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Analysis of lecithin in pharmaceutical products and diet integrators using a new biosensor operating directly in non aqueous solvent

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Abstract

One of the first examples of a bienzymatic organic phase enzyme electrode (OPEE) is described. It was obtained using two enzymes (phospholipase D and choline oxidase), both immobilised in kappa-Carrageenan gel and, as electrochemical transducer, an amperometric gas diffusion electrode for oxygen. The response of the biosensor was recorded and its sensitivity, linearity range, response time evaluated. Lastly drugs and diet products containing lecithin were analysed using the new biosensor device working in an organic mixture.

Keywords: Lecithin; Drugs; Analysis; Bienzymatic OPEE

1. Introduction

Pharmaceuticals containing lecithin are administered to patients suffering from different disorders, for instance, in the cases of hypercholesteremia, hypertriglyceridemia and hyperlipoproteinemia, as well as acute and chronic hepatitis and various types of hepatoxicosis, in dislipidemias, in atherosclerosis or as a coadjuvant in the therapy of cerebral metabolic alterations due to neuroendocrine disorders.

On the other hand, in recent years, a large number of diet integrators containing soya lecithin have come on the market in the form of granules and tablets. These integrators are used by a large number of consumers and are acknowledged as having the property of reducing blood cholesterol and of retarding the onset of age-related pathologies, such as atherosclerosis. Lastly, integrators containing large percentages of lecithin are ingested as a dietetic support and in all cases of organic wasting.

There has been a consequent strong increase in the demand for rapid and preferably cheap methods of determining lecithin content in drugs and diet integrators, often as alternatives to the classic (chromatographic and spectrophotometric) methods [1–8].

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In the last few years a significant number of enzymatic sensors have been developed by well-known researchers in the modern biosensor field, as well as by our research group [9]. These sensors can be used to determine numerous species present in drugs or foodstuffs [10–12], including also lecithin [13–19]. In the latter case, the most serious drawback is the low solubility in aqueous solutions of both lecithin and several real matrices containing lecithin.

The aim of the present research was thus to examine the possibility of constructing a bienzymatic sensor for lecithin determination capable of operating in organic solvents, in which lecithin is more readily soluble, as well as to evaluate the possibility of applying it to the analysis of drug matrices that are poorly soluble or insoluble in water.

This new OPEE (organic phase enzyme electrode) [20–23] was used to determine the lecithin content of several pharmaceutical products and diet integrators, operating in a chloroform–hexane–methanol (1 + 1 + 0.02 by volume) mixture.

2. Experimental

2.1. Apparatus

The following apparatus was used: a Metrohm G41 VA-Detector potentiostat, a model 868 Amel recorder connected to a Mitek-MK 5001 digital multimeter, an electrode for oxygen determination supplied by Universal Sensor (New Orleans, USA). The electrode used was of the gaseous diffusion amperometric type (Clark electrode).

Since the original cap of the commercial electrode used was unsuitable for operation in organic solvents (which very quickly corrode it), it was replaced with a similar cap of the same size made of Teflon. Also, the original rubber O-ring used to fix the gas-permeable membrane was also replaced with a small Teflon ring. The O_2 determination electrode and the modified cap are shown in Fig. 1.

The gas-permeable membranes were supplied by Radelkis (Budapest).

The dialysis membrane used was a D-9777 type supplied by Sigma (St. Louis, MO).

The tests were run in a 15 ml thermostatted glass cell provided with a forced circulation water jacket (supplied by Marbaglass, Rome) connected to a Julabo model VC 20B thermostat (Germany).

The solvent mixture used in the tests were kept under constant stirring using a magnetic microstirrer from Velp Scientifica (Italy).

The comparative spectrophotometric tests were performed on a Perkin Elmer Lambda 15 (UV–vis) spectrophotometer provided with 1 cm optical path quartz cuvettes.

