Determination of Phosphorylcholine with an Alkaline Phosphatase Reactor Using Flow Injection System

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Summary: A flow injection spectrophotometric method is developed for the quantitative determination of phosphorylcholine utilizing alkaline phosphatase reactor (immobilized on controlled-pore glass). The measurement is based on the rate of formation of "Molybdenum blue", a heteropoly molybdenum (V) species at 660 nm. The detection limit is 10 μM at a sampling rate of upto 45 hr^{-1}, with a relative standard deviation of lower than 1% (n = 10).

Introduction

Flow-injection methods based on packed-bed reactors with immobilized enzymes are one of the most commonly used analytical tools for the quantitation of various clinically and biochemically important analytes [1-4].

"Alkaline phosphatase" represents a group of relatively non-specific enzymes which hydrolyse a variety of phosphate esters under alkaline conditions [5]. Phosphorylchloine is an important intermediate in the biosynthesis of phosphatidylcholine. The compound is also formed in human body as a result of hydrolysis of lecithin and sphingomyelin by enzymes i.e. lecithinase (PL-C) and sphingomyelinase respectively. Lecithin to sphingomyelin ratio in amniotic fluid is routinely determined in clinical laboratories to assess the fetus lung maturity [6]. Though the determination of phosphorylcholine levels for clinical diagnosis is a very rare test, however, this has attracted much attention as a result of increasing interest in phosphatidylcholine metabolism [7]. The previously published procedures [8] for phosphorylcholine determination involve the use of HPLC which is comparatively slow and the use of sophisticated instrument, generally is not available in most of the laboratories in Pakistan.

In the present work a system based on immobilized enzyme column in a flow-injection system was developed for the rapid determination of phosphorylcholine by using the following reaction scheme:

$$\text{Phosphorylcholine} \xrightarrow[\text{Phosphatase}]{\text{Alkaline}} \text{Choline} + \text{Phosphate}$$

$$\text{Phosphate} + \text{Amm. molybdate} \longrightarrow \text{Amm. phosphomolybdate}$$

$$\text{Amm.phosphomolybdate} \xrightarrow{\text{reducing agent}} \text{Molybdenum blue.}\ \lambda = 660\ \text{nm}.$$

Results and Discussion

Initially the activity of immobilized alkaline phosphatase was examined by inserting the packed reactor in the flow system as shown in Fig. 1. A stream of Tris-HCl buffer (0.1 M, pH 8.0) along with ammonium molybdate and reducing agent were made to flow through the manifold.

Preliminary experiments were carried out to find the optimized pH for the activity of the immobilized enzyme. Phosphorylcholine solution 1 x 10^{-3}M was prepared in 0.1 M Tris-HCl buffer of varying pH values ranging from 8.0 - 9.5. The peak absorbance obtained at the detector was plotted against pH shown in Fig. 2. The immobilized alkaline phosphatase exhibited its highest activity at pH 9.0 and was therefore used subsequently. The optimized pH for soluble alkaline phosphatase is rather 9.8 [11,12]. The immobilzed enzyme packed in glass column was utilized for about 300 hr without any appreciable change in its activity. The enzymatic activity was completely preserved after three months storage at 4°C.

Other experimental parameters for the manually operated three channel manifold were optimized by univariate approach. The effect of ammonium molybdate and ascorbic acid on the absorbance of the molybdenum blue were examined. The results shown in Fig. 3, gave the most suitable response at 5 x 10^{-3}M and 0.9% (w/v) respectively and therefore were used subsequently.

