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A stable and selective electrochemical biosensor for the liver enzyme alanine aminotransferase (ALT)

Mamun Jamal[a], Oliver Worsfold[a], Timothy McCormac[b], Eithne Dempsey[a,*]

[a] Centre for Research in Electroanalytical Technologies (CREATE), Department of Science, Institute of Technology Tallaght, Tallaght, Dublin 24, Ireland
[b] Electrochemistry Research Group, Dundalk Institute of Technology, Dundalk, County Louth, Ireland

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ABSTRACT

An electrochemical method to determine alanine aminotransferase (ALT) activity over its normal and elevated physiological range was developed based upon detection of L-glutamate at a glutamate oxidase-modified platinum electrode. Measurements were carried out in the presence of ALT co-substrates L-alanine and α-ketoglutarate and current response from either the oxidation of hydrogen peroxide or the re-oxidation of the mediator ferrocene carboxylic acid was employed. The enzyme electrode was tested over a 6-month period and found to retain 79% of its original activity towards ALT detection with >200 measurements performed over this time. Signals associated with interfering electroactive species (ascorbic acid and uric acid) were eliminated using background subtraction at a denatured glutamate oxidase enzyme electrode. The sensitivity of the device was found to be 0.845 nA U^{-1} L ALT with t_{90} = 180 s, linear range 10–1000 U L^{-1} and LOD of 3.29 U L^{-1} using amperometry at E_{app} = 0.4 V vs. Ag/AgCl at 308 K (35 °C).

1. Introduction

The measurement of liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (γGT) in physiological fluid provides valuable information in the diagnosis of liver disease. Under normal circumstances, these enzymes reside within the cells of the liver, but when the liver is injured/diseased they spill into the blood stream and elevated levels signal liver damage such as hepatitis, myocardial infarction and jaundice (Dafour et al., 2000).

Among the most sensitive and widely tested of these liver enzymes are the aminotransferases, which include ALT and AST. Normal levels of these enzymes are 5–40 and 7–56 U L^{-1}, respectively in blood and serum. Following severe liver damage, ALT levels increase to >50 times the normal level with moderate elevation at 96–240 U L^{-1} and high elevation 240–960 U L^{-1} (Huang et al., 2006).

A recent review (Huang et al., 2006) outlines some of the main detection strategies such as colorimetric, chemiluminescence, chromatography and electrochemical techniques which have been employed for ALT and AST determination. Conventional spectrophotometric assay based methodologies which are routinely performed in clinical laboratories require complex reagents, are costly and require trained operators. Therefore, there is a recent growing demand for development of *in situ* healthcare devices (Dafour et al., 2000) and electrochemical sensors have proven advantages for such applications. A number of biosensors for ALT monitoring have been reported recently based on electrochemical detection e.g. Chang et al. (2003) reported an ALT sensor based on glutamate oxidase (GlutOx)-modified palladium electrode, while a kinetic based differential pulse voltammetric (DPV) method for the determination of ALT activity in human serum with a gold microelectrode without modification was reported by He and Chen (1997). In the latter case, ALT reacts with the reagents to produce NADH which is oxidised to NAD^+, resulting in a quantitative current. Sensitivity was however pH dependent. An immunosensor method for ALT detection was developed by Xuan et al. (2003) using monoclonal antibodies to human recombinant ALT. The anti-ALT antibody immunosensor system showed sensitivity of 26.3 nA/(ng ml^{-1}) with a limit of detection of 10 pg ml^{-1}.

Multi-enzyme detection was demonstrated by Moser et al. (2002) who reported a rapid liver enzyme assay with miniaturized liquid handling system comprising a thin film biosensor array while Song et al. (2007) reported an electrochemical biosensor array for liver diagnosis using a silanization technique on nanoporous silicon electrode. These systems and others were based on determination of hydrogen peroxide as a result of glutamate production and some demonstrated limitations at elevated ALT ranges with operational stability of the sensor ranging from 1 to 6 months. All operate on

* Corresponding author. Tel.: +353 1 4042862; fax: +353 1 4042404.
E-mail address: Eithne.dempsey@ittdublin.ie (E. Dempsey).

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the principle outlined in the equations below.

