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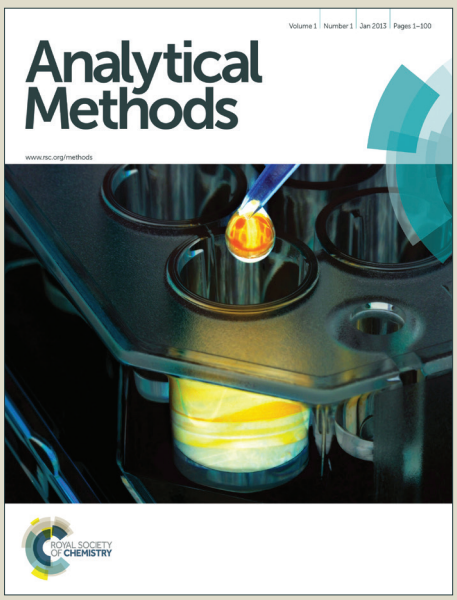

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Development of a Disposable Bile Acid Biosensor for use in the Management of Cholestasis

D Lawrance[a], C Williamson[a], M G Boutelle[b], A E G Cass[c]

[a] Maternal and Fetal Disease Group, Institute of Reproductive and Developmental Biology, Imperial College London, Du Cane Road, London, W12 0NN, UK

[b] Department of Bioengineering, Imperial College London, South Kensington, SW7 2AZ, UK

[c] Department of Chemistry, Imperial College London, South Kensington, SW7 2AZ, UK

Corresponding Author:

Tony Cass

6th Floor, Flowers Building

Imperial College London

South Kensington

London SW7 2AZ

Tel: +44 (0) 207 594 5195

E-mail: t.cass@imperial.ac.uk

Abstract

Measurement of serum bile acid concentration is a valuable tool for the management of intrahepatic cholestasis of pregnancy (ICP) and is also useful in the monitoring of other liver diseases. This study describes the development of a disposable bile acid biosensor, based on commercial screen-printed electrodes, towards a point-of-care measurement system for serum bile acids. Here a common serum bile acid, taurocholic acid, was used to calibrate the biosensor in 80% (v/v) human serum, at clinically relevant levels, using chronoamperometry. A good correlation between the concentration of the bile acid and the measured currents is reported. The limit of detection was below the threshold for management of ICP (40μM). The linear range of the biosensor exceeded 150 uM for taurocholic acid with a relative standard deviation between measurements of below 5%. We propose this as a simple and rapid method for quantifying bile acids in human serum, which could be used in the clinical management of ICP.

Keywords

Cholestasis; NADH; Meldola's Blue; Dehydrogenase; Amperometric; Bile acid

Introduction

Bile acids are 24-carbon steroids, synthesised in the liver as the end products of cholesterol catabolism. They are primarily responsible for emulsification of fats, thereby aiding in the absorption of lipid-soluble nutrients in the gut [1]. Bile acids are recycled by the body in a continuous process, known as enterohepatic circulation, whereby 95% of bile acids excreted from the gall bladder are reabsorbed in the small intestine. Efficient recycling maintains an appropriate level of bile acids ready to be released after meals and also protects other tissues from exposure to a high level of bile acids [2].

Measurement of circulating bile acids is important in the diagnosis and monitoring of liver diseases such as primary biliary cirrhosis and chronic hepatitis [3]. Moreover measurement of bile acid levels in a rapid and simple manner would be highly expedient for the bedside management of a pregnancy-specific liver disorder, namely intrahepatic cholestasis of pregnancy (ICP). Generally diagnosis of ICP is made by exclusion of other causes of biliary obstruction. There are two key maternal features: (1) Pruritus, i.e. itch without an obvious dermatological cause, often of the hands and feet and (2) abnormal liver function typically characterised by increased total serum bile acids (TSBA). ICP is usually a transient condition in pregnant women that resolves after delivery. However it is associated with adverse pregnancy outcomes, including spontaneous and iatrogenic preterm labour, fetal hypoxia, meconium staining of the amniotic fluid (a sign of fetal distress) and intrauterine fetal death [4].

