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HPLC detection of choline and acetylcholine in serum and urine by an immobilized enzyme reactor followed by chemiluminescence detection

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Abstract: A method using HPLC has been developed for the detection of choline (Ch) and acetylcholine (ACh) using an immobilized enzyme reactor which converts Ch and ACh into hydrogen peroxide and betaïne. The formed H_2O_2 is quantified by means of a solid-state peroxyoxalate chemiluminescence detector based on an immobilized fluorophore and addition of oxalate from a solid bed. The conditions necessary for chemiluminescence detection are obtained by using a make-up flow of acetonitrile after the enzyme reactor. Precipitation problems due to the poor solubility of salts in the final acetonitrile–water mixture are circumvented by adding a crown ether to the make-up flow. The reproducibility of the method was calculated to be 3.4–3.7% RSD. Detection limits are in the sub-picomole range and a linear range of at least three orders of magnitude is found. Measurements in urine and serum reveal no matrix effects.

Keywords: *Immobilized enzyme reactor; chemiluminescence detection; choline; acetylcholine; HPLC.*

Introduction

The combination of liquid chromatography and immobilized enzyme reactors (IMERs) appears to be of growing interest for the detection of biological substances in various complex matrices. Especially the detection of acetylcholine (ACh) and choline (Ch) have received much attention in the recent literature [1–4].

One of the main advantages of the use of IMERs is their high specificity. This can occasionally permit the use of less selective devices such as UV detectors for detection

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and quantitation of the products. Specificity and sensitivity can often be enhanced further by coupling IMERs to fluorescence, chemiluminescence or electrochemical detectors.

In this paper we present the combination of a highly selective IMER in combination with a selective and sensitive H_2O_2 monitor based on peroxyoxalate chemiluminescence. Ch and ACh are separated on a cation exchange column and subsequently converted to betaïne and hydrogen peroxide in an IMER which is packed with immobilized acetylcholinesterase (AChE, E.C. 3.1.1.7) and cholineoxidase (ChO, E.C. 1.1.3.17). This IMER can be combined with electrochemical detection of the formed peroxide as described by Damsma *et al.* [3]. In the present study we describe the quantitation of the formed H_2O_2 by our solid state chemiluminescence detector [5–7], which consists of an immobilized fluorophore (immobilized on glass beads) and a bed of solid bis-(2,4,6 trichlorophenyl) oxalate (3-aminofluoranthene TCPO).

The main problem in using peroxyoxalate chemiluminescence in conjunction with IMERs is the incompatibility of the optimal media for either reaction. In principle enzymic reactions function well in aqueous solvents while the chemiluminescence reaction requires an organic solvent. This is because the oxalates used in the chemiluminescence reaction hydrolyse rapidly in aqueous media and, competing with the actual chemiluminescence reaction cause a considerable loss in chemiluminescence efficiency. In the present study this problem was solved by adding a make-up flow of acetonitrile after the IMER. This also provides the possibility of adapting the carrier stream to more optimal chemiluminescence conditions. The addition of a crown ether to the make-up flow presents inorganic salts, needed for the separation of ACh and Ch, precipitating in the ultimate organic reaction medium.

Experimental

Chemicals

TCPO was prepared according to Mohan and Turro [8] and recrystallized three times from Uvasol® benzene (Merck, Darmstadt, FRG). It was kept under reduced pressure for several hours both to evaporate to dryness and to remove traces of trichlorophenol. Acetonitrile (HPLC, gradient grade, Baker, Deventer, The Netherlands) was purified on an alumina column as previously described [7]. Tetramethylammonium (TMA) nitrate was prepared from TMA iodide (Aldrich, Beerse, Belgium) by an ion exchange procedure; the final product was tested with $AgNO_3$. Triethylamine (TEA) analytical grade was purchased from Merck (Darmstadt, FRG). Analytical grade 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) was obtained from Aldrich (Beerse, Belgium). 3-Aminofluoranthene was immobilized on glass beads covalently linked via 3-glycidoxy-propyltrimethoxysilane as described previously [6]. ACh chloride and Ch chloride were obtained from Sigma (St Louis, USA). AChE and ChO were bonded covalently to CNBr-activated sepharose 4B as reported by Damsma *et al.* [3]. This procedure was extensively described in the instructions supplied by Pharmacia. A 6 mg mixture of AChE and ChO in a ratio of 550:175 U was added per 1 ml of sepharose gel (similar to refs [1] and [3]). In brief, the procedure was as follows: the coupling gel was prepared by swelling the freeze-dried powder with 1 mM HCl; the gel was then washed with a small amount of buffer (0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl); finally the gel was coupled to the enzymes (6 mg in 3 ml of the above buffer). The coupling reaction takes place overnight at 4°C. At this temperature the product is stable for at least a month.

