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Analytical comparison of an enzyme-amperometric method for chlorocresol determination in ointments with colorimetry and liquid chromatography*

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Abstract: The direct determination of chlorocresol in *n*-hexane extracts of commercial ointments was successfully performed using an enzyme-amperometric probe for analysis of phenols and working in previously characterized and optimized non-aqueous solvents. The analytical data obtained were compared with those found by using classical HPLC or chemical spectrophotometric method for determination of phenols.

Keywords: *Enzyme sensor; n-hexane solvent; chlorocresol analysis; liquid chromatography; colorimetry; ointments.*

Introduction

Small amounts of chlorocresol (0.1%, w/w) are contained in commercial ointments used for various skin affections. A direct method for chlorocresol determination in these pharmaceutical formulations is not available, as it is very difficult to obtain a complete solubilization of the sample using either aqueous or non-aqueous solvents, so a preliminary extraction of the chlorocresol from the matrix is required. This procedure is difficult to use when performed with aqueous solvents, due to the formation of emulsions, while better results can be obtained using non-aqueous solvents; i.e. good extraction can be achieved by *n*-hexane. Some reports on the use of biosensors for the analysis of phenol, in aqueous solutions have been published [1–3]. Recently a new enzyme sensor for the analysis of phenols that is able to work in *n*-hexane has been tested [4, 5], and this has been used for the direct determination of chlorocresol in the *n*-hexane extracts of ointments. Results were compared with those obtained using a HPLC method and with another chemical spectrophotometric method for phenol determination.

Experimental

Apparatus

For the enzyme-amperometric measurements a gas diffusion oxygen electrode Orion model 97-08 was employed, the original membrane of which was substituted by an oxygen gas permeable membrane from IL cat. 19010 and the O-ring by a suitably constructed one in Teflon; measurements were performed using an Orion Research Ionalyzer model 901, coupled with an Amel model 868 recorder. Temperature was maintained at 25°C by a Julabo VC 20B thermostat. Glass thermostatted cells were furnished by Marbaglass (Rome, Italy).

Spectrophotometric measurements were carried out using a Perkin Elmer Lambda 15 spectrophotometer, HPLC measurements were performed using a Perkin Elmer series 10 liquid chromatograph, coupled with an LC 90 UV spectrophotometric detector and the LCI-100 laboratory computing integrator.

Materials

Tyrosinase (polyphenol oxidase; EC 1.14.18.1 from mushrooms; 3870 U mg) was supplied by Sigma (St Louis, MO, USA) and 4-Cl-3-cresol from Fluka AG (Buchs, Switzerland). Organic solvents for HPLC and all other chemical reagents, of 'analytical grade', were from Farmitalia-Carlo Erba (Milan, Italy), *n*-hexane from Aldrich Chimica s.r.l. (Milan, Italy). The commercial kit with reagents for spectrophotometric test was supplied from Poli Diagnostic (Roma; cat No. 7076).

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Sample pretreatment

The analysed formulations were three pharmaceutical commercial ointments, used for skin affections and containing a small percentage (0.1%, w/w) of chlorocresol and many other substances (see Table 1).

One gram of each ointment was treated four times with 10 ml of *n*-hexane, under stirring.

Table 1
Pharmaceutical formulations composition of the analysed ointments

Ointment 1	
Chlorocresol	0.100%
Clobetasone	0.05%
Glycerylmonostearate	9.60%
Cetylstearyl alcohol	4.80%
White wax	2.00%
Autoemulsifying glyceryl monostearate	2.00%
Dimethicone	1.00%
Glycerol	25.00%
Sodium citrate	0.05%
Citric acid	0.05%
Purified water	55.35%
Ointment 2	
Chlorocresol	0.100%
Betamethasone valerate	0.12%
Polyoxyethylene cetylstearic ether	1.80%
Cetylstearyl alcohol	7.20%
White vaseline	15.00%
Liquid paraffin	6.00%
Sodium phosphate monobasic	0.26%
Sodium hydroxide	0.0006%
Purified water	69.52%
Ointment 3	
Chlorocresol	0.100%
Gentamicin phosphate	0.16%
Polyethylene glycol monocetylether	1.80%
Cetylstearyl alcohol	7.20%
White vaseline	15.00%
Liquid paraffin	6.00%
Sodium phosphate monobasic	0.30%
Purified water	69.40%

Each *n*-hexane aliquot was analysed to optimize the extraction process. Thereafter, all the *n*-hexane extracts were collected and the resulting solutions analysed. Different dilutions were needed owing to the different calibration ranges of the three methods.

The percentage of chlorocresol determined in each extraction with *n*-hexane, is shown in Fig. 1 for two of the three examined drugs. It can be seen that, for both pharmaceutical preparations considered, after four extractions, 100% of the chlorocresol contained in the ointments was recovered.

