Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 6, pp. 765–770, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Short Communication

The determination of choline in pharmaceutical preparations by means of an enzyme sensor

LUIGI CAMPANELLA,* MAURO TOMASSETTI, MARIA PIA SAMMARTINO and PAOLA STEFANONI

Dipartimento di Chimica, Università di Roma "La Sapienza", P.zza A. Moro 5, Roma, Italy

Keywords: *Choline determination; enzyme sensor; pharmaceutical preparation.*

Introduction

Previous attempts to produce enzymatic sensors for the determination of choline have involved the immobilization of choline oxidase enzyme in polymeric matrices and combining these with an oxygen electrode of the amperometric Clark type. Firstly, the enzyme was chemically immobilized in nylon [1, 2]; subsequently a simpler immobilization technique was developed involving the physical entrapment of the enzyme in a cellulose triacetate membrane [3]. This method proved to be superior to the earlier immobilization techniques insofar as the enzyme is not denatured, the final enzymatic activity is higher and the operational life is longer. The method also was shown to be suitable for the analysis of biomedical matrices [4]. As many pharmaceutical preparations commercially available (injections and syrups), especially those active against anaemies, hypovitaminic states and haepatic diseases, contain low concentrations of choline, an attempt was made to apply the new sensors to the assay. The results obtained are presented here, discussed and compared with those given by a similar enzymatic method, but with spectrophotometric, rather than amperometric, detection.

Experimental

Materials

All the reagents were of analytical grade. Choline oxidase (EC 1.1.3.17. from alcaligenes, 10 U mg^{-1}) was purchased from Sigma (St. Louis, MO, USA); peroxidase (EC 1.11.1.7. from horse-radish) from Boehringer (Mannheim, FRG). Choline chloride, triton X-100, glycine, aminophenazon, phenol and 95% ethanol, from Farmitalia Carlo Erba (Milano, Italy); cellulose triacetate from Fluka (Buchs, Switzerland); and Tris buffer from Merck (Darmstadt, FRG). The standard choline solution was prepared by

*To whom correspondence should be addressed.

dissolving 140 mg of dry choline chloride in 100 ml of distilled water. Choline test solutions were prepared by diluting the standard with 0.1 M glycine buffer at pH 9 containing 1 mg cm^{-3} Triton X-100.

The analysed dosage forms were all commercial pharmaceutical preparations. Their nominal percent choline content and their commercial form (injections or syrup), are indicated in Tables 1 and 2.

Apparatus

The apparatus for the analysis under flow conditions, is as described previously [5, 6]. The oxygen Clark electrode cell, consisting of a silver anode, a platinum cathode, an inner solution of KCl 0.1 M, a Teflon membrane (12.7 μm thick), was produced by Instrumentation Laboratory; as was the thermostatted cell and the apparatus for the measurement under flow conditions, which was fabricated from I.L. model 213 pH Blood analyzer with simple modifications [4]. The temperature was controlled by a Lauda thermostat. The experimental measurements were recorded by an Amel 868 recorder. For the measurements under flow conditions, a Gilson peristaltic pump with Technicon tubing (2 mm, i.d.) was used, ensuring a constant flow rate of 2 ml min^{-1}.

The cellulose triacetate membrane, with the immobilized enzyme, was applied to the Teflon membrane of the commercial Clark electrode and both then fixed to the electrode body by means of an O-ring. The apparatus for the measurements under flow conditions, has been described in detail previously [6].

The spectrophotometric measurements were performed by means of Perkin–Elmer 320 spectrophotometer, with 1.0 cm cuvettes.

Immobilization of the enzyme

The choline oxidase was immobilized by entrapment in cellulose triacetate membrane by a recently developed procedure that has been described previously in detail [3, 4]. The method consists of the diffusion of the enzyme, for about 30 h, from a solution where it was contained at the concentration 100 U ml^{-1}, in 0.1 M glycine buffer at pH 9, into a cellulose triacetate membrane [3] followed by drying of the resulting membrane, for about 30 h at 5°C. This results in a strong contraction of the polymeric membrane, within which the enzyme remains entrapped with no danger of denaturation, as no contact with organic solvents occurs. The membrane, after having been washed, was attached by an O-ring to the gas permeable membrane of the Clark oxygen electrode. The resulting specific activity of the immobilized choline oxidase, measured as previously reported [1, 2], was found to be 41 nmol min^{-1} cm^{-2}.

