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Determination of inorganic phosphate in drug formulations and biological fluids using a plant tissue electrode*

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Abstract: A suitable biosensor for the analysis of inorganic phosphate was developed by coupling the enzyme glucose oxidase to a slice of potato (*Solanum tuberosum*) containing the acid phosphatase, and using an amperometric Clark electrode as an indicating sensor. Phosphate concentration can be determined from its inhibition on the acid phosphatase activity. Measurements were carried out at steady-state conditions and the available concentration range of phosphate, repeatability and accuracy, on phosphate standard solutions, were experimentally determined. Finally the sensor was used in pharmaceutical and biological matrices. The results were compared with those ones obtained by Bartlett's spectrometric method.

Keywords: *Phosphate; analysis; tissue-enzyme hybrid biosensor; drugs; biological fluids.*

Introduction

The determination of inorganic phosphate content is frequently necessary for a great number of biological matrices of biomedical and environmental interest. Previously we have investigated several methods, particularly by spectrometric, or potentiometric techniques [1, 2]. Recently an interesting method was proposed by F. Schubert *et al.* [3], using a biosensor, to measure inorganic phosphate in fertilizer and urine samples. With the aim of testing this method for phosphate determination in pharmaceutical commercial samples and in biological fluids such as urine and milk, we have developed a plant tissue hybrid amperometric sensor by a simplified procedure. Analytical characterization of the sensor, both in standard phosphate solutions and in the biopharmaceutical matrices mentioned above is now reported. A comparison, with Bartlett's spectrometric method [4], was also carried out. Results of this study are summarized in this paper.

Measurements are based on the inhibition, by phosphate, of the acid phosphatase (AF) activity contained in a thin slice of *Solanum tuberosum* and catalysing glucose-6-phosphate (G-6-P) hydrolysis. A second enzyme, the glucose oxidase (GOD), catalyses the oxidation of the glucose, produced from hydrolysis of G-6-P to gluconolactone, with oxygen consumption. The presence of phosphate, causes the inhibition of the G-6-P hydrolysis, lowers the glucose concentration and increases the oxygen concentration in the buffer solution. The phosphate concentration is evaluated, by means of a calibration graph, using KH_2PO_4 aqueous standard solutions. The biosensor consists of a Clark commercial oxygen electrode, superimposed with two enzymes (GOD and AF), both immobilized in the potato slice tissue.

Experimental

Sensor assembly

A thin slice of potato tissue (≤0.1 mm thick), was dipped in an aqueous GOD solution (60 U ml^{-1}), left in the solution, at 4°C, for 24 h and then cut into discs of about 0.6–0.8 cm dia. This slice and a superimposed dialysis membrane, were both secured to the Clark electrode body by means of a rubber o-ring. The sensor was stored at 4°C, when not used. Its lifetime is generally longer than 2 weeks, with a decrease in sensitivity of about 30% after 15 days from its assembly.

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Measurements and apparatus

Amperometric measurements were carried out by a commercial oxygen Orion electrode coupled with an Orion Research Ionalyzer 901 and an Amel model 686 recorder, under magnetic stirring, in a 10-ml glass cell, thermostatted at 25°C by forced water circulation. The buffer used was a 0.1 mol l^{-1} sodium citrate–citric acid aqueous solution, at pH 6.0. To obtain the calibration graph, the sensor was dipped into 10 ml of the buffer solution, containing G-6-P at a concentration of 4.6×10^{-3} mol l^{-1}. After stabilization of the current, increasing volumes of the phosphate solution were added with stirring and 5 min after each addition, the relative increase of the current proportional to the increase in the oxygen concentration was measured.

The samples (commercial pharmaceutical preparations, human urine and bovine milk) were analysed in the same way, by adding the sample, appropriately diluted if necessary, to the buffer solutions above containing G-6-P, and placed in the thermostatted cell.

Bartlett's spectrometric method [4] was used as previously described and optimized [1]. Briefly, samples to be analysed were strongly acidified with perchloric acid and heated (mineralization pretreatment), then total phosphorus was determined as inorganic phosphate using Fiske–Subarrow reagent [5], reading the absorbance at 820 nm against the blank reagents. Spectrometric measurements were performed with a Perkin–Elmer Lambda 15 UV–vis spectrophotometer and 1.0 cm path-length silica cell.

Drugs and biological samples

The analysed drugs were submitted to different pretreatments, depending on the commercially available form. For drugs as ampoules, five ampoules were opened and their content well homogenized by mixing in a small beaker. For solid drugs, as tablets, five of these were finely powdered in a mortar. An amount of the drug (solid or liquid), was weighed in order to obtain 50 ml of solution at a concentration in phosphate of about 10–30 mmol l^{-1} and dissolved in citrate buffer. If some turbidity was observed, the samples were centrifuged and filtered before the analysis. However, in the case of the pharmaceutical preparations reported in Table 2, with a particularly complex matrix or containing small concentration of phospholipids in hypotalamic liposomes, samples were analysed, both by the simple procedure described previously and after the acidification procedure described for the spectrometric analysis. Biological samples (urine and milk) were analysed as such.

