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REVIEW ARTICLE

Inborn errors of bile acid metabolism and their diagnostic confirmation by means of mass spectrometry

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Abstract:
Bile acids are amphipathic molecules formed from cholesterol in the liver by two main pathways, the neutral and the acidic. Biosynthesis of bile acids generates bile flow which is important for biliary secretion of free cholesterol, endogenous metabolites, and xenobiotics. Bile acids play an important role as biological detergents that make possible intestinal absorption of lipids and fat-soluble vitamins but also as metabolic regulators of lipid, glucose, and energy homeostasis. Several enzymatic steps and subcellular compartiments are involved in the biosynthesis of bile acids. In general, the synthetic pathways of bile acids consist of modification of the steroid nucleus, oxidation and cleavage of the corresponding side chain, and finally conjugation with the amino acids glycine or taurine. Genetic defects in one of these enzymes result in an accumulation of atypical bile acids or intermediates. These defects can cause beside others liver diseases that can vary from mild to very severe. Detection of elevated concentrations of unusual bile acids, intermediates and other endogenous metabolites can be performed by mass spectrometry. In this article, the most known inborn errors in bile acid metabolism and their mass spectrometric confirmation in biological fluids are concisely reviewed.

Keywords: bile acids, inborn errors, liver, metabolism, mass spectrometry
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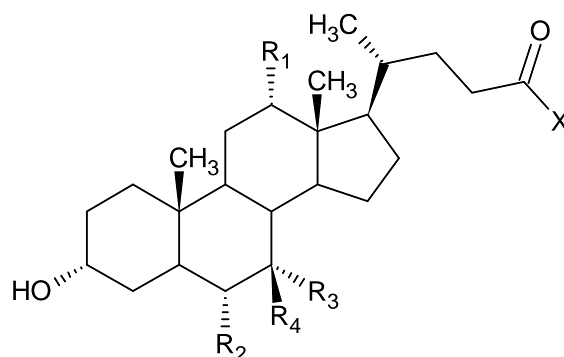
Introduction

Bile acids (BA) represent the main endogenous metabolic by-products of cholesterol and are involved in a number of metabolic processes. They are not only essential for the absorption of dietary lipids and fat soluble vitamins but also act as signalling molecules for diverse endocrine and paracrine functions. BA are ligands for G-protein coupled receptors such as TGR5 and modulators of several nuclear hormone receptors, the most important farnesoid X receptor (FXR) and consequently are needed to maintain cholesterol, lipid, and carbohydrate homeostasis [1,2]. In addition to BA, the predominant bile constituents are phosphatidylcholine (phospholipids) and unesterified cholesterol. The most abundant BA in humans are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA). The primary bile acids CA and CDCA are synthesized in the liver from cholesterol by the action of hepatic enzymes and excreted into the small intestine via the bile duct in form of glycine and taurine conjugates as illustrated in Figure 1. They are reabsorbed and transported back to the liver to enter again enterohepatic circulation [2]. Secondary bile acids DCA, LCA and UDCA are transformed from the primary bile salts by intestinal bacteria. In hepatic and intestinal diseases, synthesis and clearance of BA in liver and their intestinal absorption are disturbed, marked by the elevated levels of total BA (TBA), especially the hydrophobic BA, such as CA, CDCA, DCA or LCA and their conjugates, which may tighten the liver injury and at last lead to cirrhosis and liver failure [3-5].



	R_1	R_2	R_3	R_4
CA	OH	H	OH	H
CDCA	H	H	OH	H
DCA	OH	H	H	H
LCA	H	H	H	H
UDCA	H	H	H	OH

Free BA: X=OH

Glyco-conjugated BA: X=$NHCH_2CO_2H$

Tauro-conjugated BA: X=$NHCH_2CH_2SO_3H$

Figure 1: Molecular structures of bile acids and their glycine- and taurine-conjugates

