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Electrochemistry of Bile Acids, Cholesterol, and Related Compounds (An Overview)

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Abstract: This review summarizes electrochemical methods used for the investigation, determination, and monitoring of bile acids and their conjugates, cholesterol, phytosterols, and related compounds. In electrochemistry-related research they do not belong among frequently studied compounds, because they are inactive under variety of conditions and electrode materials. The several examples of their redox activity include electrochemical reduction of bile acids at mercury electrodes and modified glassy carbon electrodes and electrochemical oxidation of cholesterol, phytosterols, and related compounds at glassy carbon and boron-doped diamond electrodes in nonaqueous media. A brief survey of these methods and their utilization for monitoring of tested compounds in biological and pharmaceutical matrices is given.

Keywords: Bile acids; Cholesterol; Phytosterols; Dropping mercury electrode; Glassy carbon electrode; Voltammetric determination; HPLC-ED; Supramolecular assemblies.

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Introduction

The bile acids are hydroxylated derivatives of the steroid-5β-cholan-24-oic acids. In the form of their conjugates they are amphipathic end products of cholesterol metabolism with multiple physiological functions, mainly in digestion and absorption of fats. Cholesterol is a C_{27} sterol with the double bond at C_5 and an isooctane C_8 side chain.

Bile acids possess the C_3 hydroxy group of cholesterol and a C_7 hydroxy- or oxo- group, because of the rate limiting step in their biosynthesis by cholesterol-to-cholesterol 7α-hydroxylase transformation [1]. Chemical structures of cholesterol and common bile acids are shown in Fig. 1 (see overleaf).

There are two default compounds in bile acids chemistry: The first is the C_{27} bile acid with a carboxyl group at C_{27} and 3α and 7α-hydroxy substituents on the steroid nucleus (which might be termed cholestanoic acid). The second is the C_{24} bile acid with a carboxyl group at C_{24} and again with 3α and 7 -hydroxy substituents (termed chenodeoxycholic acid, CDCA). During bile acid biosynthesis from cholesterol, the default compounds may undergo additional hydroxylations on the steroid nucleus, the side chain, or both to give the spectrum of naturally occurring bile acids. Two additional modifications occur in C_{24} bile acids. The first is epimerization of the 7α-hydroxy group, forming a 3α,7β-dihydroxy bile acid termed ursodeoxycholic acid (UDCA). UDCA is a major bile acid in nutrias, bears, and beavers. The second is oxidation of the C_7 hydroxy group to give a 3α-hydroxy, 7-oxo-bile acid [1].

In most C_{24} and C_{27} bile acids, the cyclopentanophenanthrene (steroid) nucleus, composed of rings A, B, C, and D, is saturated. During bile acid biosynthesis, there is isomerization of the $\Delta^{5,6}$ double bond of cholesterol to a $\Delta^{4,5}$ double bond which is then reduced to give 5β bile acids in which the A and B rings are in *cis* configuration (denoted by a 5β hydrogen atom).

Bile acids that are synthesized from cholesterol in the hepatocyte are termed primary bile acids. The most common primary C_{24} bile acid, in addition to CDCA, is cholic acid (3α,7α,12α-trihydroxy). When primary bile acids are modified by bacterial enzymes by removal, oxidation, or epimerization of the nuclear hydroxyl groups, the resulting bile acid is termed a secondary bile acid. A common way of their formation is the removal of the hydroxy group at C_7 by anaerobic bacteria. That leads to the formation of a new class of bile acids termed 7-deoxy bile acids. The most common C_{24} 7-deoxy bile acids are lithocholic acid (3α-hydroxy), formed by bacterial 7-dehydroxylation of CDCA, and deoxycholic acid (3α,12α-dihydroxy), formed by bacterial 7-dehydroxylation of cholic acid. C_{27} bile acids also undergo bacterial modification of their hydroxy groups.

After their biosynthesis from cholesterol, bile acids are conjugated. C_{27} bile acids are conjugated exclusively with taurine. In such conjugation, the carboxyl group of the bile acid is linked to the amino group of taurine in amide linkage. For C_{24} bile acids five types of conjugation could be recognized.

