Focusing Review

Development of Highly Selective Analytical Systems for Biological Substances Using Chromatography Combined with Mass Spectrometry ––With Special Reference to Bio−Analytical Studies of Bile Acids––

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Summary

For the diagnosis of disease, reliable analytical methods for biologically important compounds are essential. Due to the existence of these compounds in complex matrices, such as blood, urine, or tissue, chromatographic procedures are preferable. In this article, we review highly selective and sensitive chromatographic methods coupled with mass spectrometry for the analysis of biologically important materials, especially bile acids and related compounds. We also discuss immunoaffinity extraction employing highly specific antibodies.

Keywords: gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry, immunoaffinity extraction, bile acid, drug, biological material, stable isotope labeled compounds, immobilized antibody

1. Introduction

Bile acids are C_{24} carboxylic acid derivatives containing the steroid nucleus with a side chain at the C−17 β position. The common bile acids have an A/B−*cis* (5 β) configuration, a hydroxyl group at the 3 α, 7 α, 7 β and/or 12 α position, and a carboxylic acid group at the C−24 position, the terminus of the side chain. In healthy human body fluids, five kinds of bile acids, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA), are usually present along with their glycine and taurine conjugates (Fig. 1). Among these acids, CA and CDCA are primary bile acids and the others are secondary bile acids.

Bile acids are the main metabolites of cholesterol and the primary bile acids are synthesized in the liver by hepatic enzymes. The 7 α−hydroxylation of cholesterol is the first and the rate−limiting step in bile acid biosynthesis. The 7 α−hydroxycholesterol then undergoes modification at the A and B rings, and a 3 α,7 α−dihydroxy−5 β−cholestanol derivative is generated. Further oxidation of the side chain produces the cholestanoic acid derivative (so−called C_{27} bile acid). The cholestanoic acid is then *trans*formed into CA and CDCA by the action of β−oxidation enzymes located in hepatic peroxisomes (Fig. 2).

The bile acids are excreted into the small intestine *via* the bile duct as their glycine and taurine conjugates. In the intestinal lumen, bile acids assist in the lipolysis and absorption of fats by forming mixed micelles, and then return to the liver through the ileum−proximal colon. During this enterohepatic circulation, bile acids are *trans*formed into DCA, UDCA, and LCA by the action of intestinal bacteria. This enterohepatic circulation is also responsible for the low concentration of bile acids in the peripheral blood. In contrast, in hepatobiliary diseases, changes in the synthesis and clearance of bile acids by the liver and absorption by the intestine cause changes in the level and metabolic profile of bile acids in biological fluids. In turn, this leads to increases in the body fluid levels of

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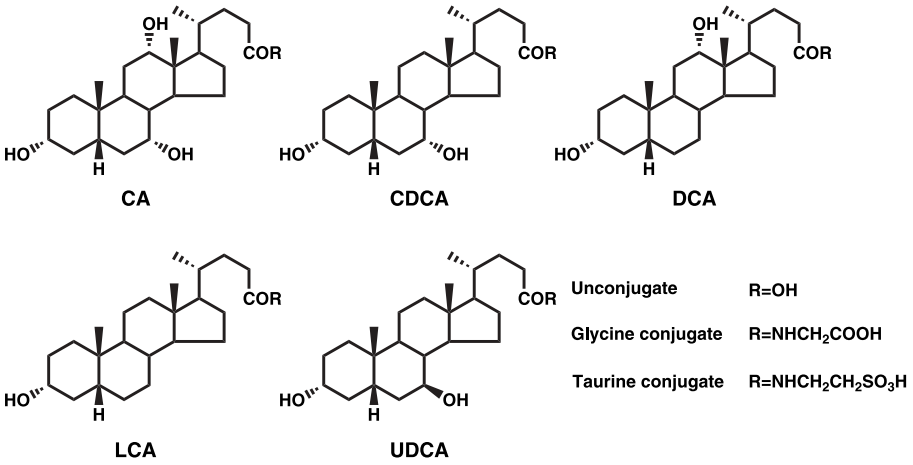


Figure 1. Structures of common bile acids.

