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A new enzyme electrode for quantification of salicylic acid in a FIA system

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Abstract

This work presents an amperometric biosensor incorporated into a flow configuration comprising salicylate hydroxylase that catalyses the irreversible hydroxylation of salicylate to catechol in the presence of NADH and molecular oxygen, and tyrosinase that further oxidises catechol giving *o*-quinone which is electrochemically reduced at −100 mV vs. Ag/AgCl yielding catechol and entering the catalytic oxidation and electrochemical reduction cycling which results in signal amplification and, consequently, low limits of detection. Additional incorporation of glucose dehydrogenase in the enzymatic sequence results in regeneration of NADH provided that glucose is present in the carrier stream and incorporation of a dialysis membrane provides operational stability to the biosensor. The analytical characteristics of this catalytic and electrochemical transduction sequence in a FIA system are: a limit of detection of 3.5 10^{-6} M (S/N = 3), a sensitivity of 22.6 nA $\mu M^{-1} cm^{-2}$, no loss of response at least after 5 h of continuous operation, and a sample frequency of 15 h^{-1}. Monitoring of salicylate after ingestion of 500 mg of acetylsalicylic acid has been followed in non-pretreated urine samples and the amount of salicylate in several drugs has been also successfully quantified.

Keywords: Salicylic acid; Multienzymatic system; Carbon paste; Electrochemical detection; Flow injection analysis

1. Introduction

Salicylic acid is widely used as keratolic, antimicrobial and antifungical agent in many pharmaceutical preparations. The analysis of salicylic acid in biological fluids is also relevant because it is the main metabolite of Aspirin® (acetylsalicylic acid) which is widely used as an antipyretic, analgesic and anti-inflammatory drug. Salicylate intoxication has been reported in therapeutic chronic inflammatory diseases such as rheumatoid arthritis [1]. Additionally, 20% of children drug intoxication cases are due to accidental ingestion of aspirin mainly in flavoured formulations [2].

The literature reports many techniques for quantification of salicylic acid. Colorimetric and fluorimetric methods, although simple and cheap, are not selective for salicylates and they require pretreatment of samples to avoid interferences [3–5]. Salicylate-ion selective electrodes have been recently described but they have not been used in

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real samples [6,7]. A liquid chromatographic assay (UV detection) for liquid and semisolid formulations has been reported with previous chloroform extraction [8].

A single enzyme reaction has been also used for selective detection of salicylate. In all cases, salicylate hydroxylase (SH, EC 1.14.13.1) catalyzes the hydroxylation and subsequent decarboxylation of salicylic acid in the presence of NADH and O_2 producing catechol, CO_2, NAD^+ and H_2O, according to reaction (**1**):

$$\text{Salicylic acid} + \text{NADH} + \text{O}_2 + 2\text{H}^+ \xrightarrow{\text{SH}} \text{Catechol} + \text{NAD}^+ + \text{CO}_2 + \text{H}_2\text{O} \quad (1)$$

There are many means to transduce this reaction allowing direct or indirect correlation to salicylic concentration. Consumption of NADH followed at 340 nm with salicylate hydroxylase in solution [9] or depletion of O_2 by an oxygen electrode with immobilised enzyme [10] have been correlated successfully with the standard colorimetric test [3], but in both cases either NADH and enzyme or NADH, respectively, had to be added to the reaction mixture assay. A potentiometric sensor to measure the formation of CO_2 [11], and several amperometric electrodes measuring catechol have been described [12–15] allowing quantification of salicylic acid. A reagentless amperometric screen-printed biosensor has been also reported incorporating the enzyme and NADH into the carbon based electrode and used as disposable strip [16].

