Clinical significance and methods of bile acid determination

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List of abbreviations:

BA: Bile acid
TBA: Total bile acid
GOT: Glutamate-oxaloacetate transaminase
GPT: Glutamate-piruvate transaminase
SAP: Serum alkaline phosphatase
GGT: Gamma-glutamyl transferase
ICP: Intrahepatic cholestasis of pregnancy
HPLC: High-performance liquid chromatography
LC-MS/MS: Liquid chromatography- tandem mass spectrometry
RIA: Radio-immuno-assay
ELISA: Enzyme-linked-immunosorbent-assay
NAD: Nicotinamide adenine dinucleotide, **NADH:** Hydrogenated NAD
LoQ: Limit of quantitation
iNOS: Nitrogen-oxid syntase

In Hungarian laboratories, the determination of bile acid (BA) is not yet among the laboratory's routine markers. No professional objections can be raised against this because its measurement is necessary for non-frequent illnesses. As with the less frequently requested laboratory markers, it is also advisable to centralize the tests in compliance with logistical standards that guarantee the completion of the results within at least 48 hours. These are, e.g., the samples of pregnant women because a positive result can have urgent therapeutic consequences. In our country, the currently used analytical methods allow the routine measurement of total bile acids (TBA). According to the data submitted to QualiCont, six domestic and two foreign laboratories sent TBA results.

This study aims to summarize the possible analytical methods of bile acid measurement based on up-to-date foreign and domestic bibliography in addition to the textbook data (1, 2) and draw attention to the knowledge required to interpret the results depending on the method used, the clinical and the sampling conditions.

Physiological and pathophysiological background

Cholesterol is an essential component of mammalian membrane cells. All our cells - including liver cells - can synthesize cholesterol from precursors. From the point of view of cholesterol homeostasis, the cholesterol → bile acid transformation taking place in the endoplasmic reticulum of the liver is of particular importance. The liver physiologically produces 12-18 g of bile acid per day, of which 95% actively reabsorbs in the terminal ileum. The primary bile acids (cholic acid, chenodeoxycholic acid) conjugate with taurine and glycine, become water-soluble, are excreted through the bile canaliculi and the bile duct into the duodenum, and are stored in the gallbladder. The intestinal microbiome converts these into secondary bile acids (deoxycholic acid, lithocholic acid), some reabsorbed and returned to the circulation (enterohepatic circulation). Forms conjugated with glycine and taurine are further converted into tertiary bile acids (Tauro-chenodeoxycholic acid, glycol-chenodeoxycholic acid), which are present in bile in approximately the same concentration. More than 90% of bile acids are deoxycholic acid, cholic acid, and chenodeoxycholic acid. ***(Figure 1)***

Bile acids enter the gallbladder during fasting, where their concentration increases tenfold. After a night of fasting, 95% of all bile acids stay concentrated in the gallbladder. That explains why

their levels in the intestinal tract, vena portae, liver, and plasma are shallow under healthy conditions after 10-12 hours of fasting. Bile acid levels in the intestinal lumen also decrease during meal breaks. Thus, the plasma bile acid level depends on the enterohepatic circulation, which is why its concentration increases by at least 50% compared to the fasting value 90-120 minutes after a meal (1, 2). ***(Figure 2)***