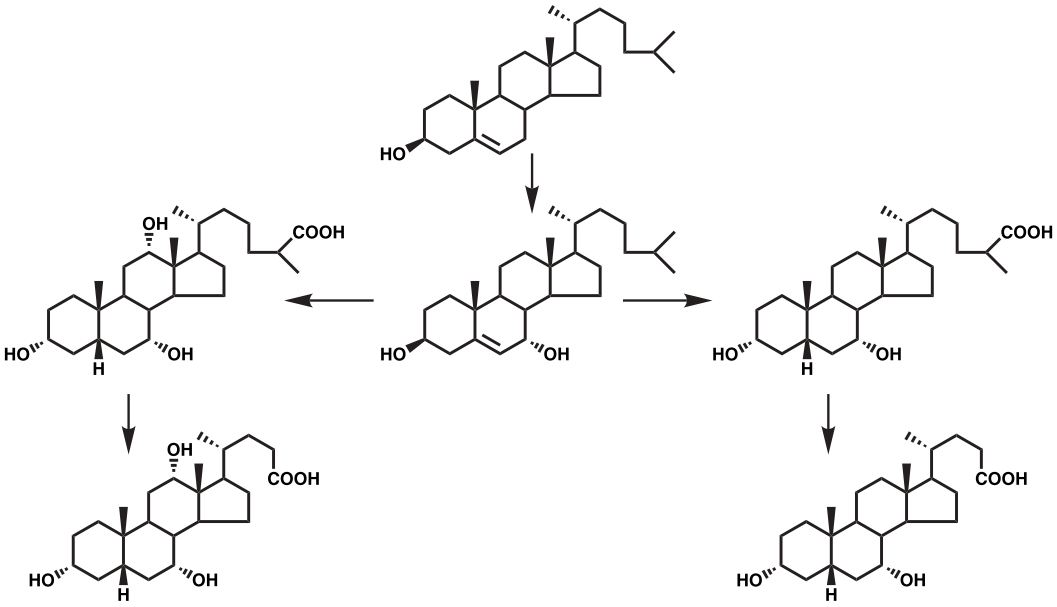


Figure 2. Biosynthesis of primary bile acids.

sulfates, glucuronides, and N−acetylglucosaminides. In addition, these changes result in the formation of uncommon bile acids containing a double bond, a carbonyl group, an A/B−*trans* structure, and hydroxyl groups at the C−4 and 6 positions.

For the diagnosis of liver diseases, reliable analytical methods for the bile acids in biological fluids are needed. For such purposes, chromatographic procedures combined with or without mass spectrometry (MS) are preferable. Although a reliable derivatization procedure to convert bile acids into their corresponding volatile forms is needed, gas chromatography (GC) with MS is an effective technique for the profile analysis of common and uncommon bile acids in biological fluids and tissues. Liquid chromatography (LC) combined with the use of a pre−column derivatization technique or immobilized enzyme can also be applied to the analysis of unconjugated, glycine−conjugated, and taurine−conjugated common bile acids in biological fluids. Recently, it has been suggested that LC/MS may be the most promising method for the separation and determination of bile acids, because this technique can be applied to a wide variety of bile acids including glucuronides, sulfates, and other conjugates.

In this article, our work on bio−analytical studies of bile acids using GC/MS, pre−column derivatization LC, and LC/MS will be briefly reviewed.