Assay methods

Enzymatic–amperometric method. The enzymatic–amperometric method involves the use of a choline sensor together with the determination of oxygen consumption, according to the following enzymatic reaction:

$$\text{Choline} + 2O_2 + H_2O \xrightarrow{\textit{choline oxidase}} \text{betaine} + H_2O_2.$$

Enzymatic–spectrophotometric method. The spectrophotometric method is based upon the above reaction, followed by colour formation:

$$H_2O_2 + \text{phenol} + \text{4-aminophenazon} \xrightarrow{\textit{peroxidase}} \text{red dye}.$$

Table 1
Comparison between the values obtained for the choline concentration, determined on the pharmaceutical preparation by the enzymatic–amperometric and enzymatic–spectrophotometric methods. Each value is the mean of at least three determinations. Dosage forms diluted with ethanol

Drug no.	Pharmaceutical form	Nominal value (as % by wt), a	Found value (as % by wt), by enzymatic–amperometric method, b	$(\frac{b-a}{a})\%$	Found value (as % by wt) by enzymatic–spectrophotometric method, c	$(\frac{c-a}{a})\%$	$(\frac{c-b}{b})\%$
1	Syrup	0.125	0.13	+4.0	0.10	−20.0	−23.1
2	Injection	1.25	1.33	−2.4	1.26	+0.8	+3.3
3	Injection	0.06	0.065	+8.3	0.054	−10.0	−16.9
4	Injection	0.06	0.066	+10.0	0.062	+3.3	−6.1
5	Syrup	3.0	3.16	+5.3	2.79	−7.0	−11.7

Table 2
Comparison between the values obtained for the choline concentration, determined on the pharmaceutical preparation by the enzymatic–amperometric and enzymatic–spectrophotometric methods. Each value is the mean of at least three determinations. Dosage forms diluted with water

Drug no.	Pharmaceutical form	Nominal value (as % by wt), a	Found value (as % by wt), by enzymatic–amperometric method, b	$(\frac{b-a}{a})\%$	Found value (as % by wt) by enzymatic–spectrophotometric method, c	$(\frac{c-a}{a})\%$	$(\frac{c-b}{b})\%$
1	Syrup	0.125	0.12	−4.0	0.13	+4.0	+8.3
2	Injection	1.25	1.24	−0.8	1.23	−1.6	−0.8
3	Injection	0.06	0.063	+5.0	0.062	+3.3	−1.6
4	Injection	0.06	0.065	+8.3	0.066	+10.0	+1.5
5	Syrup	3.0	2.98	−0.7	2.79	−7.0	−6.4

The absorbance is measured at 500 nm. Two blanks are also read at the same wavelength and their absorbance values subtracted from that of the sample.

Pretreatment of the analysed pharmaceutical preparation

The commercial dosage forms were pretreated as follows; in the case of injection solutions, five were opened and their contents combined; in the case of syrup, this was well homogenized. Appropriate amounts of the pharmaceutical preparations were weighed, in order to obtain 50–100 ml of an aqueous, or ethanolic solution, containing choline at a concentration of about 10^{-3} M.

In the case of turbidity, samples were centrifuged and filtered before analysis. In the case of the spectrophotometric assay, the solutions were examined directly, whilst in the case of the amperometric detection, they were analysed after dilution with glycine buffer. For the reproducibility and accuracy tests, a pool of dosage forms obtained by mixing together equal volumes of four solutions from different pharmaceutical preparations, was examined.

Choline determination

In order to determine choline by the enzymatic–amperometric method, a calibration curve was constructed, according to the following procedure: choline standard solutions (2.5×10^{-5}–2.0×10^{-4} M) in glycine buffer 0.1 M, pH 9, were allowed to pass through the thermostatted cell at 25°C containing the enzyme electrode. When only buffer was flowing through the cell the value of the current, read on the I.L. model 213, was adjusted to an arbitrary value of 200 units (a.u.). When one of the standard solutions contacted the sensor, a rapid decrease of the current was observed which was recorded once a constant value was obtained. The recorded differences of the current were proportional to the depletion of oxygen in solution and therefore to the choline concentration. The linear calibration plot obtained, over the reported concentration range, was found to fit the equation $y = 521.6\ 10^3x - 0.804$, where x is the concentration of the standard choline solutions and y the current variation (a.u.). The correlation coefficient, r, was found to be 0.9999.

In order to determine the choline concentration in pharmaceutical preparations, 2–4 ml of the ethanolic, or aqueous solutions was prepared as mentioned earlier and diluted to 50 ml with a 0.1 M glycine buffer solution pH 9. The resulting solution, at 25°C, was passed through a thermostatted cell containing the enzyme sensor. Values were measured as previously described for the standard solutions.

In order to determine the choline in dosage forms by the enzymatic–spectrophotometric method, 100 µl of the aqueous, or ethanolic solutions, were obtained and analysed by the procedure described previously [2, 4, 7], with the exception of the subtraction of the absorbance values of the reagent blank test and of the sample blank test, in glycine buffer. This double subtraction was required because with real matrices interferences can come from turbidity and the colour of the sample. The choline concentration was determined by means of a calibration curve [4].