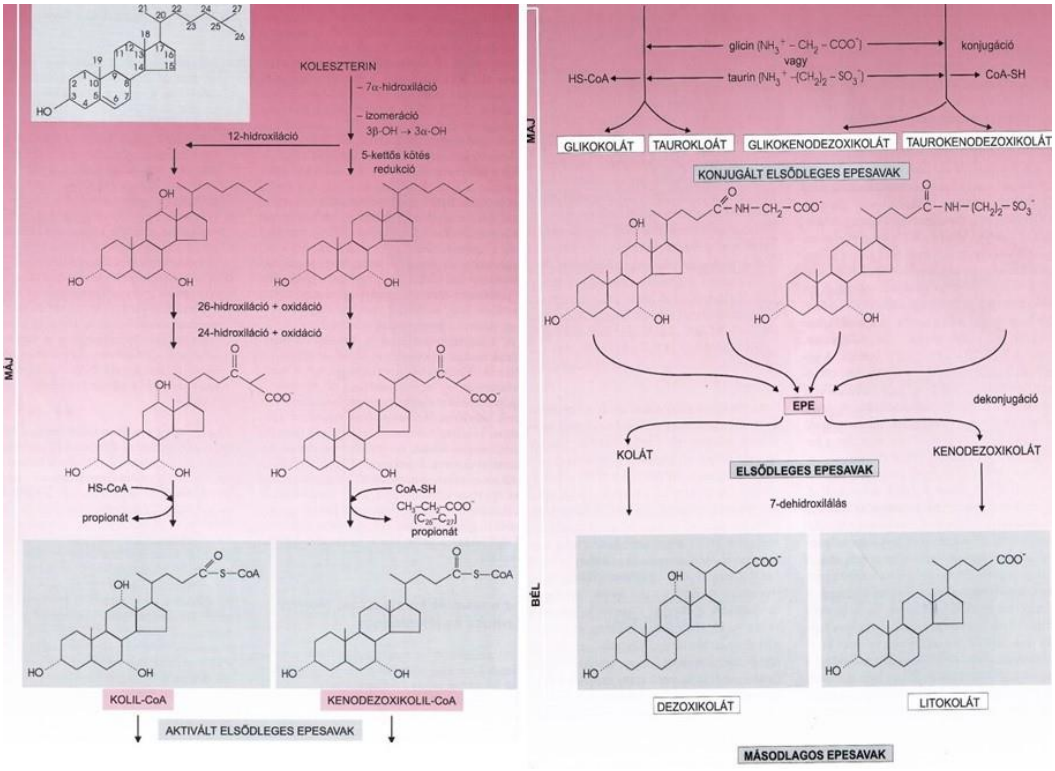

Figure-1: Synthesis and metabolism of bile acids (Veronika Ádám: Metabolism of lipids) (3)

Translation of Hungarian texts: MÁJ: Liver, BÉL: Bowel, EPE: Bile, KOLESZTERIN: Cholesterol, hidroxiláció: hydroxylation, izomeráció: Isomerization, kettős kötés redukció: Double bond reduction, oxidáció: Oxidation, AKTIVÁLT ELSŐDLEGES EPESAVAK: Activated primary bile acids, KONJUGÁLT ELSŐDLEGES EPESAVAK: Conjugated primary bile acids, MÁSODLAGOS EPESAVAK: Secondary bile acids, konjugáció: Conjugation, vagy: or, dekonjugáció: De-conjugation, dehidroxilálás: De-hydroxylation.

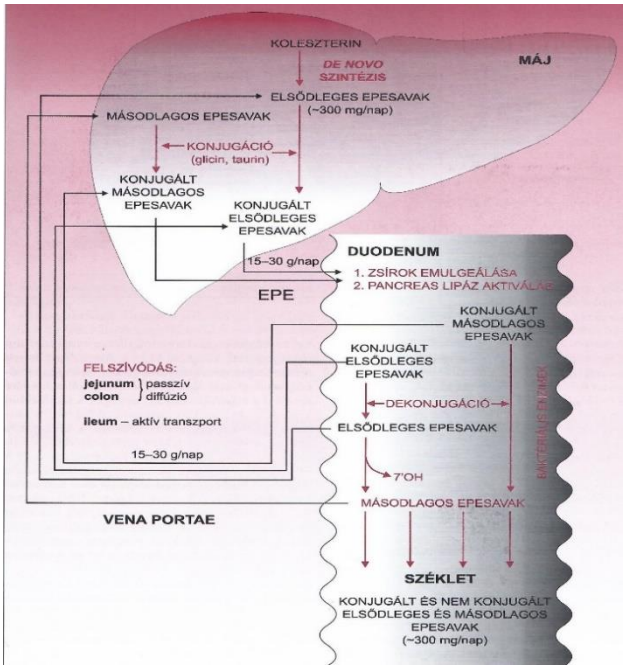

Figure-2: Enterohepatic cycle of bile acids: (Veronika Ádám: Metabolism of lipids) (3)

Translation of Hungarian texts: MÁJ: Liver, KOLESZTERIN: Cholesterol, DE NOVO SZINTÉZIS: De novo synthesis, ELSŐDLEGES EPESAVAK: Primary bile acids, nap: day, MÁSODLAGOS EPESAVAK: Secondary bile acids, KONJUGÁCIÓ: Conjugation, DEKONJUGÁCIÓ: De-conjugation, KONJUGÁLT MÁSODLAGOS EPESAVAK: Conjugated secondary bile acids, KONJUGÁLT ELSŐDLEGES EPESAVAK: Conjugated primary bile acids, EPE: Bile, ZSÍROK EMULGEÁLÁSA: Emulsification of fats, SZÉKLET: Stool, FELSZÍVÓDÁS: Absorption.