Whilst pruritus is hard to quantify accurately, TSBA are quantifiable and have been shown to correlate with the frequency of adverse pregnancy outcomes, with a demonstrated increase in the frequency of complications in pregnancies with TSBA > 40 µmol L^{-1} [5]. Therefore ICP may be classified as mild if the TSBA levels rise to 14-39 µmolL^{-1} and severe if they exceed 40 µmol L^{-1} (healthy, non-pregnant individuals will have levels around 1-6 µmolL^{-1}). The incidence of ICP varies

widely between geographical regions, with historical rates in South American countries reported to exceed 10% of preganancies [4], however, across Europe the rate is lower with ICP typically affecting 1 in 200 pregnancies. At present there is no point-of-care (POC) test to measure TSBA thus clinical diagnosis is reliant on a laboratory-based colorimetric assay, which is not available in every hospital and where time to result can range from a few hours to a week. At present there is no consensus about the optimal way to manage pregnancies complicated by ICP. Current approaches include treatment with the drug ursodeoxycholic acid which is effective at reducing pruritus and some liver function tests [6] and induction of labour at 37-38 weeks of gestation [6a, 7]. Given the relationship between raised TSBA and adverse pregnancy outcome, it would help clinical decision-making if serum bile acid results were available rapidly, particularly when the condition is suspected in the 3rd trimester of pregnancy.

Electrochemical biosensors confer advantages of low costs, portable instrumentation, simple handling procedures, fast sampling times and small sample volumes [8]. Consequently there has been much interest in the development of electrochemical biosensors, especially of the amperometric variety, for clinical analyses. A number of reports have looked at using disposable biosensors for serum measurement of various analytes including alcohol [9], glucose [10] and lactate [11]. However, to our knowledge, amperometric measurement of bile acids in whole human serum using a disposable biosensing system has not yet been reported. The electrochemical measurement of bile acids is based on the electrooxidation of reduced nicotinamide adenine dinucleotide (NADH), which is generated by the enzyme 3α-hydroxysteroid dehydrogenase (3α-HSD), in the specific oxidation of bile acids:

$$\text{3α-hydroxy bile acid} + NAD^+ \overset{3\alpha\text{-}HSD}{\leftrightharpoons} \text{3-keto bile acid} + NADH + H^+ \qquad \text{Scheme 1}$$

The process of NADH electrooxidation is well-studied due to its role as a co-substrate in a plethora of NAD^+-dependent dehydrogenase reactions and for this reason many electrochemical NADH-sensing architectures have been developed [12]. In this study we have utilised a well-known mediator,

Meldola's Blue (MB), incorporated into commercial screen-printed electrodes (SPEs) to enable the electrooxidation of NADH at a low potential (+0.1 V). Earlier studies involving the immobilisation of 3α-HSD on glassy carbon (GC) electrodes [13] and on chemically modified-GC electrodes [14] were important in demonstrating the utility of electrochemical measurement over traditional optical methods. More recently, electrochemical detection of bile acids using disposable platforms in different mediums such as calf serum [15] and urine [16] have been reported although no limit of detection was reported in the former, whilst the ability to detect bile acids in undiluted samples was not achieved in the latter.

Materials and methods

Reagents

3α-hydroxysteroid dehydrogenase lyophilised powder (EC 1.1.1.50) was purchased from Diazyme Laboratories, CA, USA. β-NADH, β-NAD^+, ethylenediaminetetraacetic acid (EDTA), sodium pyrophosphate, tauro/glycocholic acid sodium salts, tauro/glycochenodeoxycholic acid sodium salts, human serum (male, type AB), sodium phosphate dibasic heptahydrate, sodium phosphate monobasic, bovine serum albumin (BSA) and TRIZMA® (tris[hydroxymethyl]amino-methane) hydrochloride were purchased from Sigma-Alrich. Ambion® nuclease-free water (not DEPC-treated) was purchased from Fischer Scientific. Purified water was obtained from a Triple Red (Triple Red Ltd., Bucks, UK) system. All reagents were reagent grade and used as received without further purification.