2. Gas chromatographic and mass spectrometric analysis

Capillary gas chromatographic separation combined with mass spectrometric detection is a powerful method for the profile analysis of bile acids in biological matrices. Commonly, after hydrolysis and/or solvolysis, the liberated bile acids are subjected to GC separation as methyl ester−trimethylsilyl (TMS) or dimeth-

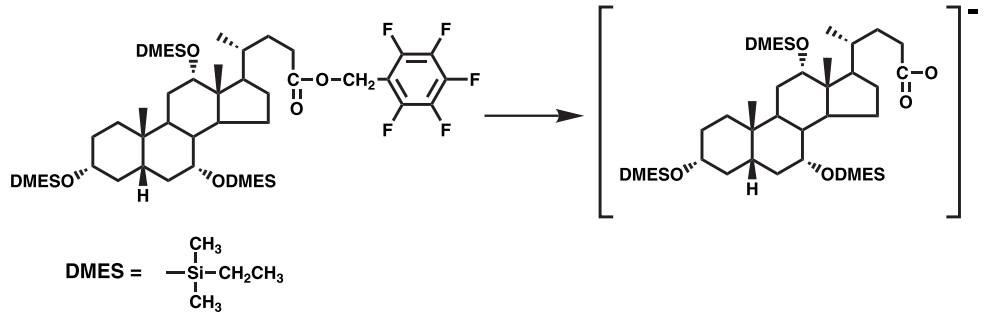


Figure 3. Structure of the dimethylethylsilyl ether−pentafluorobenzyl ester derivative of cholic acid.

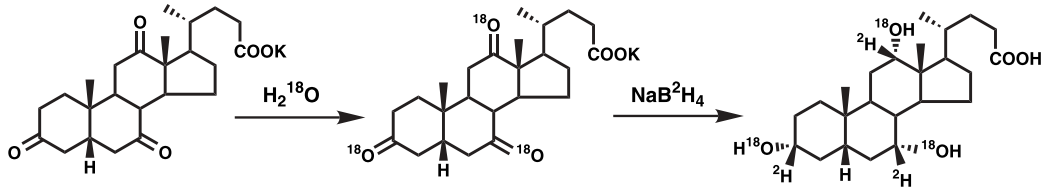


Figure 4. Synthesis of stable isotope labeled cholic acid.

ylethylsilyl (DMES) ether derivatives and are then introduced into a mass spectrometer using an electron impact ionization (EI) mode. Unfortunately, this method has a detection limit of a few pg, and is therefore insufficient to sensitively determine bile acid levels in the liver tissue. For this purpose, negative ion chemical ionization (NICI) detection provides much higher sensitivity. Derivatization of the carboxyl group into its pentafluorobenzyl (PFB) ester generates characteristic carboxylate anions, $[M-PFB]^-$, which facilitates the highly sensitive detection of approximately 2 fg (4 attomole) of bile acids at a signal−to−noise ratio of more than 10 using the NICI mode (Fig. 3) [1]. This method is 1000 times more sensitive than EI−MS, and permits the profile analysis of bile acids in the liver tissue [2] and the determination of C_{27} bile acids, which are specific diagnostic indicators of the cerebro−hepato−renal syndrome, Zellweger syndrome, in newborn infant urine [3]. The dimethylethylsilylation of the hydroxyl group on the steroid nucleus enables the excellent GC resolution of common bile acids on a methylsilicone fused silica capillary column.

The reversed isotope dilution technique in combination with mass spectrometric detection provides high accuracy, specificity, and sensitivity for the trace analysis of biologically important materials. Although ^2H labeled compounds are widely used for this purpose, introduction of more than three ^2H atoms into the target compound with high isotope purity is somewhat difficult. In NICI−MS, the base peaks of PFB esters of acidic compounds contain inherent oxygen atoms, which originate from the hydroxyl and carboxyl groups. Introduction of an ^{18}O atom into the hydroxyl group can easily be achieved by an exchange reaction with the carbonyl oxygen atom [2]. Briefly, the potassium salts of oxo bile acids derived by oxidation of the hydroxyl groups of the corresponding bile acids are dissolved in $H_2{}^{18}O$ and heated at 90°C for 50 hr, followed by further labeling with ^2H by sodium borodeuteride reduction (Fig. 4). Using this procedure, the target position can theoretically be labeled with a stable isotope with high selectivity; therefore, labeled compounds having high isotopic purity can easily be obtained. For example, the ratios of the unlabeled species to the fully labeled species in the stable isotope labeled mono−, di−, and tri−hydroxylated bile acids are about 1/500, 1/2000, and 1/10000, respectively, indicating high applicability to the trace analysis of bile acids in tissue, blood, and urine specimens. These stable labeled compounds can also be applied as tracers for biosynthetic and metabolic studies of bile acids. Indeed, the existence of a redox enzyme system for 3−oxo bile acids in human red blood cells [4−7] and an epimeric enzyme system for the C−25 position of C_{27} bile acid−CoA thioester in the hepatic mitochondrial fraction have been identified using such compounds [8−11].