The main physiological functions of bile acids:

1. Facilitation of absorption and elimination of cholesterol.
2. Physiological detergents: surfactants dissolve, absorb, break down lipids and activate the lipase enzyme.
3. Maintain a healthy microbiome (4,5).
4. Bile acids, as signal-hormone molecules, regulate their homeostasis and play a role in thyroid hormone signaling, glucose, and lipid metabolism, energy utilization, and cellular immunity.

It is also from these fine regulatory functions that under healthy conditions, the bile acid concentration of the blood is shallow (<10 µmol/L). In many diseases, however, this amount can increase significantly. (4-8)

Clinical significance

Together with other clinical and laboratory data, the knowledge of total bile acid concentration from serum or plasma has proven to be a helpful laboratory parameter in the following diseases:
1.) Cholestatic liver diseases. Disturbance of the outflow of bile causes the accumulation of TBA in the liver, which accumulates in extrahepatic tissues, blood, and urine when it enters circulation. The most characteristic clinical symptom of cholestasis is skin itching (7, 8).

2.) Bile acids are cytotoxic in high concentrations and harmful to hepatocytes and cholangiocytes. It plays an essential pathological role in various liver diseases. Clinical studies have shown that TBA in the blood and urine is 100 times higher than the average level in liver diseases. Elevated TBA levels correlate well with the degree of liver tissue damage and disease progression. To this day, liver enzymes (GOT, GPT SAP, GGT) and bilirubin are used as biomarkers of liver diseases in clinical practice. The disadvantage of these is that they are not specific for the liver and bile ducts, so their value can also be higher in non-hepatobiliary conditions (5-8). Beshee et al. (9) demonstrated an association between BA and ABCB11 gene polymorphism in cirrhotic patients with chronic hepatitis C.

3.) Overall, an increased TBA value can be measured:

- In acute and chronic hepatitis, primary sclerosing cholangitis, hepatocellular carcinoma (5-9).
- In hepatobiliary diseases: biliary obstruction, intrahepatic cholestasis, it rises only temporarily after cholecystectomy.
- In case of acute pancreatitis of various etiologies (8).
- Since the toxic level of TBA causes oxidative stress and mitochondrial dysfunction in trophoblast cells, TBA determination has a critical role in recognizing intrahepatic cholestasis (ICP) occurring in pregnancy, in assessing the progression of the disease, and in monitoring therapy (6, 7). Intrahepatic cholestasis during pregnancy endangers the fetus's health and, in severe cases, its life. Wang et al. (1) found a correlation between the dysregulation of bile acid transport and detoxification function in these pregnant women.

4.) Knowing the level of TBA can also be helpful in bile acid malabsorption associated with chronic diarrhea and in various etiologies of acute pancreatitis (8).

5.) In their latest publication, Harnisch et al. draw attention to the fact that knowing individual bile acid levels help predict the survival of patients suffering from acute respiratory distress syndrome. Significantly lower secondary bile acid levels were measured in survivors' serum (11).

Analytical methods for the quantitative determination of bile acids

1.) Only methods supplemented with a separation technique are suitable for the determination of individual bile acids (1,2):

- HPLC
- LC-MS/MS (gold standard)

2.) For the measurement of TBA, the following application methods can also be used routinely and can be installed on automated clinical chemistry platforms:

- enzymatic, colorimetric methods
- RIA
- ELISA

In Hungary, enzymatic is the mainly used method for measuring TBA, which takes place in several cycles. In each method, the enzyme 3-alpha-hydroxysteroid dehydrogenase oxidizes all bile acids (each 3-alpha-hydroxysteroid molecule) to 3-alpha-oxosteroids. Colorimetric detection is made possible by the NAD-NADH transformation.