The effect of flow rate and mixing coil length was also calibrated in order to obtain the best overall response of the system in terms of speed and sensitivity. The channel having immobilized enzyme packed reactor (Tris-HCl buffer stream) gave a significant response at a flow rate of 0.9 ml min^{-1} while in the other two

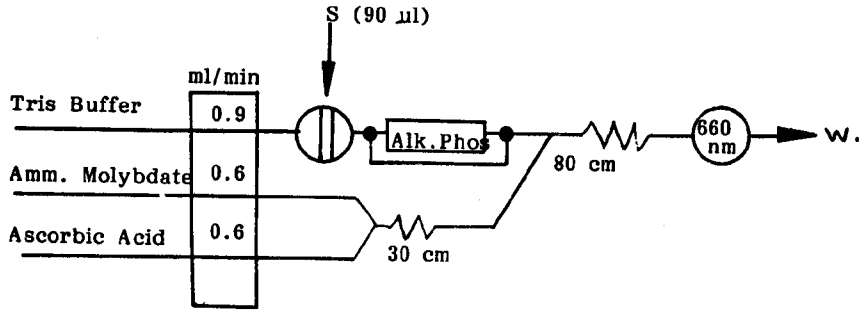

Fig. 1: Flow injection manifold equipped with peristaltic pump, immobilized Alkaline phosphatase reactor, injection valve, mixing coil and spectrophotometer (660 nm).

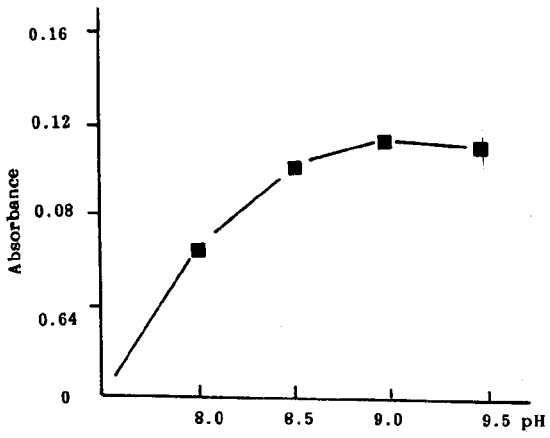

Fig. 2: Effect of pH of the carrier stream Tris-HCl buffer on the detector response.

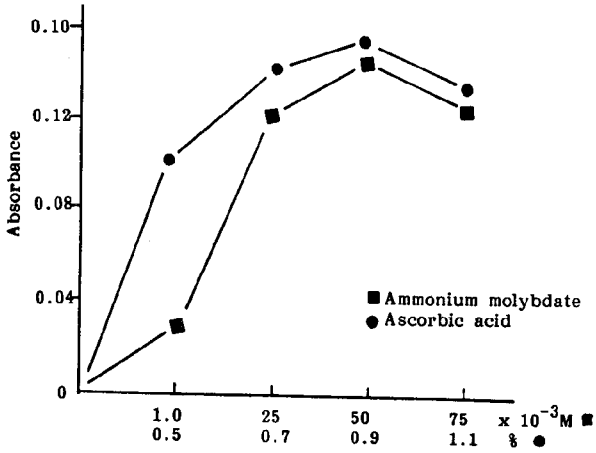

Fig. 3: Effect of ammonium molybdate (■) and ascorbic acid (●) concentrations on molybdenum blue formation.

channels maximum absorbance was achieved at a flow rate of 0.6 ml min^{-1} per channel. The mixing coil length of 80 cm which gave the optimum response was used to produce steady baseline. A sample volume of 90 µl was chosen for adequate response and less dispersion.

Analytical performance

Standard solutions of phosphorylcholine in Tris-HCl buffer (0.1 M, pH 9.0) were injected in the manifold to assess its suitability for the quantification of phosphorylcholine. The calibration data is given in Table 1. The detection limit (at S/N = 2) was 10 µM, [R.S.D. 1.8% (n = 3)]. The resulting calibration data had a correlation co-efficient of 0.99 (n = 6). The sampling rate was 45 hr^{-1} and the R.S.D. for 10 injections of 1 x 10^{-3}M phosphorylcholine was lower than 1.0%.

Table 1: Calibration data for standard phosphorylcholine solutions

Conc. (x 10^{-4}M)	1.0	3.0	6.0	9.0
Absorbance*	0.035	0.060	0.115	0.145
R.S.D.(%)	0.45	0.40	0.44	0.37

*Mean of 3 readings.