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate} \quad (1)$$

$$\text{l-Glutamate} + H_2O + \text{GlutOx(FAD)} \rightarrow \alpha\text{-Ketoglutarate} + NH_3 + \text{GlutOx}(FADH_2) \quad (2)$$

$$\text{GlutOx}(FADH_2) + O_2 \rightarrow \text{GlutOx(FAD)} + H_2O_2 \quad (3)$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 4e^- \quad (4)$$

There are relatively few reports of the use of mediators for glutamate oxidase amperometric sensors (Hale et al., 1991; Vahjen et al., 1991; Arima Jiro et al., 2002; Huang et al., 2007; Harper and Anderson, 2006) and to the best of our knowledge only one mediator based ALT biosensor system (Li et al., 2006) based on a thick film sensor using both glutamate oxidase and horseradish peroxidase coupled to an osmium redox mediator (Li et al., 2006) has been reported.

In this work, a simple dual working electrode system was developed for ALT determination (mediated and non-mediated) based upon the measurement of glutamate produced during the reaction. There was evidence of improved analytical performance and long-term stability compared with current methods of ALT detection and the use of ferrocene carboxylic acid as mediator for glutamate oxidase in the determination of ALT has not been reported, to the best of our knowledge. This mediated sensor enables oxygen independent operation at relatively low applied potential which together with the background subtraction approach enables application in the presence of physiological levels of electroactive interferents.

2. Experimental

2.1. Reagents and materials

Bovine serum albumin (BSA), monosodium glutamate, L-alanine, glutaraldehyde, α-ketoglutarate, ALT (from porcine heart), phosphate buffer saline (PBS) tablets were purchased from Sigma (Dublin, Ireland). Glutamate oxidase (GlOx) (50 units/vial) was purchased from Yamasa Corp., Japan. Nafion (perfluorinated ion-exchange resin, 5% (w/v) solution was purchased from Fluka. All other reagents were of analytical grade. Standard solutions and buffers were prepared with deionised water.

2.2. Equipment

Voltammetric and amperometric measurements were performed with a CHI 600 potentiostat and a CHI 1060 multichannel potentiostat. Scanning electron microscopy (SEM) experiments were conducting using a JEOL JSM-6390LV with a SC7620 Mini Sputter Coater from Quorum Technologies. Temperature controlled experiments employed a thermostated water jacketed electrochemical cell. A Pt electrode (0.055 cm^2) was employed as a working electrode with Ag/AgCl and Pt wire as reference and counter electrode, respectively.

2.3. Procedures

2.3.1. Surface characterisation

Surface examination of the enzyme-modified Pt disk electrode was carried out by SEM. The sample was sputtered with Au/Pd for 30 s and images were carried out a low vacuum (70 Pa) 15 kV accelerating voltage (JSM 6390 LV) and spot size of 56.

2.3.2. Enzyme immobilisation

Prior to enzyme immobilisation, platinum electrodes were mechanically polished with alumina powder of 1, 0.3 and 0.05 μm, followed by sonication in deionised water for 10 min. 20 μl of GlutOx (50 U in 500 μl of 0.01 M PBS, pH 7.4) was mixed with 2 mg of BSA, 20 μl of glutaraldehyde (2.5%) and 10 μl of Nafion (0.5%). 4 μl of this mixture was then dispensed onto the surface of a platinum electrode and allowed to dry for 45 min under ambient conditions. The electrodes were then stored at 4 °C overnight in 0.01 M PBS buffer prior to use. The optimum sensor performance was achieved using 0.16 U GlutOx per electrode, 1.0% (v/v) glutaraldehyde, 0.2% (w/v) Nafion and 2.0% (w/v) BSA.

2.3.3. Electrochemical testing

Cyclic voltammograms were performed over the potential range 0.1–0.6 V vs. Ag/AgCl at 0.1 V s^{-1} in a 10 ml cell containing PBS buffer (pH 7.4) and co-substrates L-alanine and α-ketoglutarate. For amperometric measurements various aliquots of 200 U mL^{-1} stock ALT solution (2.1 mg of ALT dissolved in 1 ml of 0.01 M PBS and stored at −20° C) was added successively following decay of the background current. An operating potential of either +0.4 V (mediated) or 0.7 V (unmediated) vs. Ag/AgCl was employed. The temperature of the solution was varied from 294 to 318 K (21–45 °C) using a thermostatically controlled cell. In the case of experiments carried out in the absence of oxygen, the solution was purged with Argon for 20 min and a blanket of gas was maintained at the surface of the solution throughout the experiment.