Methods

Enzyme-amperometric method

Three milligrams of tyrosinase enzyme were placed over the Teflon gas-permeable membrane of the Clark amperometric oxygen sensor damping with a 0.067 M phosphate buffer (pH 6.5). The enzyme was entrapped over the head of the oxygen sensor with a dialysis membrane by means of a Teflon O-ring.

Measurements were performed by adding to 5 ml of *n*-hexane solution (into which the enzyme sensor dips and which is contained in a thermostatted glass cell), 0.5 ml of *n*-hexane sample extract, or of standard solution of chlorocresol in *n*-hexane, under stirring and recording the current variations related to the oxygen consumption in solution (as Δ ppm O_2 values), caused by the enzyme reaction [5] (Scheme 1).

The unknown concentration value was obtained from direct comparison of the current variation values, relative to the unknown sample measure and the ones, relative to

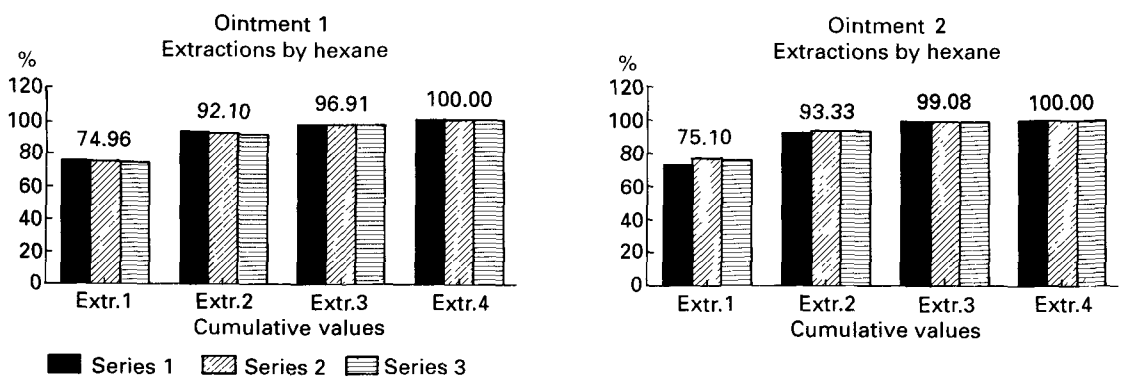
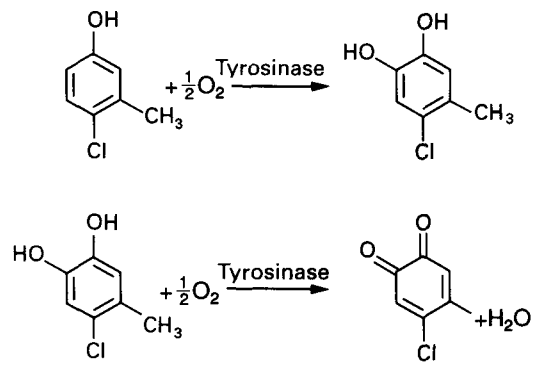


Figure 1
Consecutive ointment extractions by *n*-hexane solutions. Reported values are cumulative values after each extraction.



Scheme 1

chlorocresol standard solution at a similar concentration using the following algorithm:

$$C_s = \frac{C_{st}(\Delta \text{ppm} O_2)_s}{(\Delta \text{ppm} O_2)_{st}} \cdot \frac{V_{st}}{V_s} \cdot \frac{(V_0 + V_s + V_{st})}{(V_0 + V_{st})},$$

where C_s is the concentration of the sample, C_{st} the concentration of the standard solution, (Δ ppm O_2)$_s$ and (Δ ppm O_2)$_{st}$ are the partial pressure variations in solution, respectively, caused by the sample and the standard addition (recorded by means of the oxygen sensor and related to the current variations, evidenced by coupled recorder), V_s and V_{st} are respectively volumes of the sample and the standard added, and finally, V_0 is the initial volume of the *n*-hexane solution.

To test if the response is linear in the experimental concentration range, a calibration curve was performed before the analysis and using the same method, by performing successive chlorocresol standard additions to the *n*-hexane solution.

HPLC method

A (4.6 × 165 mm) Perkin Elmer ODS_2-C_{18} (5 μm) column and a 275 nm fixed wavelength UV detector were employed. Column oven temperature was 25°C, the mobile phase was acetonitrile–water (50:50, v/v), flow rate 1 ml min^{-1} and injection volume 20 μl.

Spectrophotometric method

Spectrophotometric measurements were performed using the 4-aminoantipyrine-ferricyanide method [6], reading the samples absorbance at λ = 460 nm, in a 1 cm path-length quartz cell, against reagents blank.

Results

The working conditions, optimized in a previous paper [5] and the analytical results for a chlorocresol solution in *n*-hexane, obtained in order to characterize the enzyme-amperometric sensor, are summarized in Table 2.

