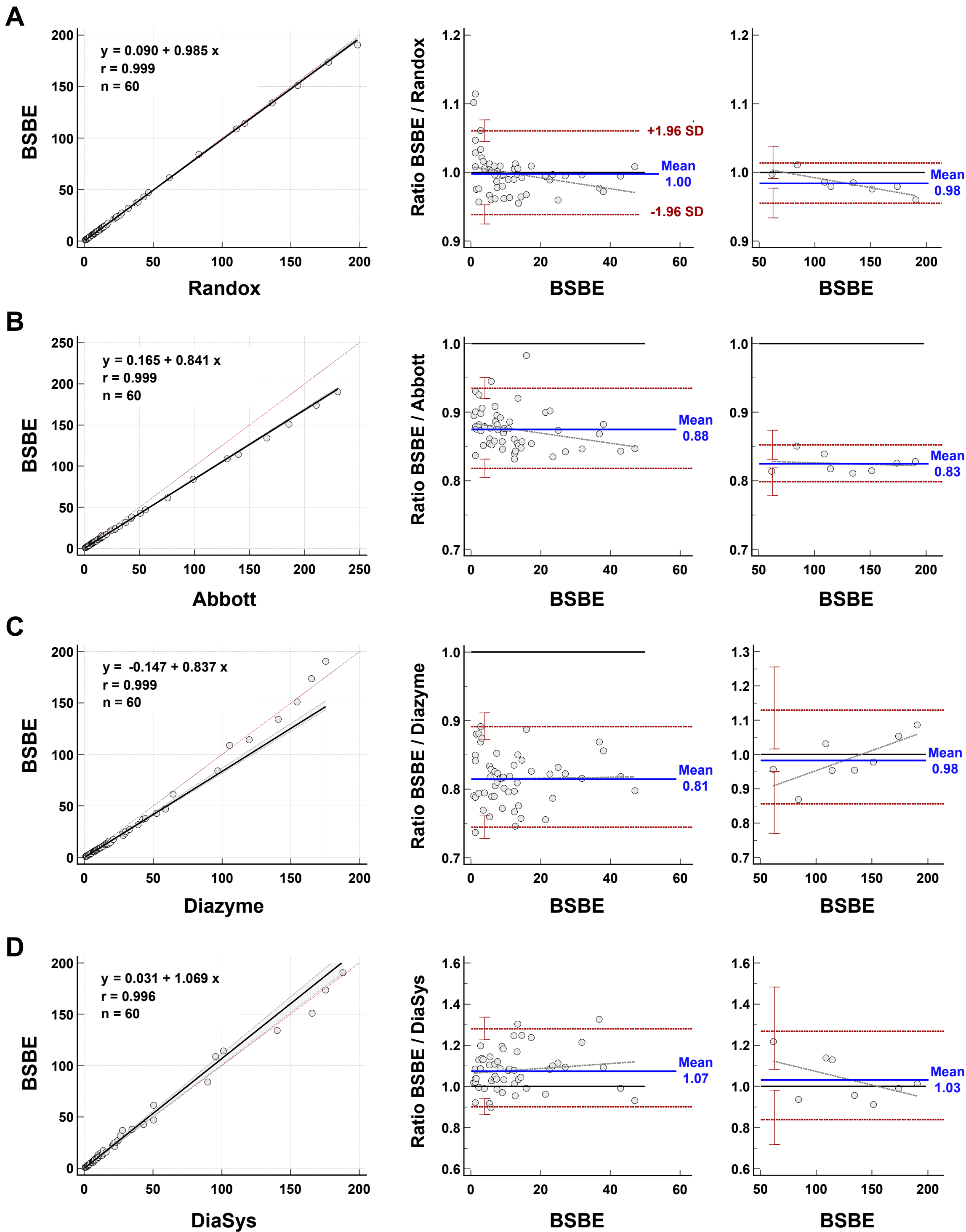


Figure 3: Method comparison. (A–D) Method comparison for patient samples (n=60; 1–200 µmol/L). Regression line (black), 95 % CI of the regression line (grey), identity line (red dotted line). Bland-Altman ratio plots (method A/B) for samples <60 (n=52) and >60 µmol/L (n=8). Zero line (black), mean difference (blue), upper/lower limit of agreement (±1.96 SD; red), regression line (grey).