Apparatus

Voltammetric and amperometric measurements were performed with a CHI 1000A multichannel potentiostat (CH Instruments, Inc., TX, USA). Carbon SPEs (DRP-110), Carbon- Meldola's Blue SPEs (DRP-610) and custom SPE connectors (DRP-DSC) were purchased from Dropsens Ltd., Oviedo, Spain.

Spectrophotometric measurements were performed with an Optimax multi-well UV-visible spectrophotmeter and a 96 well plate (VWR International). All absorbance measurements were made at a wavelength of 340 nm. A Thermo Electron Orion PerpHecT® model 310 pH meter, a Grant water bath (Grant Instruments Ltd., Cambridge, UK) and a Mettler AM100 electronic analytical balance were also used.

Working electrode modification and enzyme solutions

3α-HSD solutions were made by dissolving 10mg of the enzyme in 0.5ml of 0.1 mol L^{-1} Tris-HCl, pH 7.2 containing 0.001 mol L^{-1} EDTA. Enzyme solutions were used for both electrochemical testing and spectrophotometric analysis. All experiments were carried out at room temperature unless otherwise stated.

3α-HSD was immobilised by dissolving 10 mg of the enzyme in 0.5 ml of phosphate buffer (0.1 mol L^{-1}, pH 7.4) containing 0.5% (v/v) BSA. An aliquot of 10 µl of this solution was deposited on the carbon-Meldola's Blue (C-MB) SPE and allowed to evaporate at 4°C overnight. This modified electrode was used as a biosensor for bile acids, without any further treatment of the surface.

Electrochemical testing of the biosensors

Measurements were performed by directly applying samples to the SPEs or by using a custom SPE holder (Dropsens Ltd.), which can enclose a volume of up to 1ml over the SPE. Experiments performed using the custom SPE holder required 200 µL of human serum, 40 µL of 20 mmol L^{-1} β-NAD^+ in phosphate buffer and 10 µL of 3α -HSD solution. Experiments using the SPEs and free enzyme required a 60 µL test volume applied as a droplet. In the case of elevated pH experiments a sodium pyrophosphate buffer (pH 9) was used to make up the 20 mmol L^{-1} β-NAD solution. Potentials reported throughout this study were relative to an integrated 'on strip' Ag/AgCl screen-

printed reference electrode. All experiments were conducted at room temperature and the sensors were not electrically shielded (to replicate realistic *in situ* clinical conditions).

Results and discussion

Characterisation of carbon-Meldola's Blue screen-printed electrodes

In order to determine the suitability of the carbon-Meldola's Blue screen-printed electrodes (C-MB SPEs), for the measurement of NADH, cyclic voltammetry was used. Fig. 1 (A) shows one well-defined redox couple (a) for the C-MB SPE response in 0.1 M phosphate buffer, pH 7, which is due to the Meldola's Blue mediator incorporated into the carbon working electrode. Observed peak separations ($\Delta E = Ep_{OX} - Ep_{RED}$) were largely independent of the scan rate varying between 50 and 60 mV. Furthermore the peak currents scaled linearly with scan rate upto 0.2 V s^{-1} consistent with a reversible $2e^-$ couple adsorbed on the electrode [17]. Using a separate C-MB SPE the response to 0.5 mmol L^{-1} NADH was observed (b) and the increased anodic peak of the MB redox couple is consistent with its reduction by NADH. The increased oxidation current can be understood by considering the following reactions occurring at the electrode surface:

$NADH + MB^+ \rightarrow NAD^+ + MBH$ Scheme 2

$MBH \rightarrow MB^+ + H^+ + 2e^-$ (for $E > E^{0'}$) Scheme 3

The ratio of the anodic currents in the presence and absence of 0.5 mmol L^{-1} NADH (I_{pcat}/I_p), which is dependent on the reaction rate [18], was calculated to be 3.2, which is higher than the figure reported by Zhu et al.[19] of 2.4 obtained using Meldola's Blue-modified carbon nanotube-glassy carbon electrodes with a higher concentration (10-fold) of NADH. The comparison between the NADH electrooxidation peaks obtained using cyclic voltammetry with a C-MB SPE and an unmodified carbon SPE (c) from the same manufacturer (Dropsens Ltd.) highlights the role of the mediator in lowering the potential (E_{OX}) required for the oxidation of NADH. The plain-carbon SPE required an

operating potential of +0.478 V compared with the C-MB SPE, where the operating potential was -0.066V (ΔE = 0.544 V). Studies utilising MB as a mediator with carbon-based electrodes have reported similar decreases in the overpotential for NADH oxidation [19]. Finally, the inter-electrode relative standard deviation (RSD) was found to be 2% at 0.1 mmol L^{-1} NADH in 0.1 M phosphate buffer, pH 7 (n = 6).