Reagents

GOD (EC N.1.1.3.4) from *Aspergillus niger*, G-6-P and Fiske-Subbarow reagent [5], containing 1-amino-2-naphthol-4-sulphonic acid, for Bartlett's method [4], were supplied by Sigma Chemical Co (St Louis, MO, USA). Ammonium molybdate, potassium dihydrogen phosphate, citric acid and sodium citrate, for the buffer solution, perchloric acid and other chemicals, of the highest available purity, from Carlo Erba (Milano, Italy). Potatoes (*S. tuberosum*) were purchased from a local vegetable shop. Fosfomycin calcium, or sodium salt was supplied by Crinos S.p.A. (Como, Italy).

Results

The working conditions (temperature analysis, pH, buffer), the features of the tissue plant sensor (response time, lifetime) and the analytical results of the method (calibration graph equation, linearity range, lower detection limit, repeatability and accuracy of the measurements) applied to the standard phosphate solutions, are shown in Table 1. The measurements on authentic samples were always compared with the spectrometric procedure, with the aim of checking the new method. The results obtained using the tissue plant sensor for the analysis of commercial pharmaceutical preparations containing phosphate, or fosfomycin, are respectively summarized in Tables 2 and 3, and compared with those using Bartlett's spectrometric method. The repeatability of the measurements was evaluated (a) on standard aqueous solutions of sodium or calcium fosfomycin, (b) on commercial pharmaceutical preparations (containing sodium, or calcium fosfomycin) and (c) on pharmaceutical forms containing inorganic phosphate with and without acidification pretreatment. The results are reported in Table 4, where precision data, expressed in terms of RSD(%), are compared with those obtained with the same samples, by Bartlett's spectrometric method. Accuracy was determined by adding, respectively, known amounts of fosfomycin, or of inorganic phosphate, to the same pharmaceutical preparation containing

Table 1
(A) Analytical characterization of the plant tissue sensor in standard phosphate solutions

Temperature analysis:	25°C
pH:	6.0
Buffer:	Sodium citrate–citric acid 0.1 mol l^{-1}
Response time:	<5 min
Lifetime:	(10–16) days
Equation of the calibration graph:	$Y = 136X + 0.28$ ($Y = \Delta$ppm O_2; X = phosphate concentration in mol l^{-1})
Linearity range:	8.6×10^{-5}–6.9×10^{-3} mol l^{-1}
Correlation coefficient:	0.9987
Lower detection limit:	6.2×10^{-5} mol l^{-1}

(B) Repeatability and accuracy of the measurements on standard phosphate solutions by the tissue sensor

Repeatability of measurements, values in mmol l^{-1}

Sample No.	Nominal values	Found values a	b	c	d	Mean value	RSD (%)	Pooled SD (%)
1	0.51	0.49	0.51	0.50	0.50	0.50	1.6	
2	1.00	1.02	1.04	1.00	1.02	1.02	1.6	1.7
3	1.93	1.94	1.90	1.97	1.95	1.94	1.5	

Accuracy of the measurements, values in mmol l^{-1}

Sample No.	Nominal value (a)	Found by direct method (b)	$\frac{b-a}{a}$ (%)	Found by standard addition method (c)	$\frac{c-a}{a}$ (%)
1	0.50	0.50	0	0.50	0
2	0.99	1.02	+3.0	1.00	+1.0
3	1.98	1.94	−2.0	1.93	−2.5

Table 2
(A) Phosphate determination in commercial pharmaceutical preparations, containing phosphate. Comparison of results, obtained by the tissue sensor and by Bartlett's spectrometric method. Values are the mean of four determinations. Samples were appropriately diluted before analysis

Drug No. and their pharmaceutical form	Nominal value of inorganic phosphate (a)	Amperometric method (b)	Spectrometric method (c)	$\frac{b-a}{a}$ (%)	$\frac{c-a}{a}$ (%)	$\frac{c-b}{b}$ (%)
Without acidification pretreatment						
Ampoules*	16.2	16.7 (RSD% = 2.3)	17.6 (RSD% = 1.8)	+3.0	+8.6	+5.4
Tablets†	39.6	37.9 (RSD% = 3.1)	40.4 (RSD% = 2.5)	−4.3	+2.0	+6.6
With acidification pretreatment						
Ampoules*	22.0	22.8 (RSD% = 2.8)	23.4 (RSD% = 2.0)	+3.6	+6.3	+2.6
Tablets†	39.6	38.7 (RSD% = 2.4)	41.0 (RSD% = 2.8)	−2.3	+3.5	+5.9

*Values in mmol l^{-1}.
†Values as % by weight.