non-enzymatic reference method (Figure 4; Supplementary Figure S6). DiaSys showed the lowest variance (VAR) and lowest mean deviation (MD) from the reference value (13 %), and Abbott the highest (42 %). The other assays deviated by 25 % (Diazyme) and 27 % (Randox and BSBE), respectively (Figure 4). The primary CA and TCA were determined most

Table 1: Method comparison for patient serum samples (n=60; TBA range: 1–200 µmol/L), and conversion factors to harmonize TBA measurement results <60 µmol/L and >60 µmol/L.

Method		Spearman	Passing-Bablok regression			Bland-Altman	
		Correlation	Proportional bias	Constant bias	Cusum	Conversion factors	
		r (95 % CI)[a]	slope (95 % CI)	intercept (95 % CI)	p	mean ratio A/B (95 % CI)	
A	B		1–200 µmol/L			<60 µmol/L	>60 µmol/L
BSBE	Randox	0.999 (0.999; 0.999)	0.99 (0.979; 0.992)	0.09 (0.039; 0.132)	0.56	1.00 (0.989; 1.001)	0.98 (0.972; 0.997)
BSBE	Abbott	0.999 (0.998; 0.999)	0.84 (0.835; 0.850)	0.17 (0.074; 0.237)	0.37	0.88 (0.866; 0.883)	0.83 (0.813; 0.837)
BSBE	Diazyme	0.999 (0.998; 0.999)	0.84 (0.821; 0.868)	−0.15 (−0.451; −0.001)	0.12	0.81 (0.804; 0.825)	0.98 (0.927; 1.043)
BSBE	DiaSys	0.996 (0.994; 0.998)	1.07 (1.013; 1.112)	0.03 (−0.260; 0.264)	0.56	1.07 (1.048; 1.101)	1.03 (0.945; 1.126)
Randox	DiaSys	0.996 (0.993; 0.998)	1.10 (1.053; 1.133)	−0.09 (−0.297; 0.179)	0.95	1.08 (1.049; 1.105)	1.05 (0.960; 1.144)
Diazyme	Randox	0.998 (0.997; 0.999)	1.17 (1.137; 1.206)	0.23 (0.048; 0.519)	0.12	1.23 (1.206; 1.244)	1.00 (0.933; 1.074)
Abbott	Randox	0.997 (0.996; 0.999)	1.18 (1.158; 1.188)	−0.14 (−0.275; −0.033)	0.22	1.14 (1.126; 1.156)	1.19 (1.176; 1.211)
Abbott	DiaSys	0.996 (0.993; 0.998)	1.24 (1.201; 1.304)	−0.09 (−0.453; 0.986)	0.78	1.23 (1.195; 1.262)	1.25 (1.142; 1.369)
DiaSys	Diazyme	0.998 (0.996; 0.999)	0.78 (0.754; 0.825)	0.08 (−0.531; 0.066)	0.03[b]	0.76 (0.742; 0.775)	0.96 (0.864; 1.052)
Diazyme	Abbott	0.999 (0.998; 0.999)	1.03 (1.00; 1.049)	0.27 (0.106; 0.566)	0.03[b]	1.07 (1.062; 1.086)	0.84 (0.788; 0.894)

[a]p-value: <0.0001
[b]Deviation from linearity (p<0.05)

A (µmol/L)/CF=B (µmol/L)
B (µmol/L) x CF=A (µmol/L)

accurately and consistently (5 and 8 % mean deviation), whereas GCA, UDCA, and GUDCA were measured consistently too low by most assays (mean deviation between −13 % and −24 %; Figure 4). In contrast, CDCA, DCA, and their respective T/G-conjugates were recovered too high by most methods tested (mean deviation between +20 % and +57 %) (Figure 4).