The first is N-acyl amidation with glycine or taurine; the second is sulfation; the third is ester glucuronidation at C_{24}; the fourth is ethereal conjugation at C_3 (and possibly at other nuclear sites); and the fifth is N-acetylglucosamination at C_7 in bile acids such as UDCA with a β-hydroxy group. Bile acids, as noted, may undergo conjugation on both the steroid nucleus and the side chain [1].

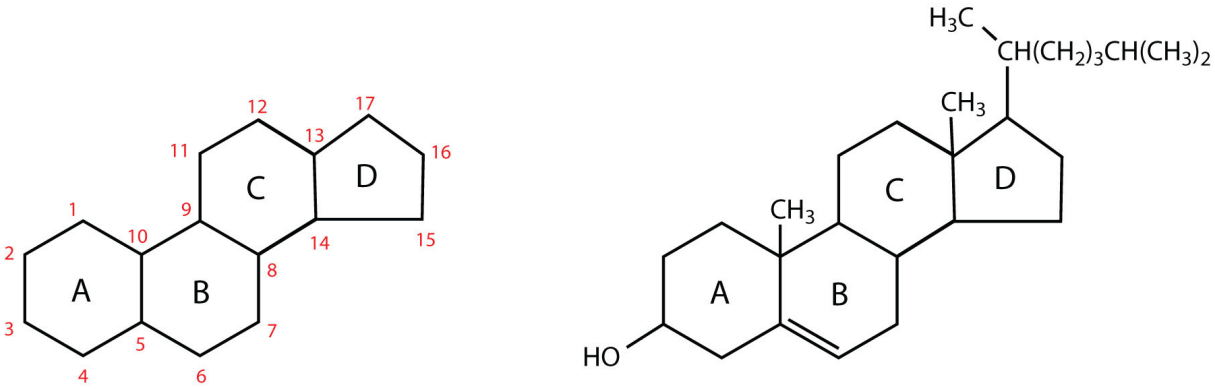
Bile acid anions are surface-active molecules due to their amphipathic character – they have a hydrophobic side (the β face of the bile acid molecule) containing no substituents and a hydrophilic side (the α face) containing the hydroxy groups. Bile acid anions self-associate over a fairly narrow concentration range to form micelles. It is convenient to name the approximate midpoint of this range the critical micellization concentration (CMC), based on a vast literature on the self-association of surfactants. For C_{24} bile acids the CMCs were intensively studied [2-4]. Typically, they increase with increasing number of hydroxy groups in the steroid molecule. Conjugation with glycine or taurine results in a slight lowering of the CMC [2]. CMC values of C_{24} acids have been tabulated, they are in the range from 9 mmol L^{-1} (chenodeoxycholic acid) to 60 mmol L^{-1} (ursocholic acid) in water at 25 °C. In the presence of 0.15 mol L^{-1} Na^+ ions (simulation of physiological conditions) slightly lower values were estimated (0.6 mmol L^{-1} to 39 mmol L^{-1}) [4]. No data exist on CMC values of C_{27} bile acids, nevertheless, their CMC values should be lower than those of corresponding C_{24} homologues because of their longer side chain.

The arched skeleton of bile acids with about ten chiral carbons and multiple hydrogen-bonding groups makes these compounds ideal for intermediate assemblies which relate to supramolecular properties, such as host guest, inclusion, reaction, chirality, recognition, dynamics, information, and so on [5].

The other important class of compounds derived from cholesterol includes the plant sterols − phytosterols. Their chemical structure differs from that of cholesterol by the presence of modified side chains at carbon C_{24} and they are metabolized differently, so that mammals are not capable of their synthesizing. Phytosterols and their esters are used clinically to reduce low density lipoprotein cholesterol and atherosclerotic risk [6].

The complex composition of naturally occurring steroid compounds and the small structural differences between individual components demand a “fit for purpose” analytical technique. The common techniques for the assay of these compounds in biological and physiological matrices include mainly chromatographic procedures, but also electrophoretic, enzymatic, and immunological methods as summarized in several reviews [7-9].