Apparatus

A schematic representation of the apparatus is shown in Fig. 1. Mobile phase (0.05 M potassium phosphate (pH 7.4) 20 mM TMA nitrate) was delivered by a Kratos Spectroflow 400 pump. A precolumn of 6 cm length and an i.d. of 4.6 mm packed with Nucleosil 5SA (Machery Nagel, Düren, FRG) placed before the injector was used as a guard column and pulse damper. Sample injection was performed with a Valco 6-port injection valve equipped with either a 20 or a 100 μl loop. A home-packed cation exchange column (75 × 2.1 mm Nucleosil 5SA) was used for the separation of ACh and Ch.

The enzyme post-column reactor (75 × 2.1 mm) was coupled directly to the analytical column by means of a Valco union. The acetonitrile make-up flow (4 mM 18-crown-6, 5 mM TEA) was delivered by a Kratos SF 400. The mixing Tee was a vortex-type as supplied by Kratos (part No. 2500-0322). The chemiluminescence monitor was coupled to the rest of the system by a Valco zero dead volume equipped with 8 μm screens (Chrompack, Middelburg, The Netherlands) in order to achieve a homogeneous mixture of mobile phase and acetonitrile in the cell [9].

Sample pretreatment

Heparin blood samples were deproteinized by means of perchloric acid brought to pH 4 with potassium acetate and centrifuged on a Beckman Microfuge B at 5500 **g** for 1 min. Urine samples were injected directly into the system without pretreatment.

Results and Discussion

Under the conditions employed in the present study (solid TCPO, immobilized fluorophore) peroxyoxalate chemiluminescence is only efficient in fluids with a high content of organic solvents such as acetone or acetonitrile. As the IMER operates optimally in aqueous conditions a make-up flow has to be added after the IMER. The introduction of large amounts of organic solvents usually causes problems. Honda *et al.* [4], for example noted the poor solubility of TCPO which precipitated in the flow lines and was a limiting factor in their system. The present system does not have this problem since solid TCPO is introduced into the system after mixing the solvents and therefore its solubility in the final reaction medium is not exceeded. Salts dissolved in the aqueous mobile phase of the separation system will not however dissolve in the final acetonitrile–water mixture but the solubility of potassium salts in the organic solvent can be enhanced by adding a crown ether which forms complexes with alkali ions. In the present study it was found that even relatively high concentrations of potassium phosphate buffers can be mixed with acetonitrile without the formation of a precipitate. Under the conditions used, 3.8 mM of 18-crown-6 was sufficient to prevent precipitation of salts after addition of acetonitrile. The presence of 18-crown-6 in the mobile phase did not influence the chemiluminescence signal.

System optimization

As the present conditions for the chemiluminescence reaction differ significantly from those employed in earlier studies, the optimization of the signal-to-noise ratio had to be reexamined. In our original work tris buffer was used to regulate the pH of the chemiluminescence reaction medium but in the present study this is not possible because of the insolubility of tris buffer in pure acetonitrile. Scott *et al.* [10] reported a catalytic

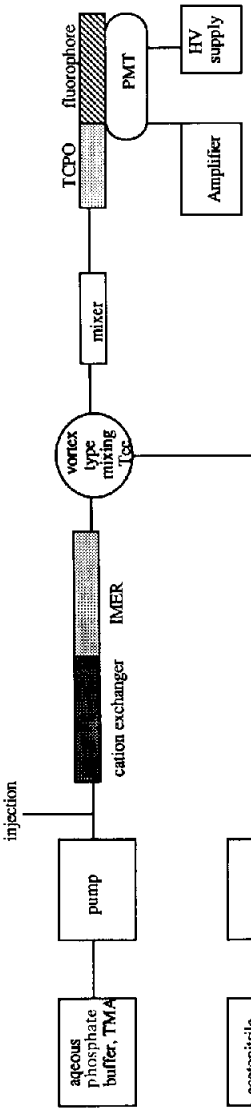

Figure 1
Block diagram of the experimental set-up.

effect of triethylamine (TEA) on the chemiluminescence signal. The rôle of the compounds like TEA and tris in the reaction mechanism is not well understood. An advantage of TEA in the present system is that it can easily be dissolved in the acetonitrile make-up flow. A concentration of 5 mM produced an optimal signal-to-noise ratio, 10-fold better than with no addition of TEA.