Bile Acid Synthesis Pathway

In general, BA synthesis can occur through two well-known pathways: the classic (neutral) pathway or the alternative (acidic) pathway [6]. The classic pathway, as depicted in Figure 2, occurs in the liver, and accounts for approximately 90% of bile acid synthesis. The alternative (acidic) pathway produces the remaining 10% of bile acids and is primarily extrahepatic (Figure 3). The classical pathway begins with the rate-limiting 7α-hydroxylation of cholesterol by the enzyme cholesterol 7α-hydroxylase (CYP7A1) and the 12α-hydroxylation of the intermediates by sterol 12 α -hydroxylase (CYP8B1), followed by side chain oxidation by sterol 27-hydroxylase (CYP27A1) [7-10]. The alternative pathway starts with hydroxylation of the cholesterol side chain by sterol 27-hydroxylase (CYP27A1) in extrahepatic sites including vascular endothelium and macrophages, followed by 7α-hydroxylation of the oxysterol intermediates by the corresponding enzyme oxysterol7α-hydroxylase (CYP7B1) [7]. Biosynthesis of cholic acid might also occur through side chain oxidation of cholesterol by the 26- and 25-hydroxylation pathway as depicted in Figure 4. The first begins with hydroxylation of C26 followed by oxidation to a carboxylic acid and cleavage of propionic acid. The second begins with hydroxylation of C25 followed by oxidation to a ketone and cleavage of acetone [11]. Feedback loops controlling gene transcription of the major enzymes in bile acid synthesis, especially CYP7A1 and CYP8B1 are well-known [12-14]. Nuclear hormone receptors such as the farnesoid X receptor (FXR), pregnane X receptor PXR, and vitamin D receptor VDR have been identified as bile acids nuclear receptors [15-16].