2.2. Reagents and materials

Phospholipase D from Streptomyces Chromofuscus (100 U mg^{-1} of solid) was supplied by Boehringer-Mannheim (Milan, Italy); choline oxidase from Alcaligenes Species (500 U mg^{-1} of solid) and L-α-phosphatidylcholine was supplied

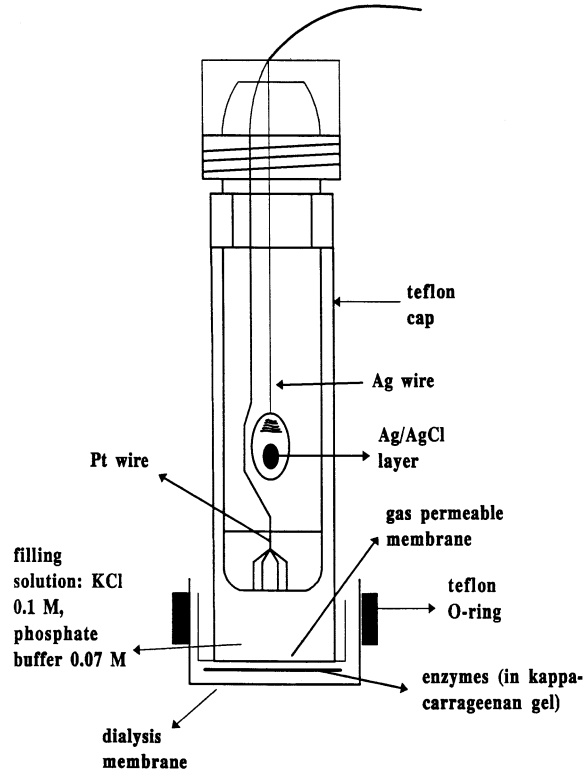

Fig. 1. Biosensor scheme.

by Sigma (Milan); kappa-Carrageenan and glycine was from Fluka (Switzerland); *n*-hexane for RPE analysis, potassium monobasic phosphate and potassium dibasic phosphate were from Carlo Erba (Milan); chloroform (stabilised with amylene) RS for HPLC was supplied by Merck (Germany); resi-analysed methanol was from Baker (Switzerland).

The water content of the organic solvents (anhydrous or saturated with water) used in the present work and expressed as a percentage by weight was determined using a model DL18 Karl Fischer titrator supplied by Mettler (Switzerland). The anhydrous chloroform resulted to contain the 0.030% by weight of water, the anhydrous *n*-hexane the 0.010% by weight, while the water saturated chloroform and *n*-hexane contained 0.090 and 0.014 by weight of water, respectively.

The cat. no. 3220 enzymatic test kit for phospholipid determination was supplied by the Poli diagnostici company (Milan).

3. Methods

3.1. Biosensor

The proposed biosensor (Fig. 1) was obtained using two enzymes, phospholipase D and choline oxidase, both immobilised in kappa-Carrageenan gel as described in the following section and a gas diffusion amperometric electrode for oxygen as electrochemical transducer. Using this biosensor, on the basis of two enzymatic reactions in series:

$$\text{phosphatidylcholine} + H_2O \overset{\text{phospholipase D}}{\rightarrow} \text{choline} + \text{phosphatidic acid}$$

$$\text{choline} + 2O_2 + H_2O \overset{\text{choline oxidase}}{\rightarrow} \text{betaine} + 2H_2O_2$$

it is possible to find a correlation between the substrate (phosphatidylcholine) concentration and the oxygen consumed in the enzymatic reaction catalysed by the choline oxidase and, consequently, with the decrease of the current intensity circulating in the measurement apparatus.

3.2. Enzyme immobilisation in kappa-Carrageenan gel

The enzymatic immobilisation method consisted essentially in immobilisation in kappa-Carrageenan gel, which had proved very effective on previous occasions [24–28].