In this overview, we have concentrated on the achievements of electrochemistry-related research of selected steroid compounds to nowadays.



(**A**) (**B**)

where:

Substance	A ring	B ring	C ring	D ring 17C(CH_3) CH_2 CH_2COR
Cholic acid	3αOH	7αOH	12αOH	R=OH
Chenodeoxycholic acid	3αOH	7αOH		R=OH
Deoxycholic acid	3αOH		12αOH	R=OH
Ursodeoxycholic	3αOH	7βOH		R=OH
Ursocholic acid	3αOH	7βOH	12αOH	R=OH
Lithocholic acid	3αOH			R=OH
Glycocholate	3αOH	7αOH	12αOH	R= $NHCH_2COO^-$
Taurocholate	3αOH	7αOH	12αOH	R=$NHCH_2$ $CH_2SO_3^-$

Fig. 1: *Chemical structure of steroid skeleton* (**A**) *and selected bile acids and their conjugates (surveyed in the table) and cholesterol* (**B**).

Electrochemistry of Bile Acids, Cholesterol, and Related Compounds

Electrochemical Reduction of Bile Acids

Electrochemical reduction of bile acids is enabled by the presence of the carboxyl group at C_{24} or C_{27}. It is known that aliphatic and aromatic carboxyl acids which are not strongly activated by electron-withdrawing groups and do not contain more easily reducible groups give a well-developed polarographic wave in aqueous [10] or nonaqueous [11, 12] solvents, but this is usually due to reduction of protons to hydrogen.

The number of studies dealing with reductive processes of bile acids is very limited, the only bare electrode material succeeding has been the classical dropping mercury electrode (DME). It was used for differential pulse polarographic determination of cholic, deoxycholic, ursodeoxycholic, chenodeoxycholic and lithocholic acid in human bile or pharmaceutical products [13]. The activity of free bile acids is characterized by the presence of a well-developed peak independent on the pH at the potential of −1150 mV to –1350 mV, ursodeoxycholic acid is reduced at about 100 mV more positive potential. Weakly acidic to basic media are suitable for the determination because of the possible interference of the signal of cholic acid and the onset of the polarographic curve due to the hydrogen evolution. Conjugates with taurine and glycine behave very similar. Due to the proximity of reduction potentials the method is suitable for determination of total bile acids in human bile down to the micromolar concentration.

Further, DME and the classical methods direct current polarography and alternating current polarography were employed to evidence the adsorption-desorption nature of the electrode processes [14, 15]. The surface-active bile salts adsorb at the DME and supress the polarographic maxima, caused by convective transport of the electroactive species resulting from a difference in charge density in different parts of DME. Polarographic maxima of oxygen (at −0.2 V) and zinc (at −1.2 V) were suppressed by selected C_{24} bile salts and used for the investigation of adsorptibility at micromolar concentrations, *i.e.* two orders of magnitude lower than CMC. Adsorption increases in the sequence monohydroxy < dihydroxy < trihydroxy sterols [15]. Electrochemical reduction of deoxycholic acid [16] was further investigated at bare glassy carbon electrode (GCE), nevertheless no reductive signal was observed in 0.05 M KH_2PO_4 aqueous methanol solution (1:1, v/v). However, at the GCE modified by multiwalled carbon nanotubes (MWCNT), there was an irreversible cathodic wave near the potential of +0.65 V. Its presence was attributed to the catalytic effect of MWCNT exhibiting large specific area, particular electronic structure and high electric conductivity, especially owes to some oxygen containing groups [17, 18]. The position of the peak is independent of pH, increases with increasing pH and also increases with the high limit potential in cyclic voltammetry, as obvious from Fig. 2. The wave was attributed to the overall four-electron reduction of the carboxyl group to the corresponding hydroxymethyl compound. Such reduction is otherwise hardly achievable and requires activation of the carboxyl group by a strongly electron withdrawing group, which is less easily reducible than the carboxyl group (this excludes the nitro group and a number of nitrogen-containing heteroaromatic groups as activating groups).