The sensitivity obtained in reaction (**1**) with direct electrochemical transduction of catechol could be easily improved by recycling such product in a second enzymatic reaction. Moore et al. (1995) have used salicylate hydroxylase coupled to glucose oxidase obtaining one order of magnitude increase in sensitivity and a limit of detection of 10 μM for a 20 μl analyte droplet [17]. This amplification is due to an electrocatalytic redox cycle that regenerates catechol after electrochemical oxidation to quinones which are efficiently reduced to catechol by glucose oxidase in the presence of glucose. The applied potential in this work, 600 mV vs. Ag/AgCl, also lead to direct oxidation of NADH which could contribute to the overall response. A second coupled enzymatic assay has been described by Bouvrette and Luong, 1996 making use of salicylate hydroxylase and tyrosinase in solution and measuring the rate of NADH consumption spectrophotometrically [18]. Similarly to the previous approach, quinones are also produced, but enzymatically with tyrosinase and according to reaction (**2**). Quinones are then reduced to catechol by reacting with NADH, reaction (**3**), resulting in an enzymatic cycling with higher rate of NADH consumption per molecule of salicylate.

$$\text{Catechol} + 1/2\text{O}_2 \xrightarrow{\text{TYR}} o-\text{quinone} + \text{H}_2\text{O} \quad (2)$$

$$o\text{-quinone} + \text{NADH} \rightarrow \text{Catechol} + \text{NAD}^+ \quad (3)$$

This coupled assay improved the detection limit for salicylate enabling quantification of nM concentrations and offering the possibility of overcoming interferences by mere dilution of the sample [18].

The approach presented here also makes use of reaction (**1**) and (**2**), but since the quinones are electroactive species susceptible to reduction at low potentials, the recycling is based on the electrochemical step, reaction (**4**), allowing simultaneously the measurement of cathodic currents and quantification of salicylate.

$$o\text{-quinone} \xrightarrow[2\text{H}^+ + 2e^-]{} \text{Catechol} \quad (4)$$

The advantage of the cycling scheme proposed in this work is mainly based on the low applied potentials, and on the use of carbon paste electrodes making possible a reagentless configuration after incorporation of a second recycling step for NADH and a dialysis membrane (see Section 3). This transduction scheme is incorporated into a monochannel FIA system where samples are directly injected with no preteatment aiming at a selective, sensitive and fast method for the quantification of salicylic acid.

2. Experimental

2.1. Reagents

Salicylate hydroxylase (salicylate-1-monooxygenase, SH, EC 1.14.13.1, lot No. 114H68771 with

1.2 U mg^{-1} protein and lot No. 23H68091 with 0.95 U mg^{-1} protein) from *Pseudomonas* sp. and tyrosinase (phenol-oxidase, TYR, EC 1.14.18.1, lot No. 24H9542 and lot No. 24H9544 with 4 400 and 3 400 U mg^{-1} protein, respectively) from mushroom were provided by Sigma. Glucose dehydrogenase (GDH, EC 1.1.1.47, lot No. 239 YD102832 with 220 U mg^{-1} protein) from *Bacillus megaterium* was obtained from Merck. Salicylic acid (2-hydroxybenzoic acid) and acetylsalicylic acid were purchased from Probus. β-Nicotinamide adenine dinucleotide reduced form (NADH), gentisic acid (2,5-dihydroxybenzoic acid), and D-glucose were supplied from Sigma. Dialysis membranes (MWCO 10 000 Da) were from Medicell. All other reagents were of analytical grade. Pharmaceutical preparations were obtained from a local drugstore. Water produced in a Milli-Q system (Millipore–Waters) was used in this work.

2.2. Preparation of carbon paste and modified carbon paste electrodes

Prior to use the graphite powder (Fluka) was pretreated at 700°C for 90 s in a muffle furnace and kept in a desiccator at room temperature. Unmodified carbon paste was made by thoroughly mixing 100 mg of graphite powder with 40 μl of paraffin-oil (Fluka) in a mortar until an uniform paste was obtained. Different modified carbon pastes were prepared by mixing the enzymes with the graphite powder before adding the paraffin-oil. The first electrode configuration was made by mixing 5.7 U of SH and 6600 U of TYR with 100 mg of graphite. Some of these electrodes were also modified with different amounts of NADH. For the reagentless configuration, 330 U of GDH was also added to the SH, TYR, NADH and graphite mixture. All the components were carefully mortared manually and mixed in an agate mortar to form a homogeneous paste after addition of paraffin-oil. The enzymatic carbon paste was stored in dry conditions at 4°C until use.