Briefly, a 2% (w/w) solution of kappa-carrageenan was prepared by dissolving 0.2 g of the polysaccharide in 10 ml of distilled water, gradually heating the solution and maintaining it under constant stirring. The warm solution was placed on a Petri dish and, after cooling, some disks (1 cm diameter) were cut from the obtained gel. After drying at room temperature, the kappa-Carrageenan gel disks can be stored as long as needed. To obtain the enzyme membrane, 25 μl of an enzymatic solution of the two above-cited enzymes was added to a disk, another gel disk was then placed on top of the first and both disks pressed together carefully. Finally, the disks were stored overnight at 5°C. The concentration of the added enzymatic solution (0.1 M glycine buffer, at pH 8.5) used in the arrangement of the enzyme biosensor was about 100 U of phospholipase D and 500 U of choline oxidase in 25 μl.

3.3. Choice of solvent

The tests aimed at developing and optimising biosensor performance were carried out using water saturated chloroform-hexane mixture (50% v/v) as solvent. This choice represented a happy compromise between the need for a satisfactory substrate solubility in the solvent considered and the need to avoid possible negative effects on biosensor response and lifetime, which depend on the nature of the solvent. The latter actually exerts a considerable effect on enzymatic activity [24,25]. We preferred to use a water saturated solvent mixture instead of an anhydrous one in order to obtain a longer biosensor lifetime [26,27]. The above solvent mixture was used for the analysis of standard lecithin solutions, while in the analysis of the lecithin content of real matrices such as dietetic or pharmaceutical products, a small percentage (1% by volume) of methanol had to be added to the chloroform–hexane mixture in

order to achieve a complete solubilisation of both the lecithin and the total drug matrix. The same solvent mixture was used both to solubilise the matrix and as a medium into which the biosensor was dipped for the analysis.

3.4. Spectrophotometric determination of lecithin

For the purpose of comparison a classic spectrophotometric detection enzymatic method was used [5,7,16,17]. The relevant kit is commercially available.

In practice the method entails adding 20.0 μl of sample to 2.0 ml of reagent consisting of Pipes buffer (1,4-Piperazine diethane-sulphonic acid) 75 mM, pH 7.9 containing phenol, 4-amminoantipyrine (4-AAP) and a non ionic detergent in the following concentrations: 7 mM, 0.5 mM and 1 g l^{-1}, respectively, as well as the enzymes phospholipase D, choline oxidase and peroxidase (respectively 2.5, 10 and 2.5 U ml^{-1}). The solution thus obtained was incubated at 37°C for 15 min and then, after cooling to ambient temperature, its absorbance was measured at $\lambda = 500$ nm in cuvettes having a 1 cm optical path. The absorbance of the sample blank, prepared by dissolving the sample in buffer alone together with that of the reagent blank were subtracted from the absorbance recorded for the samples. A phospholipid solution with a concentration of a 3.0 g l^{-1} included in the commercial kit was used as standard. However, because of the strong turbidity of the pharmaceutical solutions as well as of the sample blanks at the time of reading, it was preferred to perform the measures by acting on the first order derivative curve of the original curve.

3.5. Pretreatment of the samples to be analysed

The percentage compositions of all the authentic samples (drugs and diet integrators) analysed are shown in Table 1.

The solid samples (granules and tablets) were ground carefully in a mortar and then dissolved directly in a small volume of solvent mixture, while the capsules were carefully opened using a lancet and their content, consisting of a very dense liquid, was dissolved completely in the same solvent mixture in which the biosensor measure was performed.

In any case the sample solution was then made up to volume in a 25 ml volumetric flask and immediately analysed.