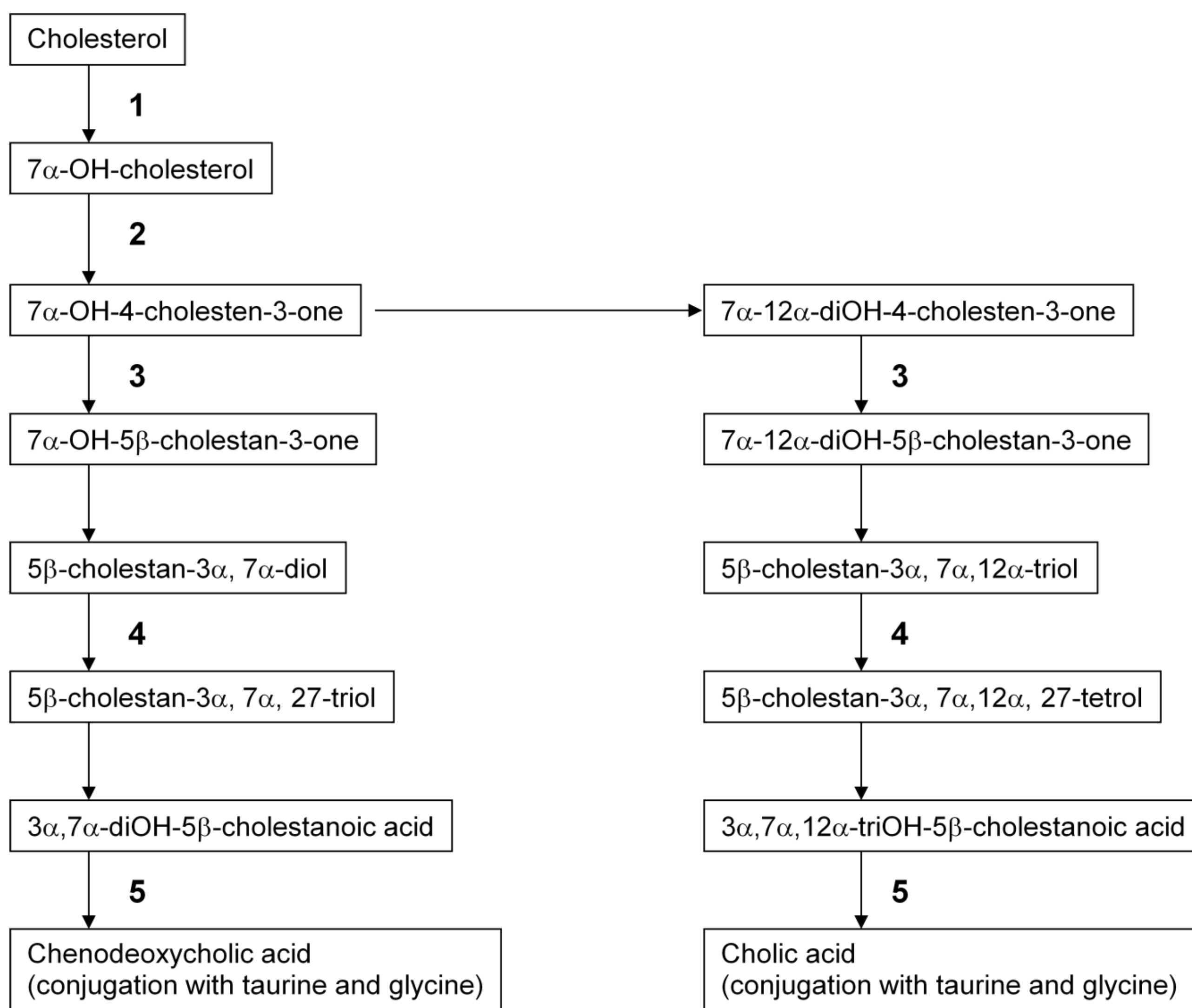


Figure 2: The classic (neutral) pathway of the bile acids synthesis. Numbers 1-5 represent following lacking enzymes in inborn errors: 1) Cholesterol 7α-hydroxylase deficiency, 2) 3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency, 3) Δ^4-3-Oxosteroid-5β-reductase deficiency, 4) Sterol 27-hydroxylase deficiency, 5) peroxisomal defects.

Analytical Detection Techniques

In cases of inborn errors of bile acid metabolism and hepatobiliary diseases, noticeable changes in the concentrations of individual BA's and their metabolic profiles in plasma, serum, bile or urine can be observed and have been considered important biochemical markers for diagnostic purposes. Due to the structural similarity, particularly for the isomers, and the lower concentrations of BA in e.g. serum or urine, high selective and sensitive analytical methods are needed for simultaneous monitoring of BA in biological samples [17-18]. In recent years, high-performance liquid chromatography (HPLC) coupled with UV detection has been applied to the analysis of some individual BA in biological fluids, with a precolumn derivatization method due to the lack of chromophore in structures of BA, which led to a more complicated sample pretreatment procedure [19]. Similarly, gas chromatography (GC) alone and gas chromatography coupled to mass spectrometry