Individual bile acids R1	R2	X		DiaSys	Diazyme	BSBE	Randox	Abbott	MR	MD	VAR	Mean ±SD		HPLC-MS/MS Target value [µmol/L]	
				Mean recovery from HPLC-MS/MS reference											
α OH	OH	G	GCA	79%	75%	71%	70%	83%	76%	24%	0.00	40.0	± 2.6	52.9	± 0.7
β OH	-	G	GUDCA	95%	70%	79%	79%	86%	82%	18%	0.01	49.8	± 5.0	60.9	± 0.1
β OH	-	-	UDCA	100%	74%	84%	85%	92%	87%	13%	0.01	47.7	± 4.7	54.8	± 0.7
α OH	OH	-	CA	103%	101%	94%	93%	110%	100%	5%	0.00	38.9	± 2.4	38.9	± 0.9
α OH	OH	T	TCA	120%	113%	102%	101%	102%	108%	8%	0.01	37.6	± 2.8	34.0	± 0.4
α OH	-	T	TCDCA	85%	112%	116%	116%	143%	114%	20%	0.03	53.8	± 8.6	47.0	± 0.3
α OH	-	G	GCDCA	79%	113%	134%	134%	165%	125%	33%	0.08	61.0	± 13.8	48.8	± 0.1
-	OH	G	GDCA	74%	130%	131%	130%	161%	125%	36%	0.08	69.0	± 15.5	55.1	± 0.4
-	OH	T	TDCA	89%	147%	132%	133%	162%	133%	37%	0.06	62.0	± 11.3	46.7	± 0.2
-	OH	-	DCA	79%	139%	142%	143%	174%	135%	44%	0.10	61.5	± 14.1	45.4	± 0.7
α OH	-	-	CDCA	98%	139%	168%	169%	205%	156%	57%	0.13	60.8	± 13.9	39.0	± 1.1
Mean recovery (MR)				91%	110%	114%	114%	135%							
Mean deviation (MD)				13%	25%	27%	27%	42%							
Variance (VAR)				0.02	0.07	0.08	0.09	0.16							
				min				max							

Figure 4: Recovery of individual bile acids from non-enzymatic reference method (HPLC-MS/MS). TBA assays are sorted by mean deviation (MD) from the target value from minimum (white) to maximum (green). Color scheme of mean recovery (MR): <100 % (blue), 100 % (white), >100 % (red). Variance (VAR) indicates the heterogeneity of measurements.

Discussion

Several TBA cycling assays are currently used in clinical laboratory routine to assess the total bile acid concentration in human serum or plasma. The systematic comparison of five 5th-generation TBA cycling assays on the same clinical chemistry platform revealed proportional differences between most TBA assays when measuring patient samples, despite excellent overall correlation (Figure 3, Table 1). Although some assay combinations showed strong linearity and constant proportional bias over the entire TBA concentration range, the correlation was best for samples with moderate TBA increase (up to 60 µmol/L). Here, the provided conversion factors (CF) may help to further harmonize TBA measurements from different routine assays (Table 1). Harmonization is more challenging for patient samples with highly elevated TBA levels since huge differences in substrate specificity can greatly affect the correlation between assays.

In general, the results presented here are in agreement with Danese et al. who reported excellent correlation and linearity up to 140 µmol/L for three 5th-generation TBA assays from Diazyme, Randox, and Sentinel [26]. However, all three assays underestimated the serum TBA concentration to varying degrees, which the authors attributed mainly to the heterogeneous composition of the proprietary calibrators [26]. In this study, CA and TCA (3α-, 7α-, 12α-OH) were measured with the highest accuracy and lowest inter-assay variance, whereas GCA was consistently underestimated by all five TBA assays compared to the non-enzymatic reference value (Figures 4 and 5A). CDCA (3α, 7α), DCA (3α, 12α), UDCA (3α, 7β), and their respective T/G-conjugates were under- or overestimated by most assays to varying degrees (Figures 4 and 5A). Overall, the DiaSys TBA assay showed the lowest mean deviation from the target value, Abbott the highest (Figures 4 and 5B). Interestingly, the enzymatic quantification of lithocholic acid (LCA), the most hydrophobic and least abundant BAs in circulation, produced the most inconsistent results, ranging from 36 µmol/L (Diazyme) to 185 µmol/L (Abbott) (Mean: 97.4 ± 55.1 µmol/L; Supplementary Table S6). Consistent with previous reports, the measurement of LCA by LC-MS/MS was the most challenging and the reference concentration could not be determined with sufficient quality (Supplementary Table S6) [28].