The electrochemical cell used was a BAS TL-5 cell (BAS, West Lafayette, IN). The working electrode with a geometric surface area of 0.0707 cm^2 was prepared by packing the end of the holder with unmodified carbon paste and leaving about 0.5 mm to be filled with the enzyme modified paste, then the electrode was carefully polished on a glassy-surface.

2.3. Instrumentation

The flow injection (FI) system consisted of a peristaltic pump (Gilson Minipuls 2), a pneumatically operated valve (Cherminet PA-875) with an injection volume of 25 μl and the flow-through electrochemical cell under three electrode potentiostatic control (Zäta Elektronic, Sweden). A Ag/AgCl (KCl sat) electrode and a glassy carbon served as reference and counter electrodes, respectively. The enzyme carbon paste electrodes were used as a working electrodes. The output of the potentiostat was displayed on a recorder (Kipp and Zönen). All parts were connected with Teflon tubing (i.d. 0.5 mm). The automatic system was an ASPEC (Gilson, Villiers-Le-Bel, France) adapted to FI conditions using the electrochemical cell described above. This system was operated by Gilson 719 and 725 software for control and data acquisition. Unless otherwise stated, the carrier was 0.1 M phosphate buffer, pH 6.0 previously filtered through 0.45 μm pore diameter Millipore membranes, and degassed for 20 min by reduced pressure. Current intensities were measured as peak heights and represent the average of three injections.

3. Results and discussion

3.1. Characterization of the enzymatic and electrochemical coupled system

The proposed detection system is based on the electrochemical reduction of quinones enzymatically produced by tyrosinase enabling a catalytic and electrochemical redox cycling. The dependence of the cathodic response on the applied potential is shown in Fig. 1 where currents are measured after 25 μl injections of 16 μM

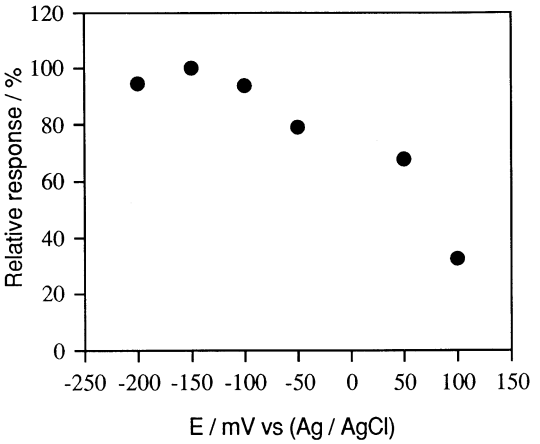

Fig. 1. Dependence of the cathodic current on the applied potencial for enzyme carbon paste electrodes with SH and TYR in a FI system injecting 25 μl of 16 μM salicylic acid supplied with 1 mM NADH. Carrier 0.1 M phosphate buffer pH 6.0 and a flow rate 0.6 ml min^{-1}.

salicylate containing 1 mM NADH. No response was obtained at any potential when salicylate was injected without NADH or after injections of 1 mM NADH (data not shown). This potential dependence for salicylate follows that obtained when injecting a substrate of tyrosinase and it corresponds to direct electrochemical reduction of quinones [19]. The slight response increase below −150 mV vs. Ag/AgCl does not merit to risk non-selective reductions and a potential of −100 mV vs. Ag/AgCl was chosen for further experiments.

