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Salicylic acid determination in cow urine and drugs using a bienzymatic sensor

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Abstract

An enzymatic biosensor was developed for salicylic acid (salicylate ion) determined using a Clark type gas diffusion electrode and two enzymes (tyrosinase and salicylate hydroxylase) entrapped in a cellulose triacetate membrane. After optimization, the method was applied to the determination of salicylic acid in cow urine. Relatively good recoveries were achieved, between about 83% and 109%, using the calibration curve, and acceptable precision (R.S.D. about 8%). The method is now being tested for the determination of salicylic acid contained in commercially available drug specialities or galenic products. So far agreement with nominal values has been found to be between 75% and 110% with a R.S.D. of less than 8%.

Keywords: Bienzymatic electrochemical sensor; Salicylic acid; Cow urine; Drugs; Analysis

1. Introduction

Salicylic acid (and its salicylate anion) is the main metabolite of acetylsalicylic acid [1], that is, of one of the drugs most widely used in the world as a painkiller and anti-inflammatory [2–4], but banned from veterinary therapeutic treatment [5]. Salicylic acid is therefore monitored in the urine and blood of animals to be slaughtered. Several methods of salicylate determination are described in literature. The most frequently used method of clinical analysis is the spectrophotometric "Trinder test" [6] based on the formation of a purple-violet complex between salicylate and Fe(III) ions that can be monitored spectrophotometrically, but it is strongly affected by interference from substances bearing enol and phenol groups [7,8]. For this reason, several other instrumental methods have been developed in the past based on gas chromatography and on HPLC [9–14], spectrofluorimetry [15–17], potentiometry with ion selective electrodes [18–22], voltammetry [23,24] and optical sensors [25,26]; the present authors have also previously developed an ISFET for salicylate [27]; however, in many cases, these methods are very time consuming (e.g. chromatographic methods), as the determination of real matrixes is feasible only after a number of sample pretreatments which are sometimes rather laborious (extraction, preconcentration, etc.). The literature also contains enzymatic methods based on the use of the salicylate hydroxylase enzyme, or the enzyme pair tyrosinase and salicylate hydroxylase [28–40]. In particular, the majority of these methods use a single enzyme, i.e. the salicylate hydroxylase enzyme (SH); in some cases, [36,37] the SH was used coupled with glucose oxidase, not with tyrosinase; lastly, in works [38–40] the tyrosinase and salicylate hydroxylase (SH) enzyme pair were used but using different detection techniques, e.g. spectrophotometric [38], or amperometric [39,40], determining the catechol with carbon paste electrodes in the latter case. In the present research it was proposed to use a dual enzyme (SH and tyrosinase) biosensor method (with only one, or both, enzymes immobilized). However, as an alternative to the above methods, a gas-diffusion amperometric electrode for the oxygen as transducer was used, since, as reported in literature [41], direct catechol detection using the carbon paste electrode and amperometric method is affected by the polymerization of the orthoquinone, which leads to turbidity and the depositing of the polymer on the surface of the electrode. Furthermore, the method we used was found to be of great interest as the use of the salicylate hydroxylase plus tyrosinase enzymatic systems, in the presence of excess NADH, increases method sensitivity, since a cycle of reactions starts during which catechol is produced, then re-oxidized to *o*-quinone; however oxygen is consumed during each cycle, and this results in signal amplification.

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Table 1
Examined pharmaceutical formulations and galenic preparations

Pharmaceutical products	Composition (at 100 ml)	
	Salicylic acid content (g)	Solvents, excipients and other compounds
Pharmaceutical formulation 1	1.0	Water, ethyl alcohol, propylene glycol, fatty acids and polyethylene glycol mixture, flumetasone pivalate 0.02 g
Pharmaceutical formulation 2	1.0	Water, ethyl alcohol, rhubarb glycosidic extract 5.0 g (corresponding to 0.3 g of 1.8 dihydroxyanthraquinone)
Galenic preparation 1	2.0	Ethyl alcohol 96°, water
Galenic preparation 2	1.0	Ethyl alcohol 96°, water, resorcine 1.0 g

2. Experimental

2.1. Instruments

- Electrode mod. 332 Amel srl (Milan, Italy).
- Analogic Recorder mod. 868 Amel srl (Milan, Italy).
- Dissolved Oxygen Meter mod. 360 Amel srl (Milan, Italy).
- Thermostable glass cell with Thermostat Julabo 58, Labospital (Rome, Italy).