Zhang et al. [22] already reported different specificities of the recombinant 3α-HSD for individual BAs under the same reaction conditions [22]. Therefore, it is reasonable to assume that the combination of several assay-specific differences, including the genetic modification of the recombinant enzyme and the composition of the reaction buffers, strongly influences the specificity and conversion rates for individual BAs – especially for samples with pronounced changes in BA composition and disease-specific overrepresentation of individual bile acids. In this case, inter-assay comparability would depend critically on the calibration to a common reference standard of well-defined BA composition rather than to proprietary and kit-specific calibrators, each spiked with different individual BAs. However, no such higher-order reference material is currently available. The heterogeneous recovery of non-proprietary calibrators and controls (Figure 2), and the observed similarities and differences in the quantification of individual BAs (Figures 4 and 5) support the following hypothesis: For most TBA cycling assays, the recombinant 3α-HSD has a strong preference for the unconjugated trihydroxylated CA, as the calibration to different proprietary standards had minimal effect on assay agreement when measuring CA alone. The highest inter-assay agreement was observed for Randox and Beijing Strong, indicating a considerable similarity in terms of enzymes, calibrators, and reaction buffers.

In healthy individuals, the liver efficiently removes excess BAs from the portal circulation [1, 4, 9]. Fasting TBA blood levels above the normal range (>10 µmol/L) are therefore an early indicator of impaired liver function and/or bile acid synthesis, secretion, or reabsorption – as seen in patients with various hepatobiliary diseases including hepatitis, liver cirrhosis, steatotic and cholestatic disorders or hepatobiliary cancer [1–3, 7–16]. However, there is no established diagnostic TBA cut-off to further differentiate between these possible causes. The results presented here indicate that all 5th-generation routine TBA assays tested are suitable to support the early detection of hepatobiliary dysfunction (complementary to other liver tests), as all enzymatic cycling methods showed excellent correlation, linear relationship, and constant proportional deviation around the clinically relevant 10 µmol/L threshold. However, the lower agreement for TBA levels above 60 µmol/L may be relevant for decision-making in other conditions such as intrahepatic cholestasis of pregnancy (ICP), a rare pregnancy-specific liver disorder associated with poor outcomes [10, 16]. According to current guidelines, ICP should be included in the differential diagnosis of any pregnant woman presenting with pruritus [29]. As normal pregnancy is associated with mild hypercholanemia, a non-fasting TBA concentration >19 µmol/L is considered diagnostic of ICP [29, 30]. TBA levels ≥40 µmol/L have been suggested as a threshold for predicting adverse outcomes, while levels ≥100 µmol/L indicate a significantly increased risk of stillbirth [10, 16, 29]. Clinicians should be aware that the agreement between different commercial 3α-HSD-based assays is lower for blood samples in the upper detection