Results

The response of the electrode, to increasing concentrations of choline under flow conditions, is shown in Fig. 1. It can be seen that, the response time is practically constant (2.5 min), being independent of sample concentration. The optimization of the

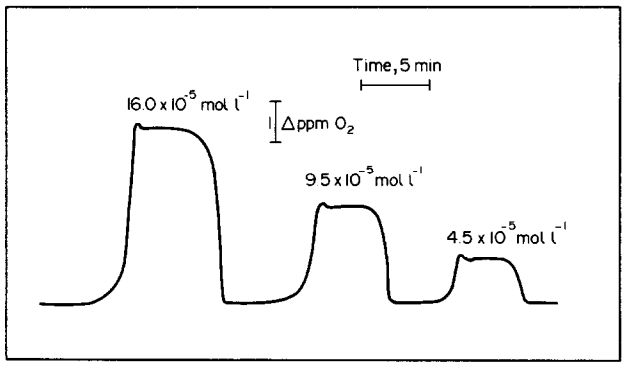

Figure 1
Response of the choline oxidase based electrode, to increasing concentrations of choline.

analytical method was performed by initially solubilizing or diluting the pharmaceutical preparations with ethanol, or distilled water, whichever was the best, whilst the final dilution was always made with glycine buffer. The precision of the determination of choline in dosage forms, after solubilization with water, or ethanol, was experimentally evaluated; the results are summarized in Table 3 and compared with those obtained by spectrophotometric detection. In the same table the estimated accuracy of the method is reported. This estimation was performed by adding increasing amounts of choline to a stock solution obtained by mixing together equal volumes of four different pharmaceutical preparations. The recoveries, obtained by both the detection techniques, are compared in Table 3. Lastly, in Tables 1 and 2, all the values obtained for the examined dosage forms, initially solubilized with water, or ethanol, with both the amperometric and spectrophotometric method, are reported. Thus it is possible to evaluate the correlation between the two methods, based on the same reaction, but using different detection systems, by analysing the percentage differences of the values between the two sets of results. The reported data, in Tables 1 and 2, also yield a comparison between nominal and experimental values of the choline concentration.

Discussion

From the data in Table 3, it is apparent that the precision of the measurements (as RSD%), in the case of the amperometric detection with prior dilution with ethanol, are consistently higher than those given by the spectrophotometric detection with initial dilution with water. Accuracy, by the standard addition method, is practically the same, independent of the mode of detection used. Correlation, between nominal and experimental values, is good in some cases, but only acceptable in some others. Generally, the values given by amperometric detection are more reliable than those given by spectrophotometric detection. Only in two cases (drug nos 3 and 4), is the correlation poor. It can be seen that in both these cases the choline concentration is particularly low, so that precision will be lower, especially in the case of spectrophotometric detection, where the minimum detection limit is at a concentration ten times higher, than in the case of amperometric detection [2, 4].

Table 3
Comparison of precision and accuracy data of the choline determinations, in pharmaceutical preparations, by enzymatic–amperometric and enzymatic–spectrophotometric methods

	Enzymatic–amperometric method	Enzymatic–spectrophotometric method
Precision (as RSD%) of choline concentration determination in a pool of dosage forms diluted with ethanol	0.6	13.4
Precision (as RSD%) of choline concentration determination in a pool of dosage forms diluted with water	4.4	6.6
Accuracy (as % choline recovery, by standard addition method) in a pool of dosage forms diluted with ethanol	98.5–91.6	100.7–89.9
Accuracy (as % choline recovery, by standard addition method) in a pool of dosage forms diluted with water	104.8–94.6	100.8–97.8

Conclusion

The proposed enzymatic–amperometric method for the determination of choline proved to be simpler, cheaper and more precise than that involving spectrophotometric detection. Accordingly, it represents a useful alternative, especially when the analyte solutions are coloured, or are turbid, or are characterized by very low concentrations of choline. Ethanol is a better solvent for dosage forms, than water; by using it, the measurements are more precise. Furthermore, ethanol exerts a protective action against bacterial attack of the enzyme. Also it is interesting to note that the enzymatic–amperometric method suffers no interference from the other chemical species. Nevertheless some problems can arise if, in the solution to be analysed, some bacterial pollution occurs, with concomitant decrease of the oxygen in solution, due to the breathing function of these bacteria. Finally, enzymatic immobilization in cellulose triacetate membranes is simpler and more suitable than other commonly adopted chemical immobilization methods [5, 6].

Acknowledgement — This work was carried out with the financial support of the Italian Ministry and of Italian National Research Council.

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[*Received for publication 20 March 1989*]