2.3.4. Stability studies

Electrode stability was evaluated using hydrodynamic amperometry at E_{app} = 0.7 V vs. Ag/AgCl by addition of 150 μM of L-glutamate at room temperature (298 K). Testing was performed everyday for the first 40 days and every 15 days for a period of 6 months. In between measurements the electrode was stored at 4 °C (277 K) in PBS.

2.3.5. Interference studies

A dual electrode system was employed where the control (inactive) working electrode employed denatured glutamate oxidase. Enzyme denaturation was achieved by heating at 373 K (100 °C) for 10 min. Mixtures of L-glutamic acid or ALT, ascorbic acid and uric acid were added (over their physiological concentration range) and current response measured at 308K (35 °C), E_{app} = 0.4 V vs. Ag/AgCl in the presence of 0.2 mM ferrocene carboxylic acid. By subtracting the current signals for active and inactive enzyme electrodes measurement of ALT activity levels was possible in the presence of background physiological levels of both ascorbic and uric acid.

3. Results and discussion

3.1. Method development

Immobilisation of glutamate oxidase (GlutOx) was achieved via cross-linking with glutaraldehyde (1% v/v) and BSA (2% w/v) onto the surface of a clean Pt electrode (0.055 cm^2). The optimum enzyme loading was determined by examining activity levels over the range 0.02–0.8 U/electrode glutamate oxidase. An enzyme loading of 0.16 U GlutOx per electrode was found to be optimal, higher loadings resulted in poor substrate response possibly due to inhibition/blocking effects with lower activity loadings resulting in poor response sensitivity. The addition of 10 μl of 0.2% Nafion polymer provided a film with optimum glutamate response stability, while higher % loadings (0.5–5%) created a diminished substrate response and lower (0.1–0.05%) levels resulted in poor film stability. The minimum concentration of redox mediator (ferrocene monocarboxylic acid) necessary for quantitative electrocatalysis was determined to

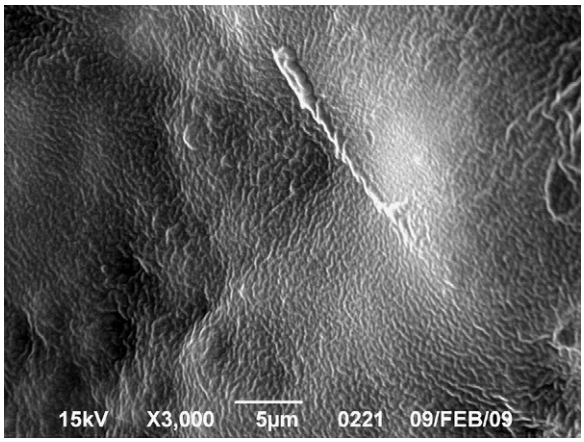

Fig. 1. Scanning electron microscope image of the glutamate oxidase-modified Pt electrode, taken at low vacuum (70 Pa), 15 kV accelerating voltage and spot size of 56 (JSM 6390 LV). Sample was sputtered with Au/Pd for 30 s.

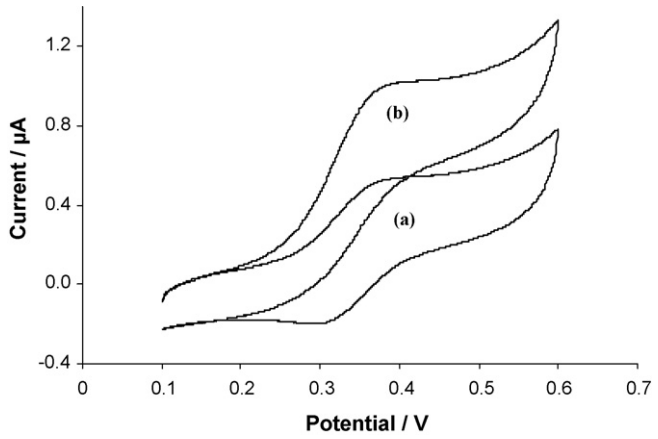

Fig. 2. Electrocatalytic response upon addition of 800 U L^{-1} ALT (a = blank; b = 800 U L^{-1}) measured at a glutamate oxidase-modified Pt electrode in the presence of 0.2 mM ferrocene carboxylic acid. Scan rate 0.02 V s^{-1} in PBS buffer pH 7.4.

be 0.2 mM which was added to the background electrolyte together with co-substrates unless otherwise specified.