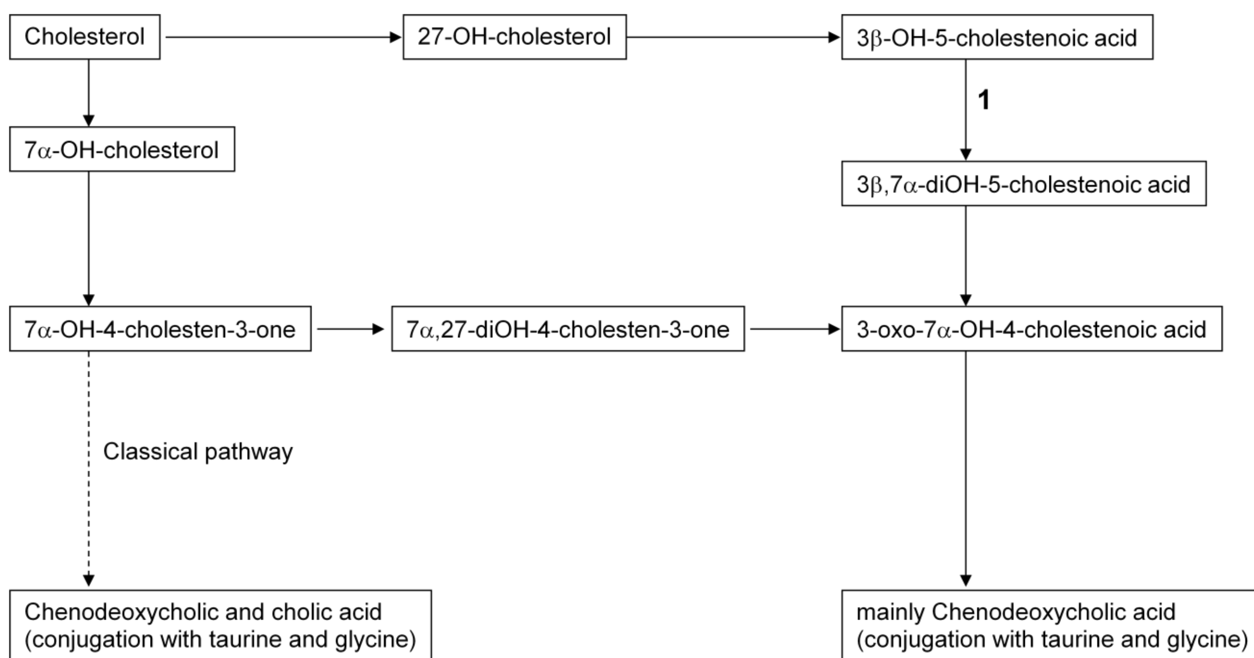


Figure 3: The alternative (acidic) pathway of the bile acids synthesis. Number 1 represents the oxysterol 7α-hydroxylase deficiency

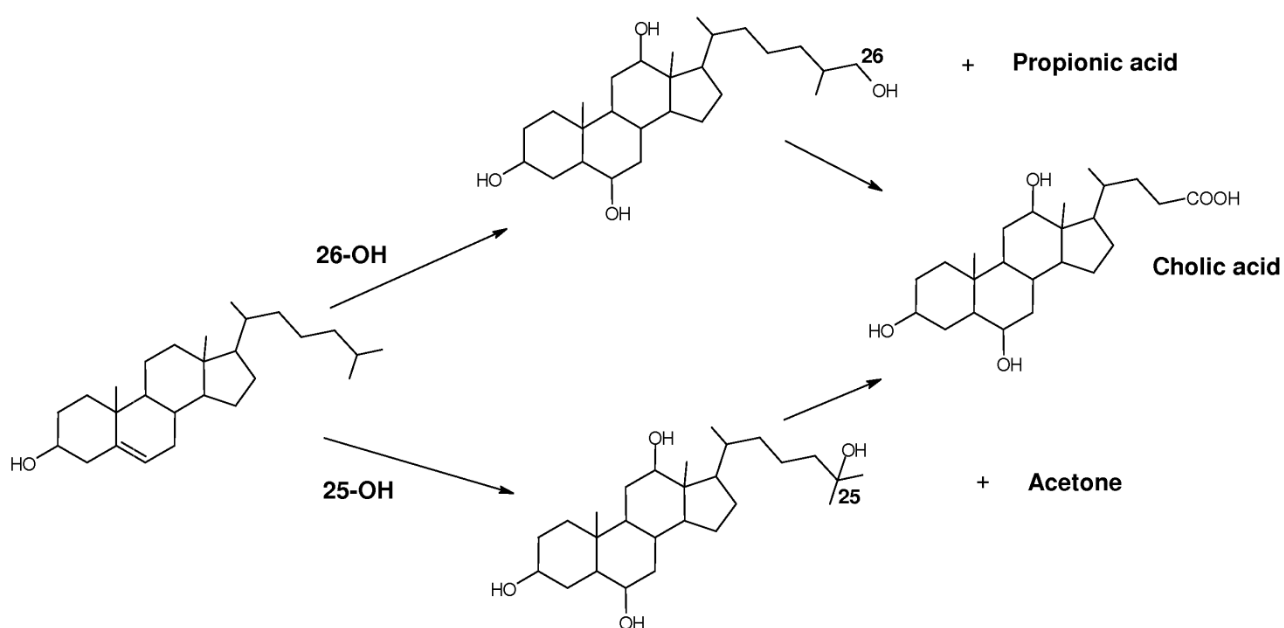


Figure 4: The 25(26)-hydroxylation pathway of the bile acids synthesis.