Biosensor reaction equilibrium

Spectrophotometric analysis of the β-NAD^+/bile acid reaction was carried out using taurocholic acid (TCA) and measuring the change in absorbance at 340nm. These measurements indicated that the reaction equilibrium is reached in under a minute in the presence of 0.5 mg ml^{-1} enzyme. The equilibrium constants (K_{eq}) for the four most common conjugated bile acids were found to be (3.6, 3.2, 2.4, 3.1) x 10^{-2} for taurocholic acid, glycocholic acid, taurochenodeoxycholic acid and glycochenodeoxycholic acid respectively. These conjugated bile acids are known to increase in ICP [20] in comparison with the unconjugated forms whereas in normal pregnancy the ratio of conjugated to unconjugated bile acids is close to parity.

Operation of the Biosensor

To establish the performance of the biosensor in human serum, a common bile acid that accounts for up to 40% of the bile acid pool during ICP [20-21], taurocholic acid (TCA), was added to serum containing 4 mmol L^{-1} β-NAD. The enzyme, 3α-HSD, was also added to initiate the reaction and a five-minute incubation period was observed prior to electrochemical measurement. Cyclic voltammograms were obtained using the biosensor system in serum in the presence of varying levels of TCA (Fig. 1. (B)) and as a result an operating potential (E_W) of +0.1 V was chosen for the amperometric studies in serum.

Current transients were obtained for different concentrations of TCA by adding serum droplets containing standard additions of the bile acid to individual SPEs (Fig. 2. (A)). The current generated at +0.1 V decays over time as described by the Cottrell equation (Eq. 1.). In practice the Cottrell equation can be simplified (Eq. 2.) and the relationship between current and $t^{-1/2}$ can be informative. For instance, a simple, diffusion-controlled redox process occurring at the electrode surface gives a linear relationship between i and $t^{-1/2}$ (Fig. 2 (B)) and deviations from this could indicate reaction processes such as surface fouling (decrease in effective surface area), catalysis or convection.

$$|i| = nFAD^{1/2}[\mathrm{NADH}] / \pi^{1/2}t^{1/2} \quad (1)$$

Where i = current ; n = number of electrons transferred from NADH to the electrode; F = Faraday constant; A = electrode area; D = diffusion coefficient of NADH; t = time

$$|i| = [\mathrm{NADH}]kt^{-1/2} \quad (2)$$

Where k = the constants from (1)

Using the Cottrell equation it is possible to calculate a concentration of NADH, which is produced in the enzymatic reaction, from the current at a given time. The amount of NADH at equilibrium estimated using spectrophotometry and amperometry are consistent: for a concentration of 100 µmol L^{-1} TCA it was found that 51 µmol L^{-1} NADH was generated (RSD = 5.8%, n = 4) as quantified by absorbance measurements and 55.3 µmol L^{-1} NADH as quantified by the current (RSD = 2.3%, n = 3).

Measurement of Bile Acids in Serum

Fig. 3. (A) displays calibration plots obtained using biosensors with adsorbed 3α-HSD, which were fabricated one day prior to experimentation. Measurements were made on successive days throughout a five day period with biosensors stored at 4°C prior to use. Each series of measurements was carried out using one SPE and a custom SPE holder. Whilst the recorded currents are subject to a large degree of noise the underlying signal does not change significantly between calibrations on successive days as determined by ANOVA ($p < 0.001$) indicating that biosensors fabricated using this

method would be viable for storage at 4°C. However, the RSD at fixed concentrations was found to be above 15%, which is generally considered high for reliable measurement.