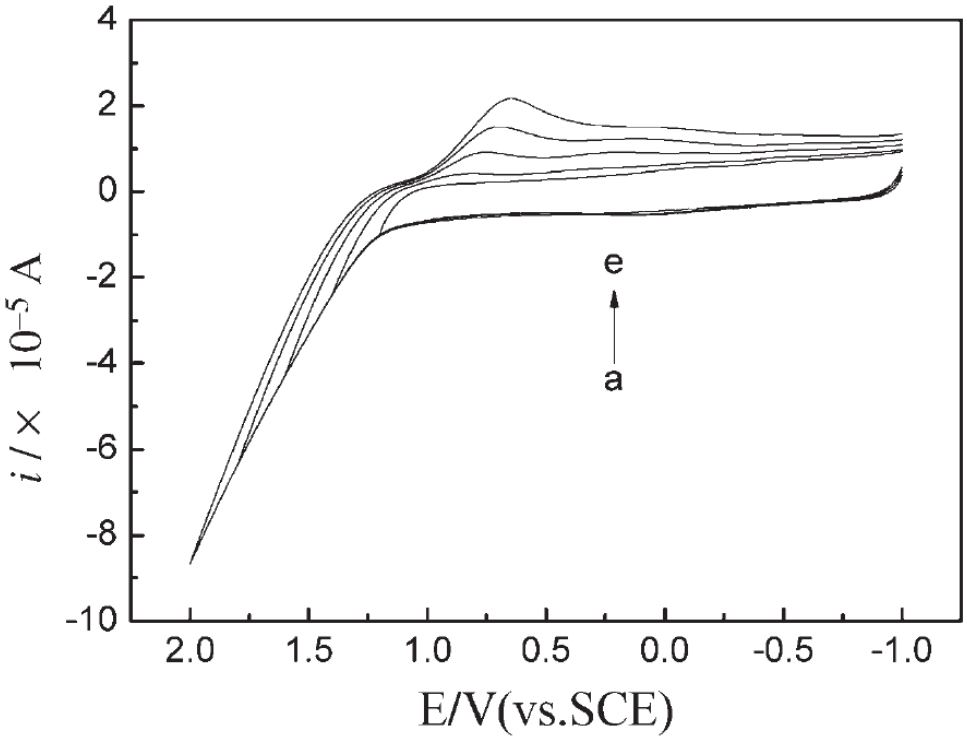


Fig. 2: C*yclic voltammograms of deoxycholic acid (DCA) at the MWCNT modified GCE.* Scan rate 100 mV s^{-1} in 0.05 mol L^{-1} KH_2PO_4 aqueous methanol solution (1:1, v/v) containing $1.8 \cdot 10^{-4}$ mol L^{-1} DCA with different high limit potential (V): a) 1.2, b) 1.4, c) 1.6, d) 1.8 and, e) 2.0 V. Reprinted and reproduced with permission from [16].

Electrochemical Oxidation of Cholesterol, Phytosterols and Related Compounds

To nowadays, few studies were performed on the electrochemical oxidation of bile acids, attention was paid more frequently to cholesterol and related compounds [18-23] and phytosterols [24]. In general, direct electrochemical oxidation of these compounds is hardly achievable and succeeds usually only in non-aqueous media [18-24] or media with high content of organic phase [25]. The other approach relies on indirect oxidation by a mediator ion [26, 27], which is a common way how to oxidize aliphatic hydroxyl groups with high oxidation potential [review [28] and references therein]. The indirect anodic electrochemical oxidation of cholic acids leads to the synthesis of the various oxo-cholic acids. The rate of the OH-oxidation is C_7>C_{12}>C_3. Medici *et al.* used chloride ions as mediators and Ti-supported lead dioxide, platinum foil and graphite electrode to oxidize the OH-functions of cholic acid and evaluated the regioselectivity of the oxidation for particular electrode materials and experimental conditions [26, 27].