Table 1: List of the most known inborn errors of bile acid metabolism
Cholesterol 7α -hydroxylase deficiency
Oxysterol 7α -hydroxylase deficiency
Sterol 27-hydroxylase deficiency
3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency
Δ^4-3-Oxosteroid-5β-reductase deficiency
α -Methylacyl-CoA racemase deficiency
Bile acid conjugation deficiency
Zellweger Syndrome (Peroxisomal disorders)

(GC-MS) have also been limited by the complexity of the derivatization steps, although they have provided high detection sensitivity and selectivity for analysis of BA [20-21]. An important drawback of the method was the need for cleavage of conjugated bile acids and derivatisation to provide the necessary volatility. The use of fast atom bombardment-mass spectrometry (FAB-MS) or electrospray ionization-tandem mass spectrometry (ESI-MS) allows direct detection of unconjugated or conjugated BA's [22].

Moreover, these techniques can not differentiate BA isomers from each other. Recently, HPLC aided with mass spectrometry has reported the determination of BA isomers and their derivatives [23-24]. However, modern separation and detection methods of BA based on HPLC-ESI-MS/MS including isomeric forms of free BA and their glycine and taurine conjugates were developed and applied [25-26]. Ultra-performance liquid chromatography (UPLC) in combination with tandem mass spectrometry showed higher power in separation and analysis speed over conventional HPLC and has been utilized for rapid analysis of individual or full BA profiles [27].

Inborn errors of bile acid synthesis

The neutral sterol, cholesterol, is converted to the two principal acidic primary bile acids, namely cholic and chenodeoxcholic acids. This conversion required several enzymatic reactions. The absence of one of these enzymes leads to different primary (enzymopathy) and secondary (peroxisomal) disorders. In Table 1, a list of most known bile acid biosynthetic defects is given.

1) Cholesterol 7α -hydroxylase deficiency

The gene CYP7A1 encodes the enzyme cholesterol 7alpha-hydroxylase and catalyzes the rate limiting step in cholesterol catabolism and biosynthesis of bile acids. There is a decrease in bile acids production via the classical pathway which is compensated by activation of the acidic sterol 27-hydroxylase pathway. The content of cholesterol in liver is increased and gallstones may result. Patients carrying this gene mutation showed abnormal serum lipids (elevated cholesterol). However, this disease can be considered rather as dyslipidemia syndrome than a liver dysfunction [28].

2) Oxysterol 7α -hydroxylase deficiency

The enzyme oxysterol 7α -hydroxylase is needed in the alternative (acidic) pathway. Lack of this enzyme causes severe neonatal liver disease which leads to accumulation of extremely cholestatic and hepatotoxic monohydroxy bile acids and can not be treated by primary bile acids. The urinary LC-MS/MS (liquid chromatography-tandem mass spectrometry) analysis showed two specific major ions at mass-to-charge ratio (*m/z*) 453 and 510. The first ion corresponds to the 3β-hydroxy-5-cholenoic acid sulfate while the second to the 3β-hydroxy-5-cholestenoic acid glycosulfate [29].