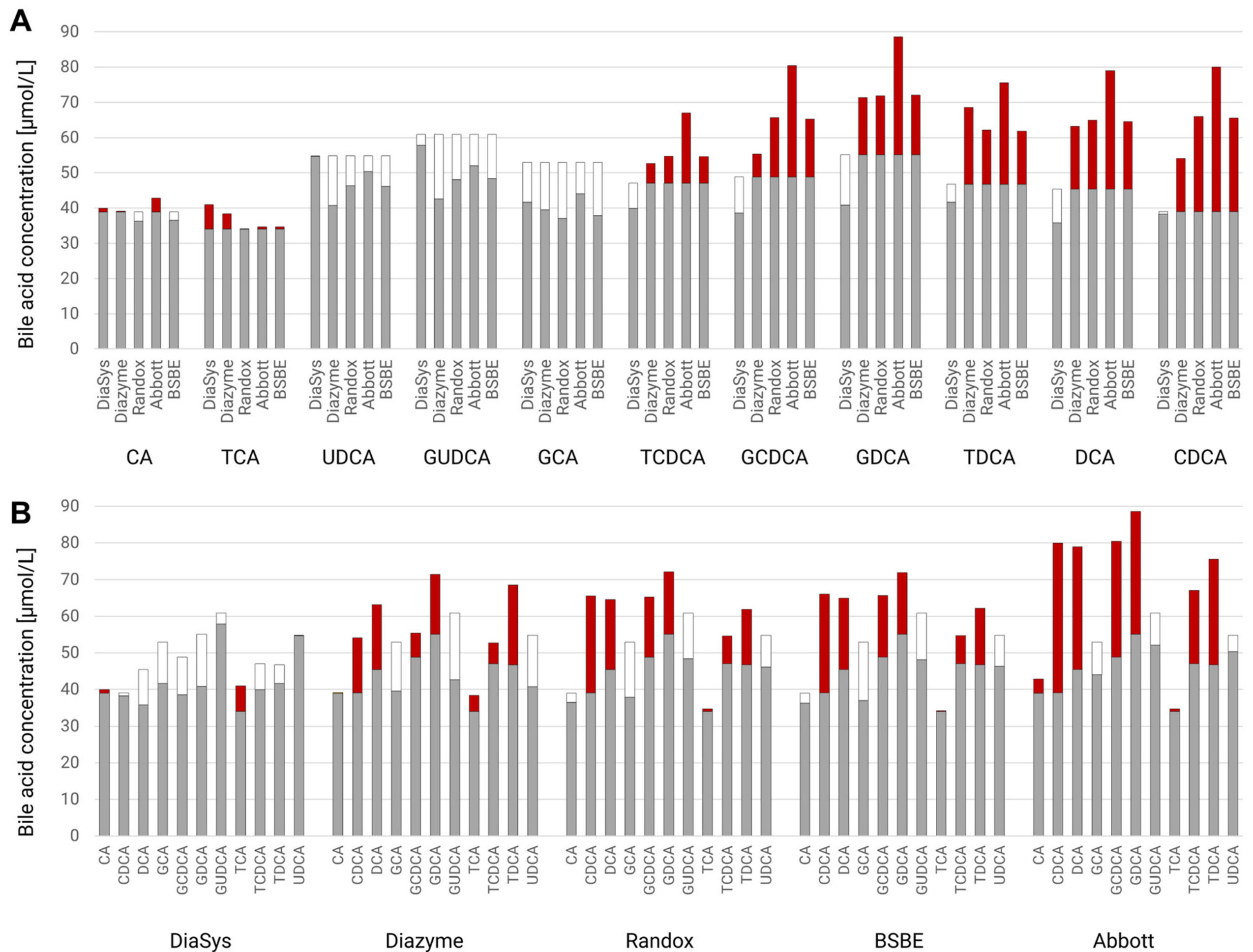


Figure 5: Degree of over- and undermeasurement. The concentration of bile acid standards [µmol/L±SD] over- (red) or under- (white) determined by TBA cycling assays compared to the non-enzymatic reference method (HPLC-MS/MS; grey). (A) Results grouped by bile acid; (B) results grouped by TBA assay and sorted by mean deviation from reference.

range (>60 mol/L), which does not affect ICP diagnosis but potentially ICP risk stratification.

There is complex crosstalk between signaling BAs (which regulate their own synthesis, transport, and metabolism, as well as triglyceride, cholesterol, glucose, and energy homeostasis) and the gut microbiome (which both shapes and is shaped by the intestinal BA pool). This complex interplay can be perturbed in a variety of ways, leading to different disease states, some of which are associated with disease-specific changes in the individual BA profile. However, unlike LC- or MS-based techniques, enzymatic methods are unable to distinguish between different individual BAs and can only detect an increase in the total BA concentration. Furthermore, a significant overrepresentation of selected bile acid species in pathological blood samples will significantly affect the 'total bile acid concentration' determined by each TBA assay, especially if each assay is calibrated against a different set of individual BAs. Patients with severely elevated serum TBA levels well above the normal range can be expected to have profoundly impaired bile acid synthesis, metabolism, clearance, circulation, signaling, or transport. The method variance and deviation of TBA results obtained by different routine assays may be higher in such highly pathological samples. However, all five TBA assays tested will correctly indicate that bile acid homeostasis in these patients is severely disturbed and requires further investigation.