2.2. Reagents

- Formic acid, potassium chloride, sodium chloride, phenol, potassium hydrogen phosphate, sodium dihydrogen phosphate, cellulose triacetate, all RPE grade, provided by Carlo Erba (Milan, Italy).
- Tyrosinase from mushrooms 6050 U mg^{-1}, provided by Fluka (Sigma–Aldrich, Milan, Italy).
- Salicylate hydroxylase 24.8 U mg^{-1}, salicylic acid (sodium salt), dialysis membrane, sodium azyde, β-glucuronidase, provided by Sigma–Aldrich (Milan, Italy).

2.3. Real samples analysed

The cow urine samples were supplied from Viterbo (Italy) by a local stockbreeder not practicing intensive breeding. The commercial pharmaceutical formulations and galenic preparations were bought in a public chemist's shop and are reported together with their composition in Table 1.

3. Methods

3.1. Principle of method

The method is based on the following reactions:

$$\text{salicylic acid} + \text{NADH} + \text{O}_2 + 2\text{H}^+ \xrightarrow{\text{salicylate hydroxylase}} \text{catechol} + \text{NAD}^+ + \text{CO}_2 + \text{H}_2\text{O} \quad \text{(a)}$$

$$\text{catechol} + \tfrac{1}{2}\text{O}_2 \xrightarrow{\text{tyrosinase}} o-\text{quinone} + \text{H}_2\text{O} \quad \text{(b)}$$

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

Via reactions (c) and (b) this triggers a cyclic enzymatic reaction which produces catechol (reaction (c)) and re-oxidizes it (reaction (b)). Reaction (c) takes place in the presence of a strong excess of NADH, which makes easier the process of orthoquinone reduction to catechol [39,42]. This increases the method's sensitivity as, for each salicylic acid equivalent present in solution, there are at least two (or more) catechol equivalents, which are then re-oxidized; this consequently increases the consumption of oxygen in solution, that is the species whose variation is measured by means of the Clark type oxygen electrode.

Lastly, when the method is applied to real urine samples containing salicylate it is advisable to have the reactions (a), (b) and (c) preceded by the reaction (a′) of deconjugation of the glucuronide conjugate of salicylic acid, which may be contained in a certain percentage in the urine:

$$\text{salicylic glucuronide} \xrightarrow{\beta-\text{glucuronidase}} \text{salicylic acid} + \text{glucuronic acid} \quad \text{(a}'\text{)}$$

3.2. Electrochemical transducer

A gaseous diffusion amperometric electrode was used as signal transducer to detect the oxygen. The amperometric system has a gold electrode as working electrode, which is separated from the silver reference electrode by insulating epoxy resin; both electrodes dip into an internal aqueous solution, i.e. phosphate buffer (pH 6.6; 0.06 mol l^{-1}) and 0.1 mol l^{-1} KCl, contained in a cylindrical hood, closed at one end with a PTFE permeable gas membrane. The gold electrode was polarized at −800 mV with respect to the anode. The amperometric system included also a temperature probe able to automatically correct any temperature changes.

3.3. Physical entrapment of the enzyme and biosensor assembly

Enzyme entrapment was carried out in a cellulose triacetate (TAC) membrane. The TAC membrane was prepared using a method previously developed in our laboratory [43]: to this end a viscous polymer solution was prepared (4% by weight) by dissolving TAC in a formic acid and water mixture (9:1 v/v).

Once the TAC was solubilized and the viscose obtained, the latter was stratified with a suitable stratifier (0.3 mm) on a glass plate. The stratified viscose was then coagulated with the plate dipping in distilled water, thus obtaining a gel like membrane. The membrane was repeatedly washed with distilled water until the washing water was no longer acid. The membrane obtained was preserved in distilled water containing a small amount of sodium azide until used.