Uncommon bile acids having a *vicinal*−glycol structure can be found in biological specimens from patients with liver diseases and newborn infants. These uncommon bile acids are very difficult to identify and determine, even using capillary GC/MS or GC/MS−MS. The unique sililating agent, N, O−bis (diethylhydrogensilyl) trifluoroacetamide, may be very useful for this purpose. This reagent reacts readily with an isolated hydroxyl group and a diaxial trans−glycol on the steroid nucleus to form the corresponding diethylhydrogensilyl (DEHS) ether. In addition, a diequatorial *trans*−glycol or an axial−equatorial *cis*−glycol can easily be transformed into the corresponding cyclic diethylsilylene (DES) derivative (Fig. 5) [12]. Accordingly, the profile analysis of common and uncom-

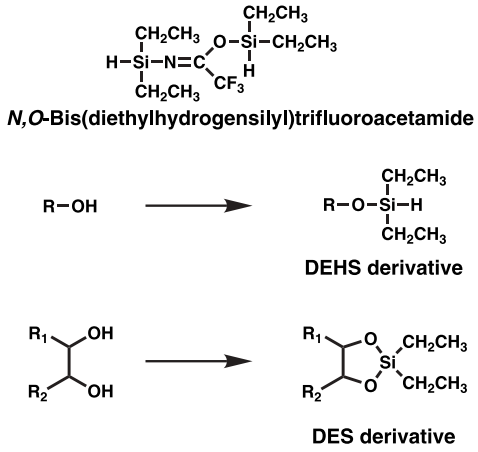


Figure 5. Derivatization of hydroxyl groups with N, O−bis (diethylhydrogensilyl) trifluoroacetamide.

mon bile acids with efficient resolution can be achieved on capillary GC/MS. Moreover, the differences in methylene unit values between TMS and DEHS or DES derivatives imply the number and nature of the hydroxyl groups on the steroid nucleus of bile acids. This derivatization method with capillary GC/MS has been applied to the identification and determination of a novel 4 β−hydroxylated bile acid in urine specimens from newborn infants [13−15].

3. Liquid chromatographic and mass spectrometric analysis

High−performance liquid chromatography (HPLC) is a powerful tool for the separation and determination of polar and thermally unstable biological compounds. Therefore, this technique appears to be useful for the analysis of bile acids without prior hydrolysis and/or solvolysis. A chemically bonded stationary phase, an ODS column, has been widely used for the separation of bile acids and related compounds, and several factors affecting the chromatographic behavior of such compounds on the column have been clarified [16, 17]. The *k* value of an organic acid on an ODS column is dependent on the acidity of the eluent and can be estimated from the p*Ka* value of the organic acid and the pH of the eluent. The p*Ka* values of bile acids are as follows: 6.0 for unconjugated acids, 4.5 for the glycine conjugates, and 1.5 for the taurine conjugates. Therefore, in a mobile phase of pH 7.0−8.0, the unconjugated, glycine−conjugated, and taurine−conjugated bile acids exhibit similar *k* values. Furthermore, the *k* values of the unconjugated and glycine−conjugated bile acids increase with a decrease in pH from 6.5 and 5.0, respectively. The *k* value of the taurine conjugate exhibits little change over the whole pH range.