The effect of pH and flow rate on the current was also studied and results are presented in Fig. 2. Maximum response for salicylate was obtained at pH 6 which is also in agreement with the optimum pH of tyrosinase in carbon paste electrodes [20]. Considering that salicylate hydroxylase has an optimum pH range in more alkaline conditions [21,22], and that reversibility of the electrochemical reduction of quinones is favoured at low pH, the pH dependence depicted in Fig. 2A shows the pH balance of the overall reactions with an optimum at pH 6.0. The flow rate dependence was studied for salicylate and catechol both supplied with NADH. Results in Fig. 2B show the expected decrease in response, for a kinetically controlled system, when decreasing the residence time (increasing flow rate) of the bulk analyte on the electrode surface. Injections of catechol in the absence of NADH were also made and the currents obtained were very similar to those in the presence of NADH indicating that reaction (**3**), a strictly chemical step, does not compete with the electrochemical transduction, reaction (**4**), under these operational conditions.

A calibration curve was obtained in the FIA system at pH 6 and a flow rate of 0.5 ml min^{-1} for 25 μl injections of salicylic acid supplemented

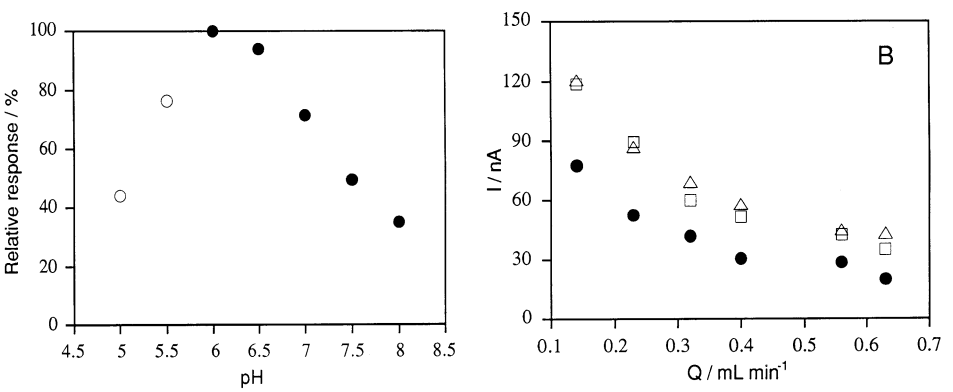

Fig. 2. pH dependence (A) and flow rate dependence (B) on enzyme carbon paste electrodes with SH and TYR in a FI system. (A) Injections of 25 μl of 16 μM salicylic acid supplied with 1 mM NADH using (○) 0.1 M acetate buffer and (●) 0.1 M phosphate buffer as a carrier at flow rate 0.6 ml min^{-1}. (B) Injections of 25 μl of (●) 16 μM salicylic acid supplied with 1 mM NADH, (△) 16 μM catechol and (□) 16 μM catechol supplied with 1 mM NADH using 0.1 M phosphate buffer, pH 6.0 as a carrier at flow rate 0.6 ml min^{-1}. The applied potential in both cases was −100 mV vs. Ag/AgCl.