3) Sterol 27-hydroxylase deficiency

This enzyme deficiency, which is also known as cerebrotendinous xanthomathosis - briefly CTX - is an inherited lipid storage disease characterized by accumulation of cholesterol and cholestanol in tissues. The most serious symptoms are, amongst others, progressive neurologic dysfunction, dementia, and the presence of cataracts, ataxia and xanthomatous lesions in the brain and tendons and hence characteristic clinical features. From the biochemical point of view, there is a significantly decrease in production of primary bile acids – cholic and chenodeoxycholic acids – and an increase in excretion of bile alcohol glucuronides in urine and faeces. The urinary mass spectrometric bile acid profile revealed the presence of molecular ions at *m/z* 613, 627 and 643 which correspond to 27-Nor-

cholestane-pentol-, cholestane-pentol- and cholestane -hexol-glucuronides. The ion at *m/z* 627 represented in the spectrum the most prominent one [30-32]. Recently, a quantification assay of 7α-hydroxy-4-cholesten-3-one, the efficient bile acid precursor to cholestanol, with LC-MS/MS as a novel approach for diagnostic testing of CTX has also been described [33].

***4)* 3β-Hydroxy-Δ^5-C_{27}-steroid oxidoreductase deficiency**

According to the published literature this metabolic disorder was the first described defect in the bile acid biosynthetic pathway and is the most common one [34-36]. This enzyme catalyzes the conversion of the precursor 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one in the classical pathway. Clinical manifestations include conjugated bilirubinemia, jaundice, elevated serum aminotrasferases accompanying by normal serum γ-glutamyl transpeptidase and fat-soluble vitamin malabsorption [37]. Rapid and definitive diagnosis of this defect can be achieved using LC-MS/MS. Urinary analysis confirmed the presence of four characteristic ions at *m/z* 469, 485, 526 and 542. These ions were identified as follows: 3β,7α-dihydroxy-5-cholenoic acid sulfate (*m/z* 469), 3β,7α,12α-trihydroxy-5-cholenoic acid sulfate (*m/z* 485), 3β,7α-dihydroxy-5-cholenoic acid glycosulfate (m/z 526) and 3β,7α,12α-trihydroxy-5-cholenoic acid glycosulfate (*m/z* 542). The most intense detected ion in the mass spectrum was at *m/z* 469 [38].

5)Δ^4-3-Oxosteroid-5β-reductase deficiency

This enzyme catalyzes the transformation of the intermediates 7α-hydroxy-4-cholesten-3-one and 7α,12α-dihydroxy-4-cholesten-3-one to the corresponding 7α-hydroxy-5β-cholestan-3-one and 7-α,12α-dihydroxy-5β-cholestan-3-one. This enzymatic process includes a reduction of Δ^4 double bond and a conversion of the 3-one moiety to 3α-hydroxy of the sterol nucleus. Lowered efficiency level in this conversion may result in increased amounts of Δ^4-bile acids. One part of 5β(H)-bile acids (Δ^4-3-oxo sterol) can be converted into 5α(H)-structures, as allocholic and allochenodeoxycholic acids, by the enzyme steroid 5α-reductase type I which catalyzes the reduction of Δ^4-double bonds in a variety of steroid substrates. The clinical features of this disorder are similar as in 3β-hydroxy-C_{27}-steroid oxidoreductase deficiency. If this disease remains untreated, patients tend to have a progressive liver damage. Elevations of 3-oxo-Δ^4-bile acids as well as allo-bile acids with reduction in primary BA were found in serum and urine [39-41]. The mass spectrum of the urinary excretion contained primarily 4 molecular ions at *m/z* 444, 460, 494 and 510. Identification of these specific ions is as follows: glycine conjugates of 7 α -hydroxy-4-cholesten-3-one (*m/z* 444) and 7α,12alpha-dihydroxy-4-cholesten-3-one (*m/z* 460), taurine conjugates of 7α -hydroxy-4-cholesten-3-one (*m/z* 494) and 7α,12α-dihydroxy-4-cholesten-3-one (*m/z* 510) [42].