Surface characterisation of the glutamate oxidase film was performed using SEM (Fig. 1), and showed evidence of uniform surface features, demonstrating effective cross-linking and protein immobilisation within the Nafion polymer layer.

According to Chang et al. (2007), the sensitivity of L-glutamate sensor may be inhibited by both α-ketoglutarate and L-alanine. Therefore, the optimum ratio of co-substrates was required to achieve maximum ALT sensitivity. This was studied by measuring the amperometric signal for the addition of 100 U L^{-1} ALT at E_{app} = 0.4 V vs. Ag/AgCl in the presence of 0.2 mM ferrocene carboxylic acid in 0.01 M PBS pH 7.4. The concentration of α-ketoglutarate in the working solution was varied over the range 0–15 mM, while the L-alanine concentration was maintained at 100 mM. The maximum response sensitivity was obtained when α-ketoglutarate concentration in the working buffer was in the range 10–15 mM (data not shown here). Subsequent experiments employed 10 mM α-ketoglutarate and 100 mM L-alanine as ALT co-substrates which were added to the background electrolyte (PBS pH 7.4).

In order to determine the optimum operating temperature for the enzyme glutamate oxidase a temperature study was performed over the range 294–318 K (21–45 °C). The maximum response to 60 μM glutamate was, as expected, close to physiological temperature at 308 K (35 °C), using amperometry at E_{app} = 0.4 V vs. Ag/AgCl, in 0.01 M PBS buffer (pH 7.4) with 0.2 mM ferrocene carboxylic acid in the presence of 100 mM L-alanine and 10 mM α-ketoglutarate. In order to determine glutamate oxidase operational stability under these conditions, the electrode was incubated for 5 h at 308 K with a 2–3% decrease in activity observed over this period.

3.2. Electrocatalyic studies

A cyclic voltammogram of the unmediated response to 1000 U L^{-1} ALT in the presence of oxygen (see Fig. S1 Supporting Information), demonstrated direct electro-oxidation of hydrogen peroxide at 0.5–0.7 V vs. Ag/AgCl, produced from the enzymatic reaction from as described by Eqs. (1)–(4) above. A cyclic voltammogram for 0.2 mM ferrocene carboxylic acid prior to and after the addition of 800 U L^{-1} ALT at the glutamate oxidase-modified Pt electrode is shown in Fig. 2. In the absence of ALT, the diffusion controlled redox features of ferrocene carboxylic acid were evident with $E_{1/2}$ 0.33 V vs. Ag/AgCl and ΔEp 75 mV. Upon addition of ALT an electrocatalytic oxidation wave was observed indicating the increased oxidation current (maximum response at 0.4 V vs. Ag/AgCl) and decreased reduction current as a result of the following proposed mechanism.

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-Glutamate} \quad (5)$$

$$\text{l-Glutamate} + \text{GlutOx(FAD)} \rightarrow \alpha\text{-Ketoglutarate} + \text{GlutOx(FADH}_2\text{)} \quad (6)$$

$$\text{GlutOx(FADH}_2\text{)} + 2\text{Fe(III)} \rightarrow \text{GlutOx(FAD)} + 2\text{Fe(II)} + 2\text{H}^+ \quad (7)$$

$$2\text{Fe(II)} \rightarrow 2\text{Fe(III)} + 2\text{e}^- \quad (8)$$

3.3. Amperometric testing and analytical performance evaluation

Electrochemical testing of the unmediated system in the presence of O_2 employed direct electro-oxidation of hydrogen peroxide produced from the enzymatic reaction at E_{app} = 0.7 V vs. Ag/AgCl. This resulted in LOD of 2.43 U L^{-1} (S/N = 3) with sensitivity of 0.642 nA U^{-1} L (308 K). The preferred mediated approach in the presence of ferrocene carboxylic acid was found to enhance sensitivity and resulted in a lower operating potential of 0.4 V vs. Ag/AgCl which was employed for subsequent amperometric experiments.

Fig. 3 shows the electrochemical hydrodynamic amperometric response for mediated ALT detection at 308 K (35 °C) for 20 U L^{-1} ALT additions, demonstrating a stable and linear response within 3 min under these conditions. The mediated chronoamperometric sensor response (see Fig. S2 Supporting Information) upon application of 0.35 V vs. Ag/AgCl with additions over 100–900 U L^{-1} ALT showed the diffusion controlled time dependent current decay which was proportional to ALT activity over the range examined (stable current achieved within 100 s of ALT addition). Quantification was also possible based upon the slope of the initial current decay over a 20 s period.