Results obtained in the analysis of ointments containing chlorocresol, concerning accuracy of measurements (as recovery by standard addition method), found using the enzyme sensor, are reported in Table 3.

In Table 4 results of the ointments analysis and precision data, using the enzyme sensor, are compared with those obtained by the HPLC and by the chemical spectrophotometric method and with the nominal values declared by producing firms of the analysed commercial ointments (Table 4).

Discussion

This study was intended to improve the

Table 2
Enzyme-amperometric sensor characterization working in *n*-hexane solution of chlorocresol

Indicating electrode:	Oxygen amperometric sensor
Immobilized enzyme:	Tyrosinase (in 1/15 M phosphate buffer, pH 6.5)
Immobilization method:	In dialysis membrane
Response time:	≤2 min
Lifetime:	≥20 days
Medium:	*n*-Hexane
Working temperature:	25°C
Linearity range to chlorocresol:	50–450 μM
Equation of the calibration graph in the linearity range:	$y = 1.5 \times 10^{-2} x + 0.68$ (x = μM; y = Δ ppm O_2; r = 0.9992)
Minimum detection limit:	20 (μM)
Precision on standard *n*-hexane solution of chlorocresol (as 'pooled standard deviation', %):	3.2
Inaccuracy on standard *n*-hexane solution of chlorocresol (by the standard addition method):	−6.2–+2.7 (% values)

Table 3
Comparison of recovery of chlorocresol in two different commercial ointments, by the enzyme-amperometric sensor, by the spectrophotometric method and by the HPLC method

Method	Ointment No.	Chlorocresol values found in the ointment (% values)	Added chlorocresol (% values)	Total chlorocresol found (% values)	% Recovery of added chlorocresol
Enzyme-amperometric sensor	1	0.100	0.089	0.195	103.2
		0.100	0.178	0.283	101.8
		0.100	0.267	0.373	101.6
Enzyme-amperometric sensor	2	0.102	0.089	0.186	97.4
		0.102	0.180	0.275	97.5
		0.102	0.267	0.368	99.7
Spectrophotometric	1	0.100	0.184	0.270	95.1
		0.100	0.296	0.374	94.4
		0.100	0.446	0.501	91.8
Spectrophotometric	2	0.108	0.033	0.138	97.9
		0.108	0.067	0.165	94.3
		0.108	0.100	0.211	101.4
HPLC	1	0.105	0.026	0.131	100.0
		0.105	0.053	0.159	100.6
		0.105	0.079	0.178	96.7
HPLC	2	0.099	0.026	0.131	104.8
		0.099	0.053	0.163	107.2
		0.099	0.079	0.192	107.9

Table 4
Precision of measurements and comparison between nominal values and values obtained, for chlorocresol concentration, determined on the examined commercial ointments, by the enzyme-amperometric sensor, by the spectrophotometric method, and by HPLC methods. Each value is the mean of at least three determinations. (The RSD% values are given in parentheses)

Ointment No.	Nominal value (% values), *a*	Found by enzyme-amperometric sensor (% values), *b*	Found by spectro-photometric method (% values), *c*	Found by HPLC method (% values), *d*	$(\frac{b-a}{a})\%$	$(\frac{c-a}{a})\%$	$(\frac{d-a}{a})\%$
1	0.100	0.100 (4.5)	0.097 (3.1)	0.105 (5.6)	0	−3.0	+5.0
2	0.100	0.103 (2.0)	0.108 (3.5)	0.099 (3.4)	+3.0	+8.0	−1.0
3	0.100	0.102 (3.5)	0.096 (3.3)	0.106 (2.5)	+2.0	−4.0	+6.0

efficiency of the enzyme-amperometric sensor, working in non-aqueous solution, in the direct analysis of some pharmaceutical formulations (ointments), for which it is very difficult to obtain a complete solubilization of the sample using common aqueous solvents. To this end, as shown in Fig. 1, four consecutive extractions with *n*-hexane, ensure the full separation of chlorocresol from ointments. Results reported in Tables 3 and 4 show that the enzyme sensor is able to directly determine chlorocresol concentration in *n*-hexane extracts, with precision and accuracy little different from those experimentally observed by employing two other classical (HPLC and spectrophotometric) methods, but using very cheap apparatus, which is easy to handle and saves time. Also the correlation of results with nominal values and among the three different methods, is satisfactory, as shown in Table 4. Finally, by comparing the response of the enzyme sensor to *n*-hexane chlorocresol solution with response to *n*-hexane phenol solution, reported in a previous paper (see ref. 5), we can observe that sensitivity (as slope of the calibration curve) is about 10 times lower and minimum detection limit is about one order higher for chlorocresol solutions; while linearity range, response time, lifetime, precision and accuracy are about the same.

Finally, the studied enzyme sensor can be considered of real interest for analytical applications in non-aqueous solutions (e.g. *n*-hexane) whenever the substance to be analysed is more soluble in this solvent compared with aqueous solution.

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