6) α-Methylacyl-CoA racemase deficiency

α-Methylacyl-CoA racemase (AMACR) is an enzyme that plays an important role in bile acid biosynthesis as well as mitochondrial and peroxisomal β-oxidation of branched-chain fatty acid. This enzyme racemizes the 25R-C_{27}-trihydroxycholestanoate-CoA's which are generated from the classical (neutral) pathway to the corresponding 25S-enantiomers. The same enzymatic reaction is performed for the conversion of the branched-chain fatty acid 2R-pristanoyl-CoA to the corresponding 2S-enantiomer which is indispensable for initiating subsequent peroxisomal β-oxidation in the fatty acid pathway. Clinical symptoms may mainly include neurological disease. However, neonatal cholestatic liver disease has also been documented. Diagnosis of this defect can be ascertained by mass spectrometric detection of obviously increased concentrations of C_{27}-trihydroxycholestanoic acids in urine or serum. Markedly elevations in plasma pristanic acid (product of phytanic acid by α-oxidation) and normal to mildly increased levels of phytanic acid in urine and serum could also be detected [43,44]. In the case of α-oxidation disorder, high augmented plasma concentration of phytanic acid (>200 μmol/L) is characteristically for the deficiencies of phytanoyl-CoA hydroxylase or of the peroxisome-targeting signal type 2 (PTS2) receptor (Refsum disease) [45]. The presence of urinary C_{27}-bile acids as taurine conjugates can be confirmed by LC-ESI-MS/MS. Three characteristically molecular ions for the deficiency of AMACR at *m/z* 556, 572 and 588 were detected. They are assigned to the taurine conjugation of C_{27}-trihydroxy- (*m/z* 556), C_{27}-tetrahydroxy- (*m/z* 572) and C_{27}-pentahydroxycholestanoic (*m/z* 588) acids. The major detected ion was at *m/z* 572 [46,47].

7) Bile acid conjugation deficiency

Two enzymes are involved in conjugation reactions of the terminal side-chain carboxylic acid in bile acid synthesis, namely the bile acid-CoA ligase and the bile acid-CoA:amino acid *N*-aceyltransferase (BAAT). The first enzyme is responsible for the formation of CoA thioester, while the second generates the coupling reaction with glycine or taurine. This rare inborn error was firstly identified by Setchell et al [48]. Characteristically for this disease is the absence of glycine and taurine conjugates in serum, bile and urine. Clinical symptoms of affected patients consisted mainly of fat-soluble vitamin malabsorption and early growth failure. The mass spectrum profiles of serum and urine samples revealed particularly two peaks at m/z 407 and 391 in the negative ionization mode corresponding to unconjugated cholic and deoxycholic acids. In addition, sulfate and glucuronide conjugates of cholic acid were also found in urine excretion [34,49].

8) Zellweger Syndrome (Peroxisomal disorders)

In general, peroxisomal disorders are categorized into two main groups: peroxisome biogenesis disorders and single peroxisomal enzyme deficiencies. Peroxisome biogenesis disorders are further divided into 4 groups: Neonatal Adrenoleukodystophy (NALD), Infantile Resum's Disease (IRD), Rhizomelic Chondrodysplasia Punctata (RCDP), and Zellweger Syndrome (ZS) [50,51]. NALD, IRD, and ZS are referred to the Zellweger spectrum, due to overlapping clinical manifestations. Clinical features in Zellweger patients include cerebral neuronal migration disorder, cranio-facial dysmorphism, pyschomotor retardation, hypotonia, chronic liver disease, and minor renal cystic changes. This disease is also known as cerebro-hepato-renal syndrome [52-54]. Analysis of urine excretions confirmed the presence of toxic polyhydroxylated C_{27}-intermedites, but predominantly as tauro-tetrahydroxycholestanoic acid (*m/z* 572). The presence of elevated very long chain fatty acids (>C22) due to lack of oxidation process in peroxisomes, increased level of pipecolic acid (Hyperpipecolic acidemia) and C_{29}-dicarboxylic acids are also typical for this disorder [55-59].

Conclusion

Genetic defects in hepatocellular bile formation result in intrahepatic accumulation of cytotoxic bile acids leading to inflammation, fibrosis and cirrhosis of the liver. In many cases, progressive and fatal conditions are the consequences for untreated patients. Early diagnosis of these inborn errors is of particular importance because affected patients, at least in some cases, can be successfully treated. Rapid and accurate confirmation of the inborn error in biofluids can be achieved by mass spectrometric techniques.

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