Kusu *et al.* published several studies on oxidation of cholesterol, and selected oxysterols, phytosterols and related compounds in non-aqueous media [18-24]. Cholesterol can be oxidized at far positive potential at GCE in acetonitrile-2-propanol (9:1, v/v) containing 50mmol L^{-1} $LiClO_4$ as supporting electrolyte [19] (see Fig. 3). The oxidation succeeded also at gold electrode, which is applicable for the electrooxidation of hydroxyl group of sugars. However, probably due to the adsorption of reaction products of cholesterol to the oxide layer of the gold electrode surface, the gold electrode did not work well in terms of repeatability [19]. Later it was proved that the oxidation of cholesterol in acetonitrile at carbon electrode proceeds *via* a four-electron, four-proton transfer to cholesta-4,6-dien-3-one [29]. That indicates that the hydroxyl group at the 3β-position is the target of electrochemical oxidation. This was confirmed by the successful electrooxidation of cholestanol having only the 3β hydroxy group as the only one in its structure [20].

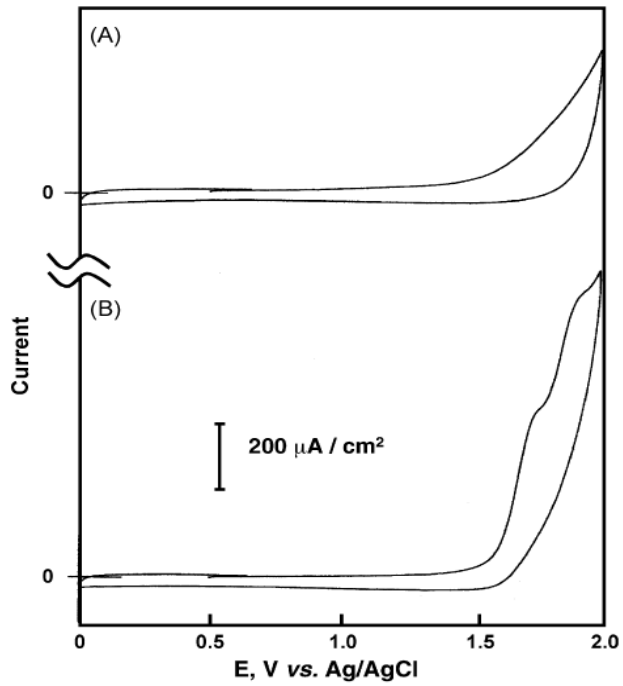


Fig. 3: *Cyclic voltammograms of cholesterol in non-aqueous media.* Cyclic voltammograms in acetonitrile-2-propanol (9:1, v/v) containing 50mmol L^{-1} $LiClO_4$ in the absence (A) or presence of 0.45 mmol L^{-1} of cholesterol (B): scan rate, 0.1 V s^{-1}; working electrode, glassy carbon; counter electrode, platinum wire; reference electrode, Ag/AgCl. Reprinted and reproduced with permission from [19].

These pioneering findings enabled direct detection of cholesterol and related compounds in HPLC and semi-micro HPLC with electrochemical detection. Fig. 4 presents the detection of cholesterol and selected oxy sterols at an applied potential of +1.9 V *vs.* Ag/AgCl after their separation in HPLC on reversed (C_{30}) phase. The detection limit for cholesterol was 0.36 μmol L^{-1} and the HPLC-ED method was applied to determine total cholesterol and free cholesterol in control human serum with the recovery of more than 90% and the relative standard deviation (RSD, n = 6) of less than 3.0% [19].