The linear range for the sensor was found to be 10–1000 U L^{-1} ALT, sensitivity 0.84 nA U^{-1} L, with LOD of 3.29 U L^{-1} at E_{app} = 0.4 V vs. Ag/AgCl (308 K (35 °C)). The intra-electrode relative standard deviation (RSD) was 6.9% (n = 4) while the inter-electrode RSD was 4.5% (n = 5) (20 U L^{-1} ALT additions). Analytical parameters for hydrodynamic amperometric experiments in the presence and absence of mediator (0.4 and 0.7 V vs. Ag/AgCl) are summarised in Table 1. This performance data compares favourably with recent reports based upon ALT/glutamate detection using direct hydrogen peroxide electro-oxidation (Chang et al., 2007), while few articles have demonstrated experimental results for ALT determination over physiological levels with such rapid response characteristics.

Table 1
Analytical performance parameters for amperometric ALT determination at room temperature 297 K (24 °C) and 308 K (35 °C) for both mediated (in presence of 0.2 mM ferrocene carboxylic acid) and non-mediated systems.

Analytical parameters	Unmediated/297 K (24 °C)	Mediated/297 K (24 °C)[a]	Unmediated/308 K (35 °C)	Mediated/308 K (35 °C)[a]
Sensitivity (nA U^{-1} L))	0.233 ± 0.01	0.671 ± 0.03	0.642 ± 0.03	0.845 ± 0.03
r^2	0.988	0.986	0.986	0.993
[a] t_{90} (s)	298	388	121	180
Linear range (U L^{-1})	10–500	10–500	10–1000	10–1000
LOD (U L^{-1})	1.33	1.76	2.33	3.29

[a] Based upon hydrodynamic amperometric response to 20 U L^{-1} ALT addition

Table 2
Comparison of current biosensor performance with other glutamate and ALT electrochemical detection systems.

Stability months	ALT sensitivity (nA U^{-1} L))	ALT range (U L^{-1})	Glutamate sensitivity (nA μM^{-1})	Interference elimination	Reference
6	0.845	10–1000	1.43	Background subtraction/mediated	[a]
<4	0.136	1.3–250	–	–	Song et al. (2007)
<6	0.595	8–250	12.2	Elevated baseline	Chang et al. (2003)
–	0.012	<1700	–	–	Kihara et al. (1984a,b)
–	0.09	5–1200	0.001	Cellulose acetate	Compagnone et al. (1992)
–	0.058	<200	–	–	Ohgami et al. (2007)

[a] Results obtained from this work.

Table 2 shows a comparison of the performance of this sensor with those ALT systems recently reported in the scientific literature. The method described here demonstrates a mediated ALT detection system with improved behaviour in relation to stability and sensitivity with extended linear range over both normal and elevated ranges.

3.4. Interference study

In real biological fluid analysis, background signals due to physiological levels of electroactive species such as ascorbic acid and uric acid create selectivity challenges. A number of strategies may be employed such as the use of charge/size exclusion membranes, lower operating potentials, overoxidised polypyrrole/insulating polymer layers or pre-electrolysis cells (Lunte et al., 1987). In this work, background subtraction was performed with the use of an inactive enzyme electrode which enabled glutamate signals to be achieved in the presence of physiological levels of two key interferents.

Mediated amperometric responses to glutamate additions (60–420 μM) at both active and inactive glutamate oxidase electrodes at 308 K (35 °C) were carried out in the presence of 45–315 μM ascorbic acid and uric acid (see Fig. S3 Supporting Information), representing low to high physiological range (each addition representing 45 μM of each interferent). Sensitivity values for the corrected signal in the presence of increasing additions of interferences were found to be on average 0.505 nA μM^{-1} at 297 K (24 °C) and 1.644 nA μM^{-1} at 308 K (35 °C). These values correlated well to the sensitivity values obtained for standard glutamate only additions over the same range (60–420 μM), 0.6 and 1.42 nA μM^{-1} for measurements at 297 and 308 K, respectively.