Experimental

All solutions were prepared in distilled/deionised water and all reagents were analytical grade (Merck, Damstradt, Germany).

Alkaline phosphatase (EC 3.1.3.1, 500 U/mg) from calf intestine was obtained from Boehringer, Mannheim, Germany, was immobilized on controlled porosity glass (pore size 10-1400 Å, 200-400 mesh) by cross linking with glutaraldehyde [9,10]. The immobilized enzyme was packed in glass column (2.5 x 30 mm) plugged with glass wool at both ends. The column was washed with a stream of 0.1 M Tris-HCl buffer (pH 8.0) and incorporated into the flow system. A 0.1 M stock solution of phosphorylcholine purchsed from Fluka Chemicals (Buchs, Switzerland) was prepared by dissolving 3.29 g phosphorylcholine in 100 ml of 0.1 M Tris-HCl buffer, pH 9.0; working standards were prepared by further dilution.

Ammonium molybdate (hepta) solution (0.005 M) was prepared by dissolving 1.54 g of the compound in 250 ml of water containing 3.5 ml of nitric acid (0.2 M). Solution of ascorbic acid 0.9% (w/v) was prepared in water containing 1% glycerol (v/v) which prevents the precipitation inside the flow cell.

The instrumentation used in the present study is schematically shown in Fig. 1. A peristaltic pump (Ismatec Reglo 100) was used to propel the Tris-HCl buffer carrier stream at 0.9 ml min^{-1}; ammonium molybdate and reducing agent streams through PTFE tubing (0.8 mm, i.d.) at 0.6 ml min^{-1}. Phosphorylcholine standards (90 µl) were injected into the Tris-HCl buffer carrier stream via a rotary valve (Rheodyne 5020), passed through the enzyme column and merged with ammonium molybdate and reducing agent in a 80 cm mixing coil length. The absorbance of molybdenum blue was monitored at 660 nm using a spectrophotometer (LKB Novaspec II Bromma, Sweden) with a flow through cell (30 µl) connected to a chart recorder.

Conclusion

The automated system reported has a high sampling capacity and is very flexible. The method presented can easily be adopted for the analysis of biological and food samples. Further attempts are in progress to extend the system to the determination of phosphatidylcholine by incorporating an extra immobilized enzyme reactor of PL-C, prior to the alkaline phosphatase reactor.

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References

1. B.A. Peterson, E.H. Hansen and J. Ruzicka, *Anal. Lett.*, **19**, 649 (1986).
2. B. Olsson, B. Stalbom and G. Johansson, *Anal. Chim. Acta*, **179**, 203 (1986).
3. C.B. Elliott, *Diss. Abstr. Int. B.*, **43**, 3966 (1983).
4. Y. Hayashi, K. Zaitsu and Y. Ohkura, *Anal. Chim. Acta*, **186**, 131 (1986).
5. M. Masoom, *Anal. Lett.*, **21**, 2381 (1988).
6. C.A. Bradley, K.E. Salhany, S.S. Entman,

R.L. Aleshiro and F.F. Parl, *Clin. Chem.*, **33**(1), 81 (1987).
7. L. Campanella, M. Tomassetti, M. R. Bruni, M. Mascini and G. Palleschi, *Food Additive and Contaminants*, **3** (4), 277 (1986).
8. A.P. Truett, R. Snyderman and J.J. Murray, *J. Biochem.*, **260**, 909 (1989).
9. P.J. Robinson, P. Dunnill and M.D. Lilly, *Biochim. Biophys. Acta*, **242**, 659 (1971).
10. M. Masoom and A. Townshend, *Anal. Chim. Acta*, **174**, 293 (1985).
11. T.U. Hausmen, R. Helger, W. Rick and W. Gross, *Clin. Chim. Acta*, **15**, 241 (1967).
12. R.Rej, *Clin. Chem.*, **23**, 1903 (1977).