For enzyme entrapment a disc of gelled film of cellulose triacetate was cut and dipped into a phosphate buffer solution

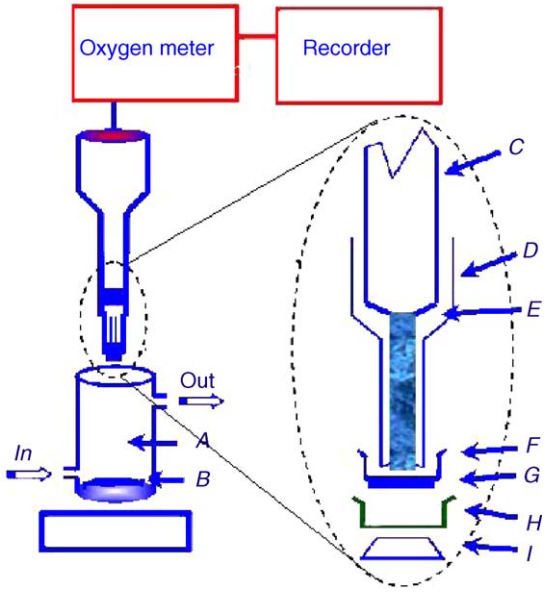

Fig. 1. Measuring system and electrochemical biosensor scheme: (A) thermostated cell; (B) magnetic stirrer; (C) electrode body; (D) electrode cap; (E) internal solution; (F) Teflon membrane (gas permeable); (G) TAC membrane with enzymes; (H) dialysis membrane; (I) O-ring.

of the enzyme or both enzymes (3 mg of enzyme in 50 μl of phosphate buffer solution, pH 7.0; 0.07 mol l^{-1}). The membrane containing the enzyme was subsequently dried at 5 °C for at least 48 h before use, the membrane was washed with buffer solution to remove the enzyme adsorbed on the surface and not entrapped in the membrane. The membrane was then fixed to the head of the O_2 electrode, allowing it to overlap the gas permeable membrane using a dialysis membrane and fixing the whole assembly to the electrode head by means of an O-ring. The biosensor assembly is illustrated in Fig. 1.

3.4. Measures

The analysis were carried out in a thermostated cell at 25 °C, containing 10.0 ml of phosphate buffer solution (pH 7.0; 0.07 mol l^{-1}); before measurements were performed, the biosensor was stabilized under continuous stirring for 20 min. pH 7.0 was chosen as a compromise value, i.e. the pH at which the two different enzymes (SH and PPO) showed a good enzymatic activity.

Two different approaches were tried; in the first, only the PPO was immobilised in the TAC membrane and the SH enzyme, the salicylate standard solutions and the NADH excess were added directly, in that precise order, to the buffer solution contained in the analysis cell. In the second approach, both enzymes were immobilised in the TAC membrane, while salicylate standard solutions and NADH were subsequently added to the solution in the cell.

Measurements were performed by dipping the biosensor (in the two different configurations) into a thermostated cell containing 10.0 ml of phosphate buffer solution (pH 7.0; 0.07 mol l^{-1}), then leaving it to stabilize under magnetic stirring. From this point on the analyses differed according to the type of biosensor used.

When only the tyrosinase (PPO) was immobilised in the TAC membrane, additions were made to the cell in the following order: the salicylate hydroxylase powder (SH) (about 4 mg), 200 μl of standard salicylate solution and a proper volume of 2.0×10^{-2} mol l^{-1} NADH solution. The final salicylate concentration in the cell ranged from 3.5×10^{-6} to 5.0×10^{-3} mol l^{-1} and the final concentration of the NADH solution was varied according to the final salicylate standard concentration so that at least 3:1 ratio of NADH/salicylate was always obtained in the cell solution.

In the second approach, in which both tyrosinase and salicylate hydroxylase were immobilized in the TAC membrane, 200 μl of the salicylate standard solution, then 500 μl of the NADH solution, were added to the buffer solution into which the biosensor was dipped. In this case too, the final concentration of the added NADH was always at least three times higher than the final salicylate solution concentration.

For both approaches the respective calibration curves were constructed using standard salicylate and phenol solutions (prepared daily).