Similar experiments were performed for ALT determination using addition of ALT (20 U L^{-1}) + ascorbic acid (45 μM) + uric acid (45 μM) at both active and denatured electrodes (Fig. 4). At high interferent levels background noise increased as a result of the direct oxidation reactions of ascorbate and uric acids at the surface with associated by-products. However, this approach demonstrates

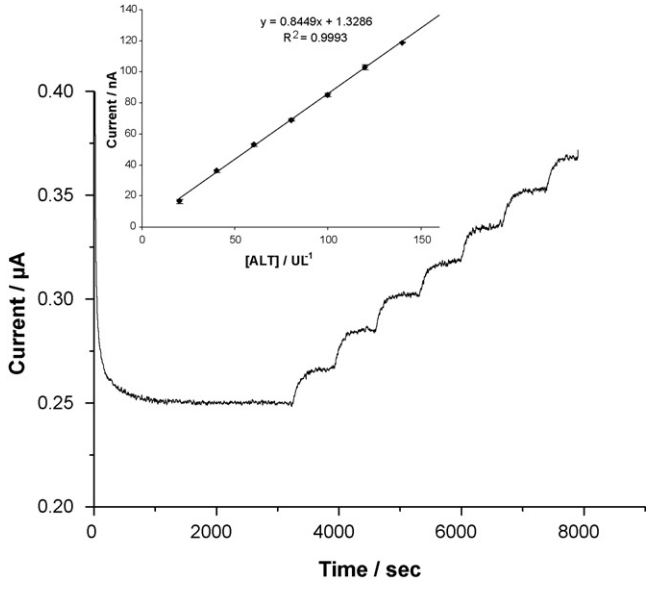

Fig. 3. Amperometric response of glutamate oxidase-modified Pt electrode for successive additions of 20 U L^{-1} ALT at 308 K (35 °C) in absence of oxygen (E_{app} = 0.4 V vs. Ag/AgCl) and corresponding concentration plot (inset). Background electrolyte 0.01 M PBS (pH 7.4), in the presence of 0.2 mM ferrocene carboxylic acid, 100 mM L-alanine and 10 mM α-ketoglutarate.

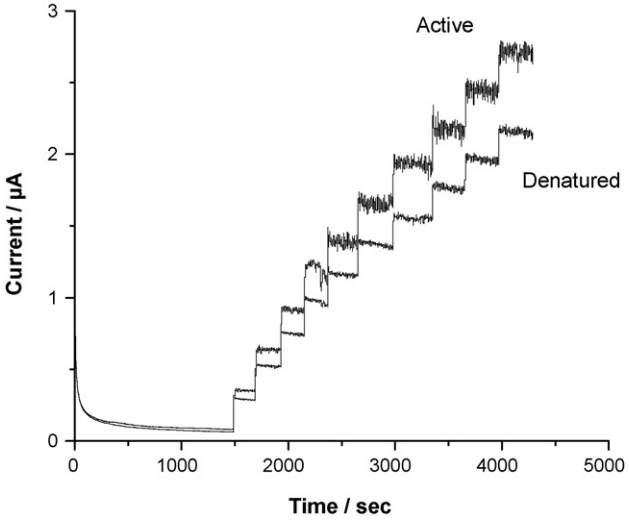

Fig. 4. Amperometic response obtained upon successive additions of 20 U L^{-1} ALT + 45 μM ascorbic acid and 45 μM uric acid using glutamate oxidase active and denatured electrode at 0.7 V vs. Ag/AgCl at 308 K (35 °C).

an ability to quantify both glutamate and ALT in the presence of such electroactive species with capability for performance in physiological fluids.

3.5. Sensor stability study

The stability of the enzyme electrode was determined over a period of 6 months with analysis carried out everyday for the first 40 days; 6 assays each day with 150 μM glutamate addition (data not shown). Over the time period examined >230 tests were performed on each electrode and in between testing the electrode was stored in 0.01 M PBS at 277 K (4 °C). The enzyme-modified electrode was found to retain 79% of its initial activity after 6 months and ca. 88% activity was retained after 40 days. The latter result was comparable to Li et al. (2006) and Song et al. (2007) (approx. 90% after 1 month) and compared well with Chang et al. (2003) (70% after 40 days).

4. Conclusion

In this article, we have reported a novel mediated, stable electrochemical ALT/glutamate biosensor with fast response time characteristics and established a method to remove interferences while maintaining a simple one-step electrode fabrication process. This represents the first step in the fabrication of a multi-analyte detection system for liver enzyme biomarker quantitation. Further work will extend the system to determination of aspartate aminotransferase activity with testing in serum/plasma samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.02.032.

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