The effect of hydroxyl groups on the retention value is dependent on the position and increases in the sequence 3−OH = 6−OH < 7−OH < 12−OH. There is no significant difference in mobility between the 5 β− and 5 α−epimers. Bile acids containing equatorial hydroxyl groups exhibit smaller *k* values than corresponding epimers having axial hydroxyl groups. Oxo bile acids show almost the same *k* values as the corresponding bile acids which have equatorial hydroxyl groups. In bile acids, the β−face of the steroid nucleus participates in hydrophobic binding to the ODS stationary phase. Therefore, introduction of a hydroxyl group on the α−face would alter only the polarity of the molecule and, hence, its solubility in the aqueous mobile phase. On the other hand, the presence of a β−hydroxyl or an oxo group on the steroid nucleus would reduce the hydrophobic surface area and increase the solubility i the mobile phase, resulting in a decrease in the retention value. Because of the intramolecular steric interaction between the 12 α−hydroxyl group and the 24−carboxyl group in the dissociated form, the resolution of two isomers, 3 α, 7 α−dihydroxylated CDCA and 3 α, 12 α−dihydroxylated DCA is influenced by the pH of the mobile phase. Unconjugated DCA has a larger *k* value than the corresponding CDCA in the higher pH region, whereas both exhibit almost identical values in the lower pH region. On the other hand, glycine− and taurine−conjugated DCAs exhibit larger *k* values than the corresponding CDCAs at pH between 4.0−7.5. The 3−sulfates and 3−glucuronides of bile acids also show the same chromatographic behavior.

LC/MS is a reliable analytical tool for the separation and determination of trace compounds in biological fluids. Among numerous interfaces, electrospray ionization (ESI) is more suitable for the detection of ionic trace compounds. In the ESI mode, it is recommended that volatile electrolytes, such as ammonium acetate, be included in the mobile phase to promote the proton transfer reaction. It is generally accepted that the ESI dispersion of a sample solution from LC would result in the production of droplets containing solute molecules. As the solvent evaporated from the droplet, the solvent charge would be retained by the solute (Fig. 6). Therefore, the ionization efficiency of organic acid derivatives is markedly influenced by dissociation of electrolytes in a buffer solution. The use of buffer solution containing an organic acid having a p*Ka* value relatively smaller than that of the acidic moiety in the target compound results in the depression of charge transfer from droplets to the acidic moiety to produce the corresponding ion [18]. Based on these observations, simultaneous determinations of bile acid 3−sulfates and 3−glucuronides [19] in biological fluids by LC/ESI−MS have been developed.

It has also been demonstrated that ester−type bile acid glucuronides, in which the glucuronic acid group is conjugated through the 24−carboxyl moiety, are biosynthesized as main glucuronides when bile acids are incubated with rat hepatic microsomal glucuronosyltransferases (Fig. 7) [20]. These acyl glucuronides have also been identified and determined in human urine by the newly devel-