The separation conditions described by Damsma *et al.* [3] involved the use of TMA chloride to regulate the retention behaviour of ACh and Ch. As Cl^- ions are known to quench the chemiluminescence signal, adversely affecting the base-line noise and the signal intensity, the use of halides should be avoided [11]. TMA chloride was therefore replaced by TMA nitrate. Contrary to Potter *et al.* [1] but in accordance with the previous study in which TMA chloride was used in combination with the same IMER [3], we found no influence of TMA nitrate on the enzyme activity. This contradiction can be explained if complete conversion in our IMER is assumed. In such a situation small amounts of inhibitor or catalyst will hardly influence the performance of the IMER. In the present case there is circumstantial evidence that conversion of Ch and ACh is complete: the method shows a large linear dynamic range (see below) and the flow-rate through the IMER, and hence the residence time, hardly influences the response towards Ch and ACh in the concentration range investigated. Exact conversion factors have not been determined because of the difficulties involved; H_2O_2 is unstable on the analytical column and has to be removed. It is not retained in the system and so analytical data involving H_2O_2 are not reliable.

Chemiluminescence background noise largely determines the detection limits of the method. In principle the chemiluminescence monitor operates independently of flow [6] but both signal and background are highly dependent on the acetonitrile–water ratio. Hence inhomogeneous mixing of these solvents will cause low frequency noise due to pump pulsations. The use of an efficient vortex type mixer [12, 13] in conjunction with a conventional mixer between the Tee-piece and the detector-cell produces a considerable reduction of the low frequency noise and a 4-fold S/N-gain. This extra mixer was constructed according to Kucera *et al.* [9] by placing an 8 μm screen in a Valco zero dead volume union. Frequency analysis of the base-line obtained with the above system shows that the ratio of fast (>5 Hz) versus slow (<0.5 Hz) noise is now 1:5. Therefore if complete mixing can be achieved, a S/N gain of five should be feasible. As would be expected, experiments with all solutes and solvents premixed and pumped through the chemiluminescence cell showed no low frequency noise.

The optimal flow rates are 0.5 ml min^{-1} for the chromatographic and 1.5 ml min^{-1} for the make-up flow. At make-up flows higher than 1.5 ml min^{-1}, noise increases faster than the signal intensity, while at lower make-up flows too low a signal intensity is observed. The chromatographic conditions employed in the present work differ only slightly from those recently used by Damsma *et al.* [14]. In this publication columns with internal diameters of 2.1 mm instead of 4.6 mm are utilized with the inherent decrease in flow rate. We have used a lower phosphate concentration with the same pH (7.4) as described by Damsma *et al.* [14]; this did not affect the separation of Ch and ACh but facilitated the CL detection because at higher phosphate concentrations precipitation will occur upon post column mixing with acetonitrile.

Analytical data

Under the above conditions an absolute limit of detection (l.o.d.) of 0.5 pmole of Ch and 0.7 pmole of ACh was calculated (S/N = 3). The concentration l.o.d.'s depend

linearly on the injection volume in the range from 20 to 100 µl. Therefore, if sample availability is not a problem the concentration–sensitivity of the method can be enhanced five-fold by using 100 µl injections. Concentration l.o.d.'s are in the order of 10 nM which is in line with results obtained earlier with this chemiluminescence reactor where l.o.d.'s of the same order of magnitude were obtained for H_2O_2.

A repetitive 20 pmole injection (20µl) showed a relative standard deviation of 3.4% for Ch and 3.7% for ACh ($N = 5$). The method was linear from 1 pmole up to at least 10 nmole ($N = 8$, $r = 0.9997$). The chemiluminescence reactor consumes solid TCPO and under the present conditions an average life-time of 4 h was found. After this period the signal shows a downward drift and the TCPO layer should be refilled as previously described [6]. As TCPO and immobilized 3-aminofluoranthene are separated by a frit, repacking of the TCPO layer can easily be performed with a microspatula. The stability of the IMER appears to be satisfactory; if it is used continuously for two weeks the sensitivity is decreased by about 25%. This means that several hundred samples can be analysed with each IMER. Because of the relatively high sample rate of the method (ca 10 h^{-1}) it was decided not to add an internal standard, but to calibrate the system before and after each sample run. The advantages of such a procedure are firstly that separation of only two analytes requires relatively simple chromatography and secondly, the performance of both enzymes (AChE and ChO) immobilized in the reactor can be verified.