In conclusion, this study highlights the complexity and challenges associated with the enzymatic quantification of total bile acids in human serum. To improve the harmonization of TBA cycling assays, particularly for samples with high serum TBA levels, the following measures will be required: 1) optimizing reaction buffers and recombinant 3α-HSD, e.g., through additives or genetic engineering, to equalize specificity for different BA substrates, and 2) calibration to a common and commutable reference

standard, e.g., a well-defined pool of different BAs, to account for remaining assay-specific differences.

Acknowledgments: The authors would like to thank Sandra Koneberg for her valuable contribution, and Sebastian Alers for providing scientific writing and editorial support, which was funded by DiaSys Diagnostic Systems GmbH (Holzheim, Germany) in accordance with Good Publication Practice (GPP3) guidelines.
Research ethics: This retrospective analysis was conducted in accordance with the World Medical Association's Declaration of Helsinki. Institutional review board approval was not required as the residual patient samples were obtained in a fully anonymized fashion.
Informed consent: Not applicable.
Author contributions: The authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Use of Large Language Models, AI and Machine Learning Tools: DeepL was used to improve grammar and readability of selected sections.
Conflict of interest: A. Masetto, H. Müller, T. Leber and M. Grimmler are employed by DiaSys Diagnostic Systems GmbH (Holzheim, Germany). All other authors state no conflict of interest.
Research funding: None declared.
Data availability: The raw data can be obtained on request from the corresponding author.