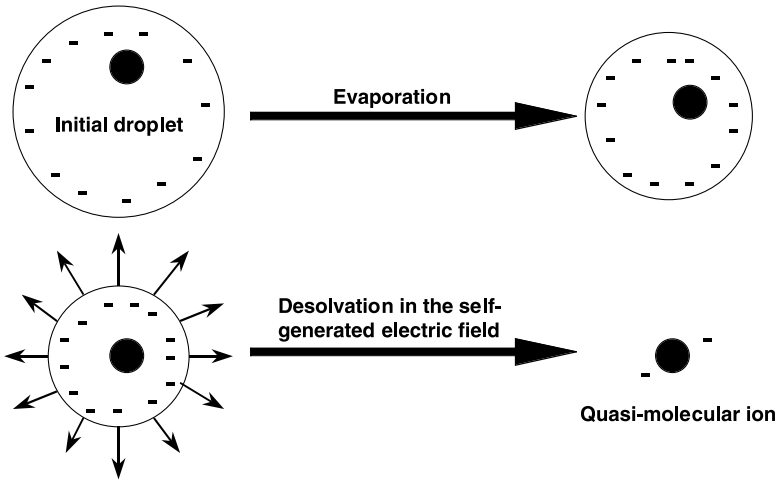
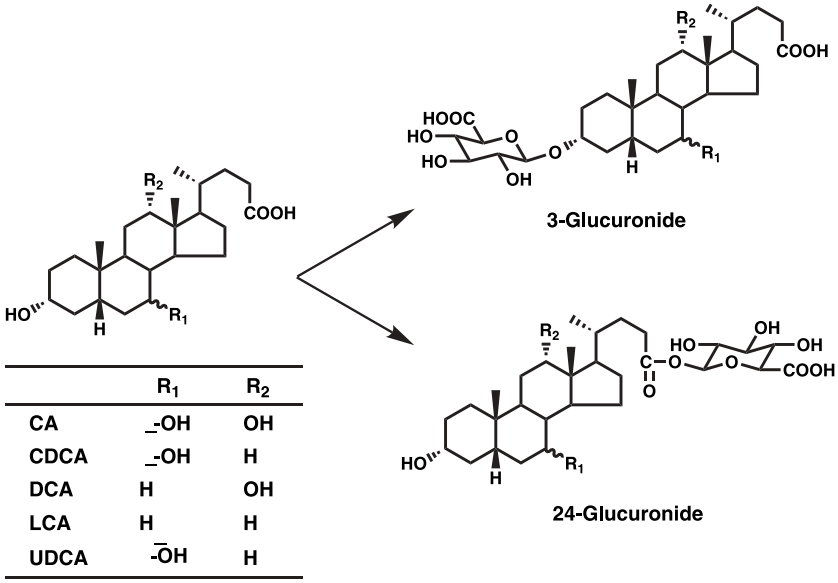


Figure 6. Ionization of the ionic compound under the ESI mode.



	R_1	R_2
CA	α-OH	OH
CDCA	α-OH	H
DCA	H	OH
LCA	H	H
UDCA	β-OH	H

Figure 7. Biosynthesis of bile acid ether and ester glucuronides.

oped analytical method employing LC/ESI−MS [21, 22]. The bile acid acyl glucuronides are readily non−enzymatically reactive with proteins through the amino group and produce protein bound bile acids, which may cause cytotoxicity and carcinogenesis [23].

It is well known that organic compounds having carboxylic acid groups are transformed into acyl CoA thioesters prior to conjugation with amino acids. It has also been demonstrated that, during the activation of the carboxylic acid group of bile acid to the corresponding acyl CoA thioester, the acyl adenylate is formed in microsomal fractions and that acyl CoA synthetase may be responsible for this process (Fig. 8) [24−26]. The acyl adenylate is very reactive with an amino group and was transformed into the amino acid conjugate with taurine under a non−enzymatic condition. The reactivity of the acyl adenylate toward the amino group can be easily controlled by pH and, hence, this functional group can be utilized as a novel reacting group for affinity labeling reagents [27].

As mentioned above, bile acids are synthesized in the liver from cholesterol by the action of hepatic enzymes. Recently, the existence of unconjugated CA, DCA, and CDCA in the rat brain was reported employing a unique pre−treatment procedure for the brain cytosolic fraction [28]. The concentration of CDCA stands out among the three bile acids and is almost 10 times higher than the concentration of pregnenolone, a very abundant neurosteroid. Moreover, CDCA is only detected when high concentrations of guanidine are added to the extract from the cytosolic fraction (Fig. 9), suggesting that the CDCA is tightly bound to protein in the brain. It is not at all clear whether these bile acids are transferred from the blood through the blood−brain barrier or biosynthesized