The experimental conditions and some features of the two biosensor types are as follows: analysis temperature, (20–30) °C; type of immobilization, physical (in TAC); signal transducer, oxygen gas diffusion amperometric electrode; pH 7.0; buffer, phosphate 0.07 mol l^{-1}; response time, 80% of the full response after 2 min, in the case of biosensor with PPO immobilised, and 70% of the full response after 2 min, in the case of biosensor with PPO and SH both immobilised in the TAC membrane; life time, about 30 days in all cases.

3.5. Recovery test in real samples

Recovery tests to determine salicylate in cow urine were carried out in the following way: initially the total phenols contained in the cow urine samples were detected using the PPO biosensor; then, using the SH + PPO biosensor, the overall contribution of the phenols and the added salicylate was determined. The added salicylate content was computed as the difference between the biosensor measure of phenols + salicylate and the measure of the phenols only, obtained using the PPO biosensor.

To perform these tests, standard salicylate solutions were added to 500 μl of urine in order to obtain samples of several different final concentrations (between 1.70×10^{-6} and 1.60×10^{-4} mol l^{-1}). After the stabilization of the biosensor signal in the phosphate buffer solution, 1 ml of the sample, i.e. the urine containing the salicylate, was added to the cell in which the biosensor was dipped; lastly, the NADH was added to the cell.

It is important to observe that the method of analysis proposed here did not need any preparation of the cow urine sample, except for filtration by membrane filter (0.45 μm) to remove any microorganisms that could interfere with the measurement.

In the case of urine samples being found positive to salicylate before performing the measurements it was found useful to subject them to a deconjugation process via β-glucuronidase as

Table 2
Analytical data of the method: calibration curve towards phenol or salicylate standard solutions, obtained by two different biosensor approaches

Immobilized enzyme	Tested substance	Calibration curve and correlation coefficient ($y = \Delta$ ppm O_2, $x = \text{mol l}^{-1}$)	Linearity range (mol l^{-1})	R.S.D.% ($n = 5$)
Tyrosinase (PPO)	Phenol	$y = (29.7 \pm 0.5) \times 10^{-3}x - (0.048 \pm 0.021)$; $r^2 = 0.9946$	2.0×10^{-6} to 1.0×10^{-4}	≤6
	Salicylic acid	$y = (54.9 \pm 2.1) \times 10^{-2}x - (0.01 \pm 0.01)$; $r^2 = 0.9917$	3.6×10^{-6} to 1.0×10^{-4}	≤7
Salicylate hydroxylase + tyrosinase (SH + PPO)	Phenol	$y = (29.3 \pm 0.5) \times 10^{-3}x - (0.06 \pm 0.03)$; $r^2 = 0.9983$	2.0×10^{-6} to 1.5×10^{-4}	≤6
	Salicylic acid	$y = (97.2 \pm 3.2) \times 10^{-2}x + (0.014 \pm 0.014)$; $r^2 = 0.9934$	4.0×10^{-6} to 1.0×10^{-4}	≤6

the salicylate is partly secreted into the urine in the glucuronide form [1]. The deconjugation pretreatment usually proved useful in obtaining all the salicylate present in non-conjugated form before quantitative analysis.

4. Results and discussion

4.1. Biosensor analytical characterization

Biosensor analytical performance was tested, first in the configuration with the tyrosinase immobilised and the salicylate hydroxylase free in solution and then with both enzymes (tyrosinase + salicylate hydroxylase) immobilized in the TAC membrane. The respective calibration curves were then constructed.

Calibration curve data for phenols and salicylate standard solutions in the two different proposed approaches are reported in Table 2. The correlation coefficient (r^2) was always satisfactory for all the identified calibration curves (always higher than 0.99). The linear range was usually between 10^{-6} and 10^{-4} mol l^{-1} and the detection limit about 0.5×10^{-6} mol l^{-1}