Application to real samples

Finally the method was tested for analyses on urine and serum samples. Untreated urine samples exhibited only one extra peak at t_0 compared to standard solutions. A chromatogram of a urine sample followed by addition of Ch and ACh is shown in Fig. 2. The early peak is caused by displacement of the buffer from the cation-exchanger, due to the high salt concentrations introduced. Separation of the early peak, Ch and Ach is good. The slopes of calibration graphs produced by spiking urine samples do not differ significantly from those obtained with standard solutions. Similar experiments were performed with serum samples, but a deproteinization-step is necessary because if untreated (filtered over a 0.45 µ filter) serum is introduced, the Tee-piece mixer and cell will become clogged after a few injections. This is due to denaturation of the proteins by acetonitrile. The problem can be avoided by the use of on-line deproteinization utilizing precolumn technology [15]. As before the experiments on serum samples did not reveal any matrix effects. A chromatogram of a pretreated serum sample is shown in

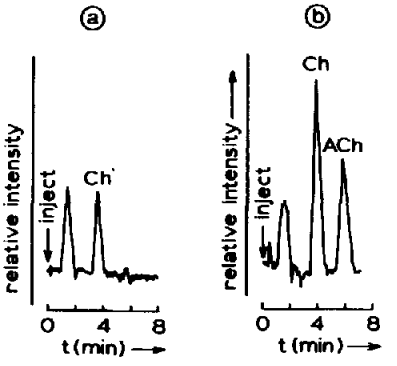

Figure 2
(a) Chromatogram of an undiluted urine sample, (b) chromatogram of a urine sample spiked with 20 pmole of Ch and ACh.

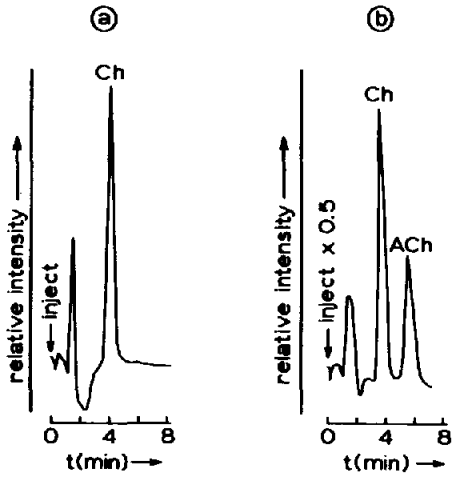

Figure 3
(a) Chromatogram of a deproteinized pooled serum sample, (b) serum sample spiked with 200 pmole of Ch and ACh.

Fig. 3, the negative peak is caused by the low pH of the sample or by chemiluminescence quenchers in the sample [16]. These negative peaks are well separated from the analyte peaks. The sensitivity of the method proves to be satisfactory for the determination of Ch in these samples; throughout this study the authors have not been able to detect ACh in real samples probably due to the fact that ACh degrades quite rapidly in the matrices under consideration. We have not attempted to study matrix effects by deleting the enzyme reactor from the system, since such an experiment would only give information on compounds responding to the CL system. The most likely enzyme inhibitors and catalysts cannot be detected in this way.

The sensitivity of electrochemical detection [2, 3] is at present about 5-fold better, but it lacks the specificity of our method because it is sensitive, for example, to catecholamines and related compounds. A sample clean-up procedure is required.

The sensitivity of our system is equal to that of the chemiluminescence system reported by Honda *et al.* [4]. However, our method is more simple because only one post-column pump is utilized and the separation conditions are the same as those used for the IMER, so that the post column pump can be used exclusively for adapting the chemiluminescence conditions.

Conclusion

A selective and sensitive method has been developed for the detection of ACh and Ch in serum and urine. The method is simple compared to other chemiluminescence detection schemes. Its simplicity and selectivity offer the possibility of a flow injection system for the determination of both the total concentration of ACh and Ch and the determination of Ch. The use of an efficient mixing unit to add the make-up flow to the system is very important, because mixing-noise is the main factor determining the sensitivity of the method. The addition of a crown ether can facilitate the compatibility of detection systems requiring a high amount of organic solvent in combination with mobile phases containing high salt concentrations.

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