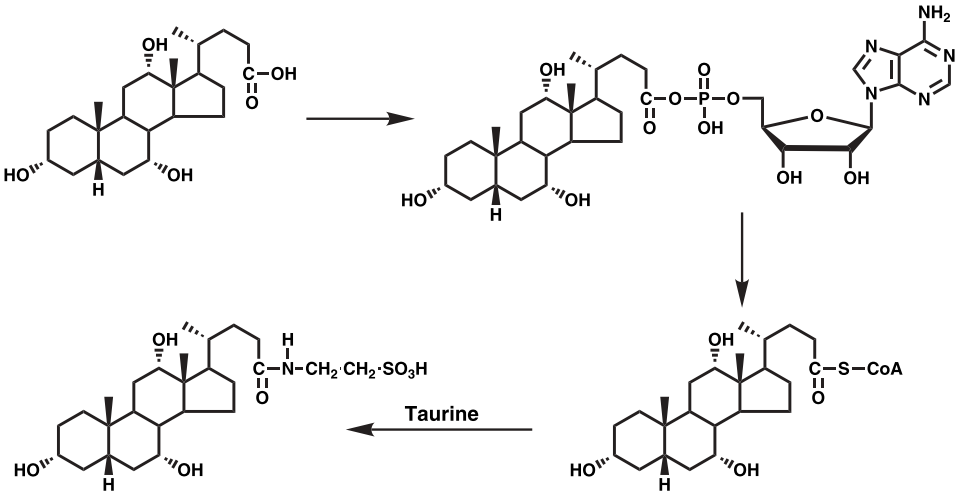


Figure 8. Biosynthesis of taurine conjugated cholic acid.

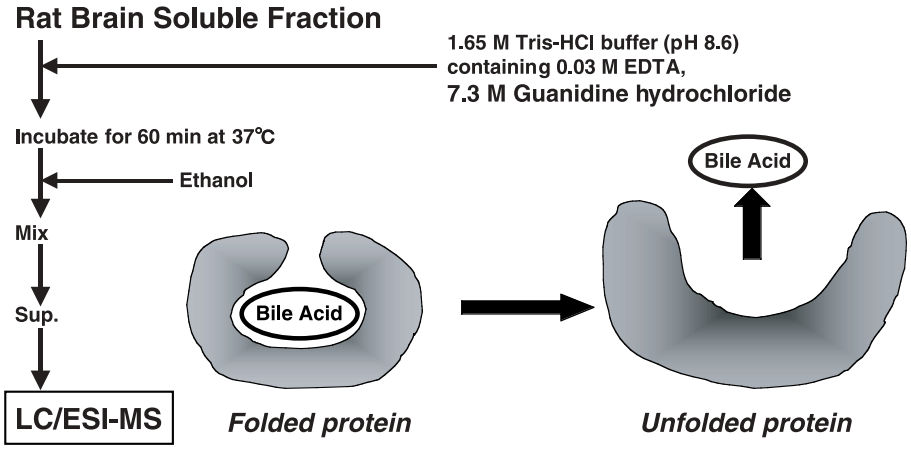


Figure 9. Procedure for the determination of bile acids in the rat brain soluble fraction.

from cholesterol or related compounds in the brain.

4. Immunoaffinity extraction employing highly specific antibodies

Although chromatographic separation combined with mass spectrometric detection is a powerful tool for the determination of trace compounds in biological matrices, tedious clean−up procedures are sometimes required just to remove interfering co−existing substances. However, LC alone is not necessarily effective for the simultaneous separation of racemates and their metabolites containing plural asymmetric centers. These problems may be solved by the use of immunoaffinity extraction, based on specific interactions between a matrix−bound antibody and soluble biochemicals [29−34]. In this technique, the simultaneous extraction of biologically important substances and their related compounds is attempted employing an immunosorbent prepared by immobilization of an antibody having a broad affinity spectrum for both a parent biochemical and its related compounds. An antibody raised against a hapten molecule usually shows significant cross−reactivity with compounds homologous around the bridge portion used for conjugation with a carrier protein, such as bovine serum albumin. Accordingly, an antibody should be prepared by immunization with the corresponding antigen, where the hapten is conjugated to a carrier protein through a position far from the discriminating structure of the target compounds. For example, bufuralol, a potent non−selective β−adrenoseptor antagonist with β_2 partial agonist properties, has an asymmetric center at the C−1 position and mainly undergoes aliphatic oxidation at C−1', generating a new chiral center and yielding 1'−hydroxylated bufuralols (carbinols), which are further transformed into 1'−ketone derivatives (Fig. 10). To discriminate the C−1 position, (1 *R*)− and (1 *S*)−1'−oxobufurarols are transformed into O−carboxymethyloxime derivatives, condensed with BSA, and subcutaneously administered to rabbits with complete Freund's adjuvant. The resulting anti−(1 *R*)−bufurarol antibody