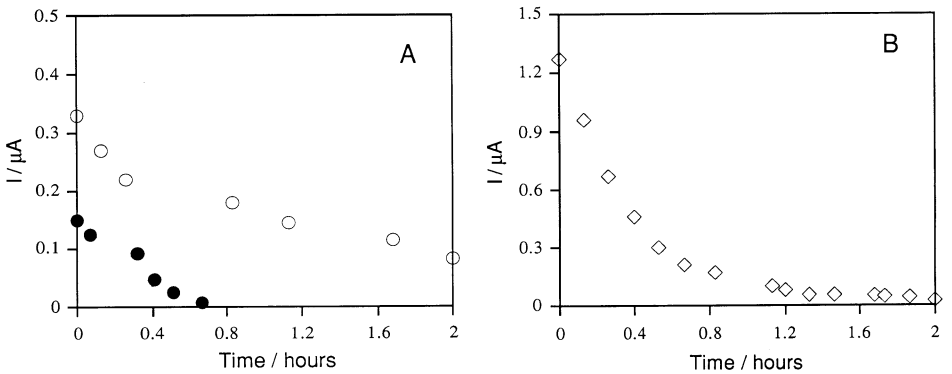

Fig. 3. Operational stability for injections of 25 μl of 1 mM salicylate; (A) enzyme carbon paste electrodes with SH, TYR and (●) 30% NADH (w/w graphite) or (○) 90% NADH (w/w graphite); (B) enzyme carbon paste electrodes with SH, TYR, GDH and 0.3% NADH (w/w graphite), the carrier stream was added with 10 mM glucose concentration. Experimental conditions: carrier 0.1 M phosphate buffer, pH 6.0 at flow rate 0.6 ml min^{-1} and the applied potential was −100 mV vs. Ag/AgCl.

with 1 mM NADH ($y = 0.0006 + 3.3693x$, where $x = \mu M$, $y = nA$; $n = 9$, $r = 0.9997$). The limit of detection (S/N = 3) was 0.5 μM and linearity up to 65 μM. The analytical characteristics of this system compare well with the performance of other strategies seen in the literature making use of either glucose oxidase for signal amplification [17] or tyrosinase and spectrophotometric detection of NADH [18]. However, the redox cycling presented in this work allows the use of low potential where the risk of non specific electrochemical reactions is diminished and it operates in a FIA system, but, in all cases NADH has to be supplemented making the salicylate analysis far from practical. Consequently, the aim of the following work is the design of a reagentless configuration.

3.2. Reagentless biosensor for salicylic acid

Contrary to NADH, NAD^+ has been extensively incorporated into carbon paste electrodes for the design of dehydrogenase based biosensors [23] and stability has been reported if either an efficient electrocatalytic cycling is incorporated to convert the enzymatically produced NADH into NAD^+ [24] or introducing a diffusion barrier to avoid the leaching of NAD^+ [25]. One reagentless salicylate biosensor including NADH into the electrode matrix has been described for a single use and, consequently, stability was not mentioned [16]. Preliminary studies with the configuration described above and containing an amount of NADH below 20% (w/w graphite) did not show any response for salicylate (data not shown). When higher amounts of NADH, 30 and 90% (w/w graphite), were included into the enzyme graphite mixture, the electrodes responded to 1 mM salicylate injections but a complete loss of response was observed after 30 min and 4 h of operation in the flow system, respectively, (Fig. 3A). As it was expected, the small molecular weight of NADH and its water solubility did not allow a stable configuration. At this point, an additional enzyme was incorporated into the electrode matrix to ensure recycling of NADH. Glucose dehydrogenase was entrapped together with the other enzymes, to ensure recycling of NADH from the NAD^+ produced in reaction (1), according to;

$$\beta\text{-D-Glucose} + NAD^+ \xrightarrow{GDH} \text{D-glucono-1,5-lactone} + NADH \quad (5)$$

A concentration of 10 mM D-glucose was added into the carrier stream and consecutive injections of 1 mM salicylate were made. Results are also included in Fig. 3B where again a complete lack of stability was observed but noticing a remarkable increase in response particularly considering that these electrodes contained 0.3% of NADH (w/w graphite) and no response was ob-

served in the absence of GDH and glucose (vide supra). Similar experiments were made introducing 0.3% of NAD^+ into the electrode instead of NADH and the system also responded to 1 mM salicylate injections confirming the effective recycling of reaction (**5**). The currents obtained with NAD^+ were 50% lower than with NADH (data not shown) but for a strict comparison the K_m and the reaction rates of salicylate hydroxylase and glucose dehydrogenase should be taken into account, which is beyond the scope of this work. Consequently, GDH and NADH were incorporated into the electrode matrix and glucose into the carrier stream. Stability was only improved when a dialysis membrane was inserted into the electrochemical cell in contact with the electrode surface. These electrodes showed operational stability at least for 5 h of continuous 1 mM salicylate injections, proving that the diffusion barrier introduced by the dialysis membrane was sufficiently efficient during this period of time. Fig. 4 presents the calibration obtained with a flow rate of 0.6 ml min^{-1} and 25 μl injections of salicylate. The regression analysis gave the following equation: $y = 0.0160 + 1.6069x$, where $x = \mu M$, $y = nA$ ($n = 8$, $r = 0.994$). The detection limit was 3.50 μM for a $S/N = 3$ and linearity up to 250 μM. Fifteen samples per hour could be determined in the FIA system. Under storage conditions no loss of response was observed, at least, for 2 weeks.