Fig. 3. (B) depicts the calibration curves for the biosensor obtained using solutions with the 3α-HSD was added in a solution containing β-NAD^+ and a fixed concentration of TCA and the concentration of serum was maintained at 80% (v/v). In this case the RSD was less than 8% across fixed concentrations, which was considerably better than with the enzyme adsorbed. This disparity may be due to the immobilisation step resulting in variable loss of enzyme activity thus preventing the reaction attaining equilibrium in the incubation period.

The line of calibration obtained at pH 9 had a gradient (sensitivity of the biosensor) of 2.95 nA / µmol L^{-1} and yielded an upper limit of quantitation greater than 150 µmol L^{-1} TCA. The limit of detection (LOD) for the biosensor was calculated using the standard convention (three times background standard deviation) and found to be 4.5 µmol L^{-1} for TCA in serum. A lower sensitivity and higher LOD were calculated using serum, adjusted to pH 7 with 0.1 M phosphate buffer, of 1.1 nA / µmol L^{-1} and 20 µmolL^{-1} TCA respectively. These measurements are consistent with K_{eq} values obtained spectrophotometrically, which were lower at pH values closer to neutrality as implied by the equation for the reaction given above. As expected, optimum conditions for the enzymatic reaction of 37°C and pH 9 [22] increase the oxidation of bile acids, thus measured NADH concentrations are higher.

Conclusion

This paper has demonstrated the use of mediator-incorporated screen-printed electrodes for quantification of bile acids in human serum within a clinical range relevant to the pathophysiology of liver conditions, and specifically the diagnosis threshold of ICP (14 µmol L^{-1}). The biosensor platform was successfully calibrated in a complex serum matrix (80% v/v) with an appropriate linearity and

limit of detection to be clinically useful. Furthermore the relative standard deviation between repeat measurements was low enough (8%) to suggest that the biosensor could be used reliably for simple and rapid determination of total serum bile acids in point-of-care setting. The reaction between conjugated bile acids and β-NAD^+, which is catalysed by 3α-HSD, was analysed by spectrophotometry and the effect of pH on the sensitivity of the assay was mirrored in the biosensor performance. Whilst adsorption of the enzyme did not affect the sensitivity of the biosensor the reproducibility of signal was worsened due to increased surface heterogeneity and/or loss of enzyme activity. This is the first time that bile acid levels have been measured in whole human serum using disposable SPEs. In the future the biosensor platform developed here could be used to measure total bile acid levels in serum from patients with cholestasis.

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Figure Legends

Figure 1 (A) Cyclic voltammograms obtained using C-MB SPEs with (a) 0.1 M phosphate buffer, pH 7 and (b) 0.5 mmol L^{-1} NADH in 0.1 M phosphate buffer, pH 7. A regular carbon SPE (c) was used to compare the response to NADH in the absence of mediator. Separate SPEs were used to obtain each voltammogram and the scan rate was 0.1 V / s. **(B)** Cyclic voltammograms obtained using the bile acid sensing sytem with varying concentrations of TCA in serum, pH 9. The black, grey, dashed and dotted lines were obtained using serum spiked with 150, 100, 50 and 0 µmol L^{-1} TCA respectively. The scan rate used was 0.1 V / s.

Figure 2 (A) Amperometric i-t curves obtained using bile acid biosensor with human serum. Serum samples were spiked with TCA to give final concentrations of 0, 25, 50, 100 and 150 µmol L^{-1} (dotted, dot-dashed, dashed, grey and black line respectively). Separate SPEs were used for each amperometric trace. **(B)** Cottrell plot obtained using the amperometric data shown in Fig. 2 (A). Current measurements from 4 s to 10 s were used in order to exclude the effects of the charging current and convection effects.

Figure 3 (A) Calibration curves constructed from bile acid biosensor measurements with immobilised 3α-HSD in human serum on consecutive days after biosensor fabrication. One biosensor was used to obtain each set of calibration data points. **(B)** Calibration curves obtained using bile acid biosensors with human serum. The effect of pH on the assay sensitivity was assessed at pH 7 (circles) and pH 9 (squares). The slopes for the lines of calibration are y = 0.107 + 0.05 (dotted line) and y = 0.295x + 0.112 (dashed line). Separate SPEs were used for each individual measurement.