4. Results

We tested the performance of the biosensor when used in chloroform–hexane (50% v/v) mixture, and also in chloroform–hexane (50% v/v) mixture containing methanol (1% by volume). In all cases water saturated solvents were used. The addition of 1% by volume of methanol improved the solubility of the lecithin and the matrix of real samples. Table 2 briefly summarises the main analytical data obtained with the biosensor working in water saturated chloroform–hexane (50% v/v) mixture and water saturated chloroform–hexane (50% v/v) mixture, containing methanol (1% by volume). From these data we can see that the highest sensitivity (expressed as the slope of the calibration graph) is obtained when the water saturated chloroform, which contains methanol (1% by volume), is used as solvent, although the use of this solvent mixture also results in a short biosensor lifetime (maximum 3 days instead of 11 days). The drugs and diet integrators tested (their composition, as supplied by the manufacturers, is listed in Table 1) were analysed immediately after weighing and dissolving in the solvent mixture. As regards the choice of the solvent, several tests were performed on sample number 1, i.e. the capsules, which were opened and their content dissolved respectively in each of the four solvents or solvent mixtures considered and reported in Table 3, then directly analysed in the same solvent. From these tests, water saturated chloroform–hexane (50% v/v) mixture containing methanol (1% by volume) was found to be the best medium, and all the drugs and integrators considered were found to be more soluble in it than in the other mixtures set out in Table 3. Furthermore, as can be seen from the same table, using this solvent mixture ensures closer agreement between experimental and nominal values. It

Table 1
Drugs and diet integrators analysed and composition, as percent by weight, declared by producer firms

Sample No.	Components	% (w/w)
1 (capsules)	**Phosphatidylcholine**	**36.69**
	Mono and diglycerides from sunflower oil	21.62
	Silicon dioxide	1.95
	Ethylvanillin	0.20
	p-Metoxyacetophenon	0.11
	Soya oil	39.43
2 (capsules)	**Phosphatidylcholine**	**31.6**
	Mono and diglycerides from sunflower oil	19.58
	Silicon dioxide	1.77
	DL-α-tocoferol	0.25
	Ethylvanillin	0.28
	p-Metoxyacetophenon	0.15
	Soya oil	46.37
3 (capsules)	**Phosphatidylcholine**	**34.68**
	Mono and diglycerides from sunflower oil	20.44
	Silicon dioxide	1.85
	DL-α-tocoferil acetate	0.69
	DL-α-tocoferol	0.23
	Ethylvanillin	0.22
	p-Metoxyacetophenon	0.12
	Soya oil	41.77
4 or 5 (tablets)	Soya lecithin	97.0
	(**Phosphatidylcholine**, phosphatidylethanolamine, phosphatidylinositol)	(**22.0**, 16.0, 14.0)
	Vitamin E	0.03
	Vitamin B_6	0.002
6 (granules)	Soya lecithin	97.0
	(**Phosphatidylcholine**, linolenic acid, linoleic acid)	(**23.0**, 5.0, 58.0)
	Moisture	1.5

was thus used to analyse all the other samples. However, also using the simpler chloroform–hexane (50% v/v) mixture rather similar results were obtained, although with the advantage of increasing OPEE life-time approximately four-fold. Table 4 shows the precision of the data, i.e. the repeatability of the measurements, obtained in the analyses of some typical drugs and diet integrator samples, using the phospholipase D and choline oxidase biosensor, when operating in the water saturated chloroform–hexane (50% v/v) mixture containing methanol (1% by volume). In Table 5 the lecithin content values obtained using the biosensor are compared with the nominal ones declared by the producer firms and with data obtained using the classical spectrophotometric method [6,16,17]. All reported experimental data are the mean of at least four determinations. It is interesting to note how the correlation between the values obtained using the biosensor and the nominal values is better than that between the latter and the values obtained using the spectrophotometric method (Table 5), in spite of the substantial improvements we made in the latter by introducing first derivative measures.

5. Conclusions

The biosensor developed by us was found to be rapid, sufficiently precise and accurate and able to work directly in non aqueous solvents without the need for any particular derivatisation of the sample which can sometimes lead to errors and inaccuracy in the analysis results.