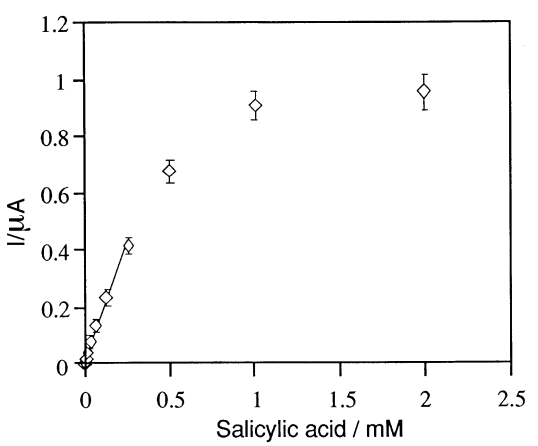

Fig. 4. Calibration curve for salicylic acid in FIA obtained with carbon paste electrodes containing SH, TYR, GDH and NADH and covered with a dialysis membrane. The carrier was 0.1 M phosphate buffer pH 6.0 containing 10 mM of glucose, at a flow rate of 0.6 ml min^{-1}. The applied potential was −100 V vs. Ag/AgCl.

3.3. Selectivity and quantification of salicylic acid in urine and commercial preparations

The biosensor described above did not show any response for 2 mM injections of gentisic and benzoic acid. Interference of acetylsalicylic acid was almost negligible, the response to salicylate being 50-fold higher for equimolar concentrations. Urine samples were directly injected into the FIA system after filtration through 0.45 μm Millipore membranes. Three of seven different samples gave signals which were always below 15 nA and were considered as blank response. After ingestion of an Aspirin® containing 500 mg of acetylsalicylic acid, urine samples of two female volunteers were collected and the average content of salicylic acid found was: 2, 60, and 58 mg l^{-1} corresponding to 15, 30, and 60 min, respectively, after ingestion. Unfortunately, monitoring was not continued after this period but these results indicate the fast and direct metabolism of acetylsalicylic acid extensively reported in the literature [26]. Consequently, and in case that the metabolism curve obtained would not follow the standard levels, the method proposed here could give a rapid evidence of disorders in the metabolism of the patient.

Additionally, five commercial pharmaceutical preparations containing salicylic acid were also analyzed with the biosensor in the FIA system and results are presented in Table 1 together with the concentrations specified by the manufacturers. The concentrations obtained are in perfect agreement with the reported values proving the validity of the salicylate FIA system described.

4. Conclusions

The catalytic and electrochemical detection principle described in this work allow direct quantification of salicylic acid in urine and pharmaceutical samples in a FIA system allowing the analysis of 15 samples per h. The coupling of two enzymatic steps with electrochemical reduction of

Table 1
Quantification of salicylic acid in five drugs in the FIA-biosensor[a]

Drug	Salicylic acid (mg ml $^{-1}$)	
	Manufacturer's specifications	FIA-Biosensor
Depurativo Richelet® (Vita-farma)	1	1.19 ± 0.2
Co-Bucal® (Smaller)	0.2	0.16 ± 0.04
Acnosan® (Bescansa)	1	1.20 ± 0.3
Diprosalic® (Scher-ing-Plough)	20	20.59 ± 1.4
Dermisdin® (Isdin)	20	20.17 ± 1.5

[a] Values specified by manufacturers are also indicated.

enzyme products results in low working potentials with minimum risk of interferences. Additionally, incorporation of a third enzymatic step ensuring regeneration of the cofactor and a dialysis membrane provide a reagentless configuration with the only need of glucose in the carrier.

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