(B) Recovery of phosphate by the standard addition method, in pharmaceutical preparations, by the tissue sensor. Values (mmol l^{-1}) are the mean of four determinations. Samples were appropriately diluted before analysis

Drug No. and their pharmaceutical form	Phosphate found in the sample	Phosphate added	Total (nominal value) (a)	Total (found value) (b)	$\frac{b-a}{a}$ (%)
Ampoules	16.7	10.0	26.7	27.4 (RSD% = 2.8)	+2.6
Tablets	12.0	10.0	22.0	22.8 (RSD% = 3.2)	+3.6

Table 3
(A) Phosphate determination in commercial pharmaceutical preparation containing fosfomycin. Comparison between spectrometric and amperometric (by the tissue sensor) method and accuracy to respect the nominal value. Values, as % by weight, are the mean of four determinations. Samples were appropriately diluted before analysis

Drugs No.	Nominal value (a)	Found value by enzyme sensor (b)*	RSD, $n = 4$ (%)	Found value by spectrometric method (c)†	RSD, $n = 4$ (%)	$\frac{b - a}{a}$ (%)	$\frac{c - a}{a}$ (%)	$\frac{c - b}{b}$ (%)
1A	86.3	84.6	2.8	90.8	3.1	−1.9	+5.2	+7.3
2A	52.6	49.7	4.7	51.1	2.7	−5.5	−2.9	+2.8
1B	90.8	93.2	3.0	98.7	2.8	+2.6	+8.8	+6.0
2B	15.6	16.4	5.7	16.4	4.5	+5.1	+5.1	0
1C	43.7	46.9	2.4	46.9	1.9	+6.8	+6.8	0

1A — powder containing sodium fosfomycin.
2A — tablets containing sodium fosfomycin.
1B — powder containing calcium fosfomycin.
2B — tablets containing calcium fosfomycin.
1C — powder containing fosfomycin associated with trometamol.
* Without acidification pretreatment.
† With acidification pretreatment.

(B) Recovery, by the standard addition method, of sodium and calcium fosfomycin, in commercial pharmaceutical preparations, containing the same antibiotics, by the tissue sensor. Values (mmol l^{-1}) are the mean of four determinations. Samples were appropriately diluted before analysis

Drugs No.	Found value	Standard added	Total (nominal value) (a)	Total (found value) (b)	$\frac{b - a}{a}$ (%)
1A	11.7	9.6	21.3	22.4 (RSD% = 2.8)	+5.2
1B	12.8	9.6	22.4	23.3 (RSD% = 3.2)	+4.0

A — powder containing sodium fosfomycin.
B — powder containing calcium fosfomycin.

sodium or calcium fosfomycin, or inorganic phosphate and determining the recovery data by the tissue plant sensor analysis. The results are shown in Tables 2 and 3. Finally, in Table 5, repeatability and accuracy data of measurements on biological fluids (human urine and bovine milk), are reported. A comparison with the spectrometric method is also shown.

Discussion

Several authors have attempted to construct biosensors for phosphate [3, 6, 7]. However, it is difficult to design an enzyme sensor based on the reaction catalysed with the acid- (or alkaline-) phosphatase only because there is no electrochemical difference between the substrate and products [7]. However it was possible to use a dual enzyme system for the electroanalytical measurement (i.e. the acid- or alkaline-phosphate and the GOD). When the glucose formed from G-6-P is sensed electrochemically, by the GOD and oxygen electrode system, it is also possible to follow the reaction, catalysed by phosphatase enzyme. Phosphate inhibition lowers glucose formation and the response to the dual enzyme electrode. This approach to the problem was studied by Guilbault *et al.* [7], by using alkaline phosphatase and GOD enzymes immobilized on the Clark sensor by a cross-linking method. Linders *et al.* [6] made a hybrid sensor with a potato tissue layer, containing acid phosphatase and superimposed an enzyme polymeric membrane, containing the immobilized GOD. This hybrid biological mediator system was coupled to an H_2O_2 sensor. Schubert *et al.* [3] made a similar system coupled to an oxygen sensor. Our approach is the same as Schubert *et al.* [3], but we have simplified the assembly of the biosensor. The GOD enzyme is allowed to diffuse into the potato slice which is then secured to a commercial oxygen sensor by means of a dialysis membrane and a rubber O-ring.

