A Facile method for on-line removal of interfering substances from biological sample

M. MASOOM YASINZAI
Institute of Biochemistry, University of Baluchistan, Quetta, Pakistan

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Summary: Electrochemical detectors often give erroneous results when biological samples without pretreatment are analyzed for important analytes. In an experiment for the determination of choline and acetylcholine in biological samples on-line using immobilized enzyme reactors, we found that incorporation of a column of an ion-exchange resin prior to the enzyme column/s removes the interfering substances from the complex biological samples.

Introduction

Enzymes, because of their selective nature as reagents, are used for analysis mainly in clinical laboratories and the food industry. The drawbacks associated with the use of soluble enzymes such as high cost and low operational stability have been reduced with the advent of immobilized enzymes. Immobilization of enzymes provides the preparation that combines high selectivity, low cost coupled with an increase in stability. In addition if the immobilized enzyme is used in minicolumns in a continuos flow system such as FIA, handling is minimized and reproducibility is enhanced [1].

The class of enzymes known as oxidases (flavin containing) catalyse reactions where the substrate molecule is oxidized by oxygen producing hydrogen peroxide which can be quantitated amperometrically.

$$\text{Substrate} + O_2 + H_2O \xrightarrow{\text{Oxidase}} \text{Product} + H_2O_2$$

Over 40 such oxidases are known which offer the possibility of measuring a wide range of analytes of importance in clinical and food laboratories [2]. Amperometric detection of H_2O_2 is although a very simple method but is subject to interference when complex biological samples are analysed without any pretreatment. In an amperometric detector, as the anodic oxidation of the hydrogen peroxide is irreversible, the electrode operates at a high applied potential of 0.6 V [3]. At such a high potential other electroactive species in biological samples are also oxidized giving erroneous results. To avoid this a lengthy clean up is needed before introduction of the sample.

Reported here is a simple system for the removal of interfering substances on-line from complex biological samples. This is illustrated with an example of acetylcholine and choline determination in rat brain supernatant using immobilized enzyme columns in a flow injection system equipped with a flow through amperometric detector [4].

$$\text{Acetylcholine} + H_2O \xrightarrow{\text{Acetylcholinesterase}} \text{Choline} + \text{acetate}$$

$$\text{Choline} + O_2 + 2H_2O \xrightarrow{\text{Choline oxidase}} \text{Betaine} + 2H_2O_2$$

The sample is directly injected into the flow system and the interferences removed by incorporating a small column of ion-exchange resin prior to the enzyme columns. The method is simple and can be applied in case of other complex samples as well.

Results and Discussion

The flow injection system comprising of two immobilized enzyme columns was first optimized and applied to the determination of acetylcholine and choline in standard solutions. The method was applied to the determination of these two metabolites in rat brain supernatant solution prepared as described by Vecchini *et al* [5]. No pretreatment of the sample was made. Very high values for acetylcholine and choline were obtained which were due to interference at the amperometric detector level, of the other electroactive species present in this real sample. This was confirmed when rat brain supernatant was injected and passed

directly through the detector. without any enzymatic reaction. A very large signal (0.41 μA) was obtained, indicating the need for a prepurification step. Such prepurification steps involve lengthy clean up, making the method very tedious and practically non-feasible. Flow injection systems offer the possibility of a variety of designs and applications for on-line analyses [6]. Ion-exchange resins have long been used for the purification of samples. To overcome this problem of interference, the use of an ion-exchange resin column on-line was used. The resin column was incorporated between the injection valve and the detector. The samples of post microsomal rat brain supernatant were injected. A very small and delayed response at the detector was observed, demonstrating that the interfering substances are retained and released very slowly, thus not affecting the analytes of interest response.

To find whether choline/acetylcholine is retained or not by the ion-exchange column. The radiolabelled choline solution was passed through the column. Measurement of the radioactivity in the aliquots (0.5 ml) collected showed that almost 98% of the radioactivity was observed in the first eluate showing that choline/acetylcholine is not retained by the column. Therefore the ion-exchange resin column was incorporated into the flow injection system prior to the immobilized enzyme columns (Fig. 1), and the analysis of choline/acetylcholine in rat brain samples carried out by the standard addition method. A content of 292 ± 21 nmols of free choline and 67 ± 13 nmols of acetylcholine per gram wet brain was found. These results were in good agreement with those reported by other authors [7].

In summary the flow injection determination of acetylcholine/choline in biological samples using immobilized enzymes demonstrates a good principle of on-line purification of the complex samples. This saves a lot of labour and time and can be applied to many similar analytical systems.

Experimental

The flow injection system is schematically shown in Fig. 1. It is constructed from simple units such as peristaltic pump to propel the buffer stream through the system, an injection valve which can precisely introduce 20 μl of the sample into the flowing stream. The amperometric detector is simply a mini glass flow cell with two platinum electrodes to which 0.6 V potential is applied through a power supply [4].

The enzymes obtained from Sigma Chemicals USA, were immobilized on controlled pore glass by cross linking with glutaraldehyde. The detailed method is described elsewhere [8]. The immobilized enzyme derivatives were packed into the mini glass columns (2.5 x 25 mm) and then incorporated into the flow injection system as desired. The ion-exchange resin column of the size of the enzyme columns (2.5 x 25 mm, AG1-X8, formate form) was packed and incorporated into the flow injection system prior to the enzyme columns when needed. To asses the retention of choline by the ion-exchange resin, the column was connected to the injection valve and the radiolabelled choline sample (20 μl ^{3}H-Choline), the radioactivity of which was initially measured, was injected into the same buffer stream and allowed to pass through the ion-exchange column. 0.5 ml aliquots were collected and the radioactivity measured.

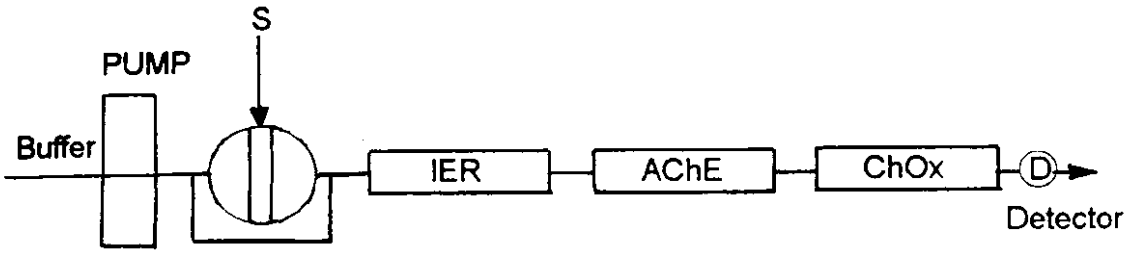

Fig. 1: Schematic representation of the flow injection system. IER, Ion exchange resin, AChE, Acetylcholinesterase, ChOx, Choline oxidase, S, Sample.

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