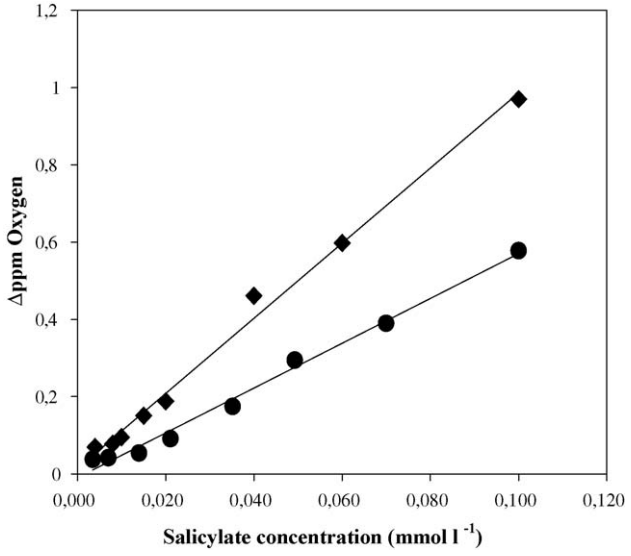

Fig. 2. Comparison between two different biosensor approaches: (●) calibration curve of biosensor with only PPO immobilized; (♦) calibration curve of biosensor with both PPO and SH immobilised.

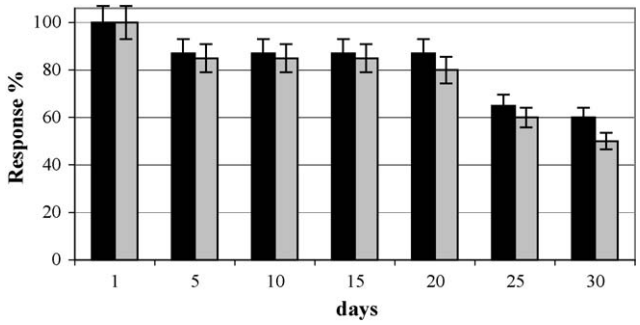

Fig. 3. Comparison of biosensor life times (in the two different configurations). The biosensor PPO data are shown in black, while those of the SH + PPO biosensor are shown in grey. Percentage biosensor response is represented by setting the experimental signal on day 1 to 100%.

also for measures in real samples (urine), i.e. five to seven times lower than the LOD of biosensor described in literature, which was applied early [39] to the analysis of the same matrices (urine and pharmaceutical preparations).

The precision of the method (in terms of repeatability using standard solutions during the entire biosensor life time) was expressed in terms of relative standard deviation (R.S.D.%), the value of which was always lower than 7%, so the precision was satisfactory for both considered approaches.

In Fig. 2 trends obtained for the two different calibration curves for the salicylate are compared. The calibration sensitivity of the biosensor is twice as high if the two enzymes are immobilized together.

Biosensor lifetime, in the two different configurations, was tested by measuring their daily response respectively towards a final phenol concentration 1.50×10^{-5} mol l^{-1}, in the case of the PPO biosensor, and towards a final salicylate concentration 1.50×10^{-5} mol l^{-1}, in the case of the SH + PPO biosensor.

Table 3
Biosensor responses towards interfering substances

Interfering substances	Final concentration of tested solution (mol l^{-1})	% Signal[a]
Benzoic acid	0.001	15%
p-Hydroxybenzoic acid	0.001	n.d
m-Hydroxybenzoic acid	0.001	n.d.

[a] Percentage experimental response by setting the biosensor experimental signal towards salicylate solution at the same final concentration to 100%.

Table 4
Salicylate determination in real urine samples

Cow urine samples	Initial salicylate concentration (mol l^{-1})	Added salicylate concentration (mol l^{-1})	Experimental salicylate concentration (mol l^{-1})	R.S.D. % ($n=6$)	Recovery %
1	0.00	1.70×10^{-6}	1.86×10^{-6}	7.5	109.0
2	0.00	1.60×10^{-5}	1.39×10^{-5}	8.6	87.0
3	0.00	1.60×10^{-4}	1.33×10^{-4}	8.3	82.7

The test showed that the highest values of the biosensor response were recorded during the first 5 days. The experimental signal decreased lightly between the first and the fifth day and then remained stable at about 87% of the starting value until the twentieth day; after this it again decreased in a more pronounced way. At day 30 however, the experimental signal was still about half that recorded on day 1. The trend of the answers of the two biosensors during their life time is shown in Fig. 3.