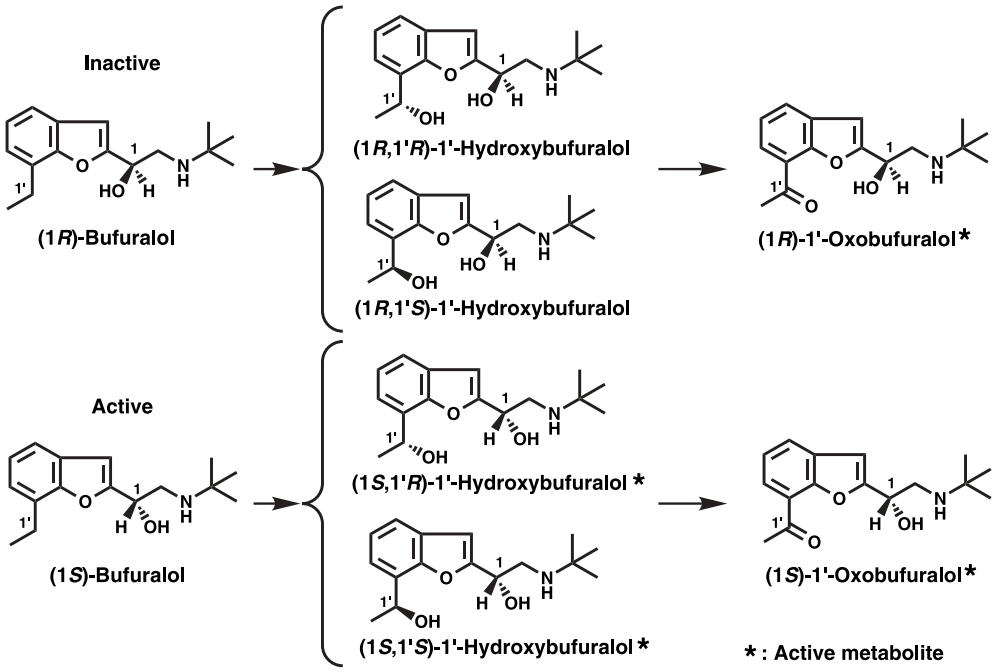


Figure 10. Metabolism of bufuralol.

shows high affinity to (1 *R*)−bufuralol and its 1'−oxygenated metabolites. However, the reactivity toward the corresponding (1 *S*)−antipodes is significantly lower. A similar phenomenon is observed for the anti−(1 *S*)−bufuralol antibody. When these antibodies are covalently coupled to agarose, the corresponding antipodes can be selectively trapped onto the immobilized antibody [35, 36].

5. Conclusions

We present highly selective and sensitive chromatographic methods coupled with mass spectrometry for the analysis of biologically important materials. Derivatization into PFB esters enables the determination of a 10^{-18} mole level of carboxyl compounds in biological fluids with capillary GC/NICI−MS. This method combined with the use of stable isotope labeled compounds is effective for metabolic and biosynthetic studies of bile acids. Liquid chromatography with electrospray ionization−mass spectrometry is very effective for the determination of water−soluble biological compounds, such as sulfates and glucuronides, and permits the analyses of very unstable metabolites such as ester glucuronides and acyl adenylates. Immunoaffinity extraction with immobilized antibody having group specific affinity prior to chromatographic separation is also useful for the simultaneous selective extraction of target compounds and their metabolites from biological fluids.

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