References

1. Fleishman JS, Kumar S. Bile acid metabolism and signaling in health and disease: molecular mechanisms and therapeutic targets. Signal Transduct Targeted Ther 2024;9:97.
2. Qi L, Chen Y. Circulating bile acids as biomarkers for disease diagnosis and prevention. J Clin Endocrinol Metab 2023;108:251–70.
3. Zhao X, Liu Z, Sun F, Yao L, Yang G, Wang K. Bile acid detection techniques and bile acid-related diseases. Front Physiol 2022;13:826740.
4. Di Ciaula A, Garruti G, Lunardi Baccetto R, Molina-Molina E, Bonfrate L, Wang DQ-H, et al. Bile acid physiology. Ann Hepatol 2017;16:S4–14.
5. Tveter KM, Mezhibovsky E, Wu Y, Roopchand DE. Bile acid metabolism and signaling: emerging pharmacological targets of dietary polyphenols. Pharmacol Ther 2023;248:108457.
6. Dutta M, Cai J, Gui W, Patterson AD. A review of analytical platforms for accurate bile acid measurement. Anal Bioanal Chem 2019;411:4541–9.
7. Luo L, Aubrecht J, Li D, Warner RL, Johnson KJ, Kenny J, et al. Assessment of serum bile acid profiles as biomarkers of liver injury and liver disease in humans. PLoS One 2018;13:e0193824.
8. Liu N, Feng J, Lv Y, Liu Q, Deng J, Xia Y, et al. Role of bile acids in the diagnosis and progression of liver cirrhosis: a prospective observational study. Exp Ther Med 2019;18:4058–66.
9. Dawson PA, Lan T, Rao A. Bile acid transporters. JLR (J Lipid Res) 2009; 50:2340–57.
10. Ma Z, Liu Y, Chai L, Jin G, Sun Y, Zhou S, et al. Metabolic changes in bile acids with pregnancy progression and their correlation with perinatal complications in intrahepatic cholestasis of pregnant patients. Sci Rep 2023;13:1608.
11. Griffiths WJ, Sjövall J. Bile acids: analysis in biological fluids and tissues. JLR (J Lipid Res) 2010;51:23–41.
12. Alamoudi JA, Li W, Gautam N, Olivera M, Meza J, Mukherjee S, et al. Bile acid indices as biomarkers for liver diseases I: diagnostic markers. World J Hepatol 2021;13:433–55.
13. Chen T, Xie G, Wang X, Fan J, Qiu Y, Zheng X, et al. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. Mol Cell Proteomics 2011;10:M110.
14. Han X, Wang J, Gu H, Guo H, Cai Y, Liao X, et al. Predictive value of serum bile acids as metabolite biomarkers for liver cirrhosis: a systematic review and meta-analysis. Metabolomics 2022;18:43.
15. Mousa OY, Juran BD, McCauley BM, Vesterhus MN, Folseraas T, Turgeon CT, et al. Bile acid profiles in primary sclerosing cholangitis and their ability to predict hepatic decompensation. Hepatology 2021; 74:281–95.
16. Manzotti C, Casazza G, Stimac T, Nikolova D, Gluud C. Total serum bile acids or serum bile acid profile, or both, for the diagnosis of intrahepatic cholestasis of pregnancy. Cochrane Database Syst Rev 2019;7:CD012546.
17. Podgórski PB, Przybyłkowski A, Nehring P. Bile composition – which components can have clinical significance? A review of the literature. J Gastrointestin Liver Dis 2023;32:110–7.
18. Trottier J, Białek A, Caron P, Straka RJ, Heathcote J, Milkiewicz P, et al. Metabolomic profiling of 17 bile acids in serum from patients with primary biliary cirrhosis and primary sclerosing cholangitis: a pilot study. Dig Liver Dis 2012;44:303–10.
19. Trottier J, Białek A, Caron P, Straka RJ, Milkiewicz P, Barbier O. Profiling circulating and urinary bile acids in patients with biliary obstruction before and after biliary stenting. PLoS One 2011;6:e22094.
20. Mayo Clinc Laboratories. Bile acid profile serum (BAPS). [Online]. Available from: https://www.mayocliniclabs.com/test-catalog/overview/62538#Clinical-and-Interpretive [Accessed 3 Sep 2024].
21. Wang X, Xie G, Zhao A, Zheng X, Huang F, Wang Y, et al. Serum bile acids are associated with pathological progression of hepatitis B-induced cirrhosis. J Proteome Res 2016;15:1126–34.
22. Zhang G-H, Cong A-R, Xu G-B, Li C-B, Yang R-F, Xia T-A. An enzymatic cycling method for the determination of serum total bile acids with recombinant 3alpha-hydroxysteroid dehydrogenase. Biochem Biophys Res Commun 2005;326:87–92.
23. Liu Y, Rong Z, Xiang D, Zhang C, Liu D. Detection technologies and metabolic profiling of bile acids: a comprehensive review. Lipids Health Dis 2018;17:121.
24. Mashige F, Tanaka N, Maki A, Kamei S, Yamanaka M. Direct spectrophotometry of total bile acids in serum. Clin Chem 1981;27: 1352–6.
25. Ducroq DH, Morton MS, Shadi N, Fraser HL, Strevens C, Morris J, et al. Analysis of serum bile acids by isotope dilution-mass spectrometry to assess the performance of routine total bile acid methods. Ann Clin Biochem 2010;47:535–40.
26. Danese E, Salvagno GL, Negrini D, Brocco G, Montagnana M, Lippi G. Analytical evaluation of three enzymatic assays for measuring total bile acids in plasma using a fully-automated clinical chemistry platform. PLoS One 2017;12:e0179200.
27. Žížalová K, Vecka M, Vítek L, Leníček M. Enzymatic methods may underestimate the total serum bile acid concentration. PLoS One 2020; 15:e0236372.

28. Haange S-B, Till A, Bergh P-O, Fauler G, Gigl M, Löfgren-Sandblom A, et al. Ring trial on quantitative assessment of bile acids reveals a method- and analyte-specific accuracy and reproducibility. Metabolites 2022;12:583.
29. Hobson SR, Cohen ER, Gandhi S, Jain V, Niles KM, Roy-Lacroix MÈ, et al. Guideline No. 452: diagnosis and management of intrahepatic cholestasis of pregnancy. J Obstet Gynaecol Can 2024;46:102618.
30. Huri M, Seravalli V, Lippi C, Tofani L, Galli A, Petraglia F, et al. Intrahepatic cholestasis of pregnancy – time to redefine the reference range of total serum bile acids: a cross-sectional study. BJOG 2022;129: 1887–96.

Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/cclm-2024-1029).