The new sensor was easier to handle and had a long lifetime, with good reproducibility and sensitivity, both in standard phosphate sol-

Table 4
Repeatability of the measurements of phosphate in calcium or sodium fosfomycin standard solution and in pharmaceutical preparations, containing the same antibiotics, or inorganic phosphate and organic phosphorus. Comparison between spectrometric and amperometric (by enzyme sensor) method. Values in mmol l^{-1}. Samples were appropriately diluted before analysis

Nominal value	Amperometric method	Mean value	RSD (%)	Spectrometric method	Mean value	RSD (%)
(a) Sodium fosfomycin in standard solutions						
42.3	41.2			44.0		
42.3	41.4	40.9	2.0	43.7	43.9	0.9
42.3	39.7			43.5		
42.3	41.3			44.4		
(b) Calcium fosfomycin in standard solutions						
24.5	23.0			25.3		
24.5	23.6	23.4	2.4	25.3	25.3	0.6
24.5	22.9			25.1		
24.5	24.1			25.5		
(c) Sodium fosfomycin in commercial pharmaceutical preparations						
7.2	6.3			7.0		
7.2	6.8	6.8	5.5	7.3	7.0	3.1
7.2	7.2			6.9		
7.2	6.9			6.8		
(d) Calcium fosfomycin in commercial pharmaceutical preparations						
4.0	4.0			4.1		
4.0	3.9	4.2	6.6	3.9	4.2	5.3
4.0	4.3			4.3		
4.0	4.5			4.4		
(e) Inorganic phosphate in commercial pharmaceutical preparations (without mineralization)						
16.2	17.2			18.0		
16.2	16.7	16.7	2.3	17.2	17.6	3.7
16.2	16.3			16.9		
16.2	16.5			18.3		
(f) Total phosphorus (as phosphate) in commercial pharmaceutical preparations (with mineralization)						
22.0	22.8			23.7		
22.0	23.1	22.8	0.9	23.9	23.4	3.2
22.0	22.7			22.3		
22.0	22.6			23.7		

Table 5
(A) Phosphate determination in human urine and bovine milk samples. Comparison of results obtained by the tissue sensor and by Bartlett's spectrometric method. Values (mmol l^{-1}) are the mean of four determinations. Samples were appropriately diluted before analysis

Sample	Amperometric method (a)	RSD, $n = 4$ (%)	Spectrometric method (b)	RSD, $n = 4$ (%)	$\frac{b-a}{a}$ (%)
Human urine (sample 1)	25.9	3.0	28.0	2.5	+8.1
Human urine (sample 2)	12.2	2.9	11.3	2.6	−7.4
Bovine milk	25.0	3.2	24.2	1.9	−3.2

(B) Recovery of phosphate, by the standard addition method, in human urine and bovine milk samples, by the tissue sensor. Values (mmol l^{-1}) are the mean of four determinations. Samples were appropriately diluted before analysis

Sample	Phosphate found in the sample	Phosphate added	Total (nominal value) (a)	Total (found value) (b)	$\frac{b-a}{a}$ (%)
Human urine (sample 1)	25.9	10.0	35.9	34.1 (RSD% = 3.5)	−5.0
Human urine (sample 2)	12.2	10.0	22.2	23.8 (RSD% = 2.9)	+7.2
Bovine milk	25.0	9.6	34.6	35.6 (RSD% = 2.3)	+2.9

utions (Table 1) and in human urine and bovine milk (Table 5), or different commercial pharmaceutical preparations (Tables 2–4). The analysis of the latter samples not investigated previously by the new sensor is of considerable interest. The repeatability of the measurements (Table 4), as well as the accuracy (as recovery data, by the standard addition method), shown in Tables 2 and 3, are undoubtedly satisfactory and the comparison with the data obtained by analysing the same pharmaceutical samples with the classical spectrometric Bartlett's method, is generally good. Also the correlation with the nominal values of the pharmaceutical preparation is acceptable, or good, especially after the acidification pretreatment [1] of the sample (Table 2). It should be noted that using the tissue sensor, it was possible to analyse the phosphate content of the fosfomycin antibiotic without any acidification pretreatment of the samples (Table 3), unlike Bartlett's method.

Finally the working conditions we used, shown in Table 1 and reported in the experimental section, are practically the same, as reported by Schubert *et al.* [3] as the best conditions. No evidence of interference by fluoride [3] was noted because it was not contained in the examined pharmaceutical dosage forms. Influence by glucose in the urine and milk matrices did not produce difficulties to the sensor response, as proved by the recovery test reported in Table 5, so it was not necessary to apply the special procedure of analysis suggested by Linders *et al.* [6]. These good results were probably also favoured by using G-6-P concentrations 10 times higher than those used by the above cited authors, with the result that the greater glucose concentration in solution exerted a buffering action toward interference of the glucose present in the biological samples.

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