Table 2
Lifetime and main analytical data, obtained for the phospholipase D and choline oxidase OPEE[a]

Solvent mixture[b] used	Lifetime (days)	Linearity range (mg l^{-1})	Low detection limit (mg l^{-1})	Equation of the calibration graph $y = a + bx$[c]	Correlation coefficient (r)	Precision (RSD%)
Chloroform/hexane (50% v/v)	11	2.1–42.4	1.05	$b = 1.475 \pm 0.004$; $a = 8.4 \pm 0.3$ $(n - v) = 6$; $t = 2.45$	0.9997	6.2
Chloroform/hexane (50% v/v) containing methanol (1% by volume)	3	1.1–66.1	0.55	$b = 8.4 \pm 0.3$; $a = 13 \pm 1$ $(n - v) = 9$; $t = 2.26$	0.9837	7.6

[a] Enzymes are immobilised in kappa-Carrageenan gel and using chloroform–hexane (1+1) by volume mixture, or chloroform–hexane–methanol (1+1+0.02 by volume) mixture, as solvent.

[b] All solvents were saturated of water.

[c] $x = (\text{mg } l^{-1})$ $y = (\text{a.u.})$ and confidence interval $(1 - \alpha) = 0.95$

Table 3
Lecithin content (as % by weight), determined using phospholipase D and choline oxidase OPEE, in the sample no. 1 (capsules)[a]

Solvent	Found lecithin content (as % w/w) RSD% ≤ 6.1 ($n = 4$)
Water saturated chloroform–hexane 50% (v/v) mixture containing 1% by volume of methanol	32.1
Water saturated chloroform–hexane mixture	31.6
Water saturated chloroform containing 1% by volume of methanol	31.8
Water saturated chloroform	31.6

[a] Sample was analysed just after solubilisation in different solvent mixtures. Lecithin nominal value in the drug: 36.7% (w/w).

Table 4
Reproducibility data obtained in the analysis of samples using phospholipase D and choline oxidase OPEE in a water saturated chloroform–hexane–methanol (1+1+0.02 by volume) mixture

Sample no.	Mean value (% w/w)	RSD% ($n = 4$)
1 (capsules)	32.1	3.6
4 (tablets)	21.9	3.3
5 (tablets)	22.1	6.1
6 (granules)	23.8	10.7

The biosensor, which employs the enzyme immobilisation method in a sandwich of two kappa-Carrageenan gel disks, proved to be an efficient probe for phospholipid determination working in organic medium. Using this biosensor we directly determined the lecithin content of some pharmaceutical matrices (drugs) and diet integrators after simple solubilisation in the organic solvent mixture. In all cases, the same solvent as for real sample solubilisation was also used as the medium in which the analyses using the biosensor were successively performed.

The data obtained using the biosensor were generally satisfactory or at least acceptable as regards precision (see the RSD% values in Table 4). On the other hand, the experimental values were found to lie sufficiently close to the nominal ones. It may thus be concluded that the proposed biosensor is a valid analytical tool when hydrophobic lecithin substrate has to be determined in authentic matrices, especially when other analytical methods (e.g. the spectrophotometric method) may be subject to interference and turbidity or take up too much experimental time.

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Table 5
Comparison of: (a) nominal values, (b) data obtained using phospholipase D and choline oxidase OPEE, (c) data obtained by the spectrophotometric method, concerning the lecithin content of different drugs samples, or diet integrators

Sample no.	Nominal value (% w/w) (a)	Value found by OPEE (% w/w) (RSD% ≤ 10.7) (b)	Value found by spectrophotometric method (% w/w) (RSD% ≤ 11.5) (c)	$\frac{b-a}{a}$ %	$\frac{c-a}{a}$ %
1 (capsules)	36.7	32.1	38.8	−12.5	+5.7
2 (capsules)	34.7	31.0	37.0	−10.7	+6.6
3 (capsules)	31.6	30.8	38.7	−2.5	+22.5
4 (tablets)	22.0	21.9	25.0	−0.5	+13.6
5 (tablets)	22.0	22.1	27.0	+0.5	+22.7
6 (granules)	23.0	23.8	21.0	+3.5	−8.7

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