On Beckman-Coulter DxC 700 analyzer:

The formation rate of the final product thio-NADH is directly proportional to the total bile acid concentration (TBA) in the sample. Detection is done colorimetrically (at 404 nm).
The lower limit of quantification (LoQ): 0.6 µmol/L, CV$\leq$ 20%
Measurment range: 0.6-180 µmol/L
The reference values measured from the samples of fasting healthy, non-pregnant women and men in both sexes: 1-6 µmol/L.

With Randox reagent, using Roche Cobas 6000 analyzer:

The NAD-NADH conversion is detected using NBT (nitro tetrazole blue), which is measured colorimetrically at 546 nm.
The published reference values are measured from samples of healthy, non-pregnant women and men on an empty stomach: in both sexes: 0-6 µmol/L.
According to the bibliography, these methods correlate well with the gold standard (LC-MS/MS) method. However, it is common to find that the TBA level is measured 20-42% below the "gold" standard method; therefore, it is essential for method-specific reference ranges to be set (11). (Although the TBA reference blood level with the enzymatic method is 0-6 µmol/L, the clinical decision limit can be <10 µmol/L.)

Interpretation of results:

The detectability and reference values given by the manufacturers are primarily determined in a small number of cases but based on the CLSI EP28-A3c protocol: Due to inter- and intraindividual variabilities, food intake, diurnal rhythm, and deviations from LC-MS/MS, it is not easy to set valid reference range (10, 13). A considerable deficiency of the methods is that the manufacturers do not provide a reference range measured from samples of pregnant women. Most of the time, the introduction of the test arises primarily in connection with intrahepatic cholestasis of pregnancy (ICP). Despite this, the relevant bibliography data is relatively poor.

The frequency of ICP is 0.1-5.6% of pregnancies worldwide. The early diagnosis of ICP is also important because the severity of fetal risks is related to increased bile acid levels (14, 15). In a normal pregnancy in the third trimester, the upper value of TBA taken from postprandial samples is recommended to be 19 µmol/L. However, if the pregnant woman has unexplained pruritus, it is considered that the increase in TBA level should be checked regularly. However, the cut-off value for high-risk ICP was defined as 40 µmol/L, while with a TBA value >100 µmol/L, stillbirth can be predicted with high probability (15). All these values were also confirmed by a meta-analysis processing a large number of cases (16).

Other authors (17) have defined the following criteria for the diagnosis of ICP in their studies:
1.) Unexplained pruritus starting from the second trimester, which disappeared after delivery
2.) TBA level measured in the mother's serum >10 µmol/L
3.) Abnormal liver function tests (GPT or GOT) >40 U/L
4.) Total bilirubin level is normal or slightly elevated, but other evidence does not prove liver dysfunction.
Mitchell et al. (15) established the sampling time and reference range in the third trimester as 30-37 weeks of pregnancy. The measurements were performed from sera, and the Beckman Coulter methods were used for each laboratory parameter (15). Liver function tests showed a positive correlation with TBA, and a significant negative correlation between nitric oxide synthase (iNOS) and TBA was confirmed (14). Their main conclusion: iNOS may promise an even better predictive value regarding the severity of ICP and the resulting adverse effects in newborns (14).

Preanalytical information:

The recommended sampling time should be in the early morning. If the change in the TBA level needs to be continuously monitored, keeping a similar time for blood collection is necessary. In the case of pregnant women, non-fasting sampling is recommended since the clinical specificity of the method promises to be better this way (15). While for non-pregnants, on an empty stomach (after 10-12 hours of fasting) in the morning, blood sampling is recommended.

Sample required for testing:

For each method, serum can be used, but depending on the method, Li-heparin, Na-heparin, or plasma collected in a tube containing K2EDTA can also be used. With the Randox reagent, heparin causes interference, so the heparinized plasma is unsuitable for TBA determination.
Stability of the sample according to the package insert given for the Beckmann Coulter method:

Serum	1 day at 20°C	1 week at 2-8°C	12 months at -20°C
Plasma	6 hours at room temperature	48 days at 2-8°C	2 months at -20°C

The description of the Randox method needs to provide exact data regarding the stability of the sample.

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