Bienzymatic biosensor responses were also evaluated as a function of several possible interfering substances (Table 3), such as benzoic acid, *p*-hydroxybenzoic and *m*-hydroxybenzoic acids. In addition it was verified that the presence of ethyl alcohol negatively affected the biosensor response to a significant degree.

4.2. Salicylate determination in real samples (cow urine)

Once the method was optimized, it was applied to salicylate determination in the real matrixes. To analyse the cow urine samples, the authors chose the PPO + SH biosensor because, by comparing the experimental data with the two biosensor approaches (Table 2), better calibration sensitivity and a better precision (see R.S.D.%) were obtained when the two enzymes were immobilized together in TAC membrane.

To validate the method, recovery tests were carried out. To this end, fixed salicylate standard solutions were added to cow urine samples. The urine samples were obviously negative for salicylate before the addition, as demonstrated by chromatographic analysis carried out at the Veterinary Medicine Laboratory (now "Dipartimento della Sanità Veterinaria and Alimentare") of the "Istituto Superiore di Sanità" in Rome.

The absence of salicylate in the bovine urines examined was also demonstrated by the authors by means of biosensor analysis, using the biosensor described.

In Table 4 the results of quantitative recovery test are shown for cow urine samples using the calibration curve (a) reported in Table 2. The recoveries, reported in Table 4, are always higher than about 80% and lower than 109%, while R.S.D.% is always of the order of 8% or lower.

Lastly, salicylate concentration was determined in two galenic preparations and two commercial pharmaceutical formulations by the enzymatic biosensor. Pharmaceutical formulation analysis was made directly on the drug (0.20 ml of sample) by analysing the preparation with the same standard procedures as described in the Section 3 for the standard salicylate solutions and using the above calibration curve to check the concentration of the salicylate it contained. Conversely, in the case of the two galenic preparations, in practice an ethyl alcohol solution of salicylate, measured volumes of the two solutions were first dried by rotavapor and then the residue re-dissolved in water and analysed by the same procedure as described in the Section 3.

Table 5
Salicylate determination in pharmaceutical products

Pharmaceutical products	Nominal value of salicylate g/100 ml (a)	Experimental value of salicylate g/100 ml (b)	R.S.D.% ($n=6$)	$\Delta\% = [(b-a)/a]\%$
Pharmaceutical formulation 1	1	0.70	8	−25
Pharmaceutical formulation 2	1	0.80	8	−20
Galenic preparation 1	2	2.20	8	+10
Galenic preparation 2	1	1.05	7	+5

However, galenic preparation No. 2 contained the same resorcine concentration of salicylate, so it was necessary to carry out the quantitative analysis as already described for the urine samples, i.e. first determining the resorcine concentration using the PPO biosensor, then obtaining the total (resorcine + salicylate) using the SH + PPO biosensor. The salicylate concentration was computed as the difference between the two concentrations found.

In Table 5, the experimental values were compared with the respective nominal values supplied by the manufacturers.

5. Conclusions

In the presence of excess NADH, the (SH + PPO) biosensor gave an excellent response towards the salicylate and using this device it was possible to obtain some good measures of the salicylate and phenol contained in the cow urine.

On the other hand, the biosensor using only immobilised PPO was also necessary for this kind of application because it allowed phenol content to be measured; this must be subtracted from the total phenols + salicylate content obtained using the SH + PPO biosensor. In the case of galenic solutions, after pre-treatment consisting of simple alcohol elimination and if necessary using also the PPO biosensor in case the sample also contained phenols, it was possible to obtain good results (see Table 5). For instance, in the case of galenic preparation No. 2 containing resorcine, $\Delta\%$ was 5 and R.S.D.% was always 7 or lower.

Finally in the case of the two commercial pharmaceutical formulations, the values obtained were always 20–25% lower than the nominal values. The authors believe that this difference is due to the presence of ethanol and glycols (in percentages

not specified by the manufacturer) which considerably lower the biosensor response. In these cases, also alcohol elimination could be tried, but considering the complexity of the pharmaceutical preparation, the operation would in any case entail a certain risk for the integrity of the whole formulation to be analyzed.

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