The phytosterol stigmasterol possessing the same oxidizable 3β-hydroxyl group as cholesterol was used as the internal standard in this study for its quantification. Thus, other studies were undertaken aimed at the simultaneous determination of phytosterols, concretely β-sitosterol, campesterol, stigmasterol, and brassicasterol in HPLC-ED adopting the experimental conditions for cholesterol detection [24]. The detection limit of phytosterols was less than 3.4 μmol L^{-1}. Phytosterols spiked into the control human and rat sera were determined by the present method with recovery rates of more than 80% and RSDs ($n = 3$) of less than 4.2 %. This method has been successfully applied to the monitoring of experimental phytosterolemia (an autosomal recessive disease causing *e.g.* primary coronare atherosclerosis [6] in rats induced by a high-phytosterol diet. Similar approach concerning the electrochemical detection succeeded in determination of seven oxysterols in oxidatively modified low-density lipoprotein by semi-micro high-performance liquid chromatography [23]. The other electrode employed by Kusu *et al*. was the boron doped diamond (BDD) electrode, that was employed in electrochemical flow cell for HPLC-ED [22]. For its performance assessment to determine cholesterol by the standard deviation of area measurements in chromatographic baseline noise was evaluated based on the function of mutual information theory without repetitive chromatographic measurements. After the selection of suitable components for the electrochemical flow cell in HPLC-ED, the detection limit of cholesterol was 9 nmol L^{-1} at detection potential of +2.2 V *vs.* an Ag/AgCl electrode, which is lower detection limit than for GCE and commonly employed HPLC-UV, LC/MS and LC/MS/MS methods [8, 28]. The HPLC-ED method was applied to determine cholesterol in various meats [22].

The presented methods enabling direct electrochemical detection of cholesterol, phytosterols and related compounds without preceding derivatization step in HPLC-ED clearly demonstrated the usefulness of the alternative electrochemical approach to their detection in various biological matrices and provide basis for development of a number of potential applications in detection of wide variety of steroid compounds.

Conclusions

Obviously, the possibilities of electrochemistry for characterization and detection of cholesterols, phytosterols, bile acids, and related compounds are rather limited. For analytical chemists, these compounds represent a challenge, because they may occur in very complex mixtures, and the most common and relatively cheap separation and detection techniques have

substantial drawbacks, *e.g.* weak chromophores with absorption around 200 nm (for HPLC-UV) and the necessity of derivatization, or hydrolysis (for gas chromatography). The most progressive method seems to be LC-MS/MS, nevertheless, there is still a need for reduces cost and improvement in analytical ruggedness and reproducibility [7,8]. Thus, all contributions to the problem of detection of steroid compounds in pharmaceutical, biological and physiological matrices are welcome. Hopefully, further research on new electrode materials and the electrochemistry of steroid compounds will lead to sensitive sensors capable of detecting these compounds in specified samples or more complex matrices after preliminary separation.

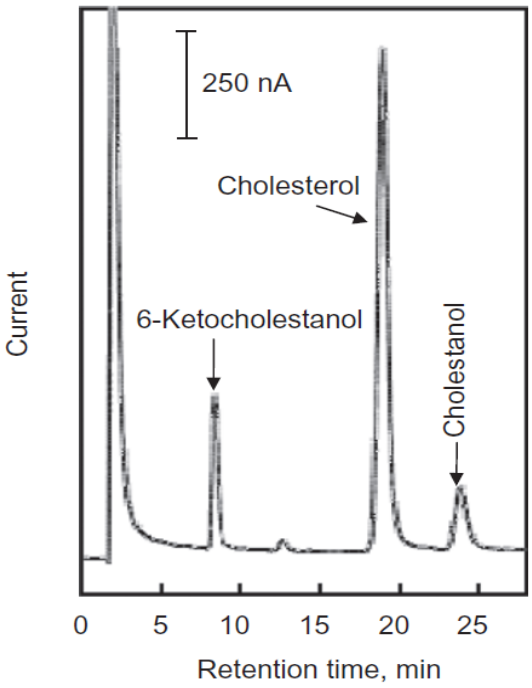


Fig. 4: *Chromatogram of standard cholestanol and cholesterol.* Cholestanol (50 μmol L^{-1}), cholesterol (150 μmol L^{-1}) and 6-ketocholestanol (50 μmol L^{-1}) were dissolved in the mobile phase and injected into HPLC-ED. The HPLC conditions: mobile phase, acetonitrile containing 10 mmol L^{-1} $LiClO_4$; flow rate, 100 μl min^{-1}; column, Develosil C30-UG-3 microbore column (250 mm × 1.0 mm, i.d. 3 μm); column temperature, 50 °C; applied potential +2.8 V *vs* Ag/AgCl; injection volume 5 μL. (Reprinted and reproduced with permission, [20].)

Acknowledgements

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