Tris(2,2’-bipyridine)ruthenium(II) electrogenerated chemiluminescence of alkaloid type drugs with solid phase extraction sample preparation

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An electrogenerated chemiluminescence (ECL) method for the determination of pethidine, atropine, homatropine and cocaine is described. The optimum conditions were found to be similar for all of these compounds although the ECL emission intensity for cocaine was an order of magnitude lower than for pethidine due to their different chemical structures. Linear calibrations were obtained for all the compounds at pH 10 in borate buffer (0.05 mol l\(^{-1}\)) at 1.3 V. Limits of detection of \(6.8 \times 10^{-6}, 2.2 \times 10^{-7}, 3.2 \times 10^{-7}\), and \(6.5 \times 10^{-7}\) mol l\(^{-1}\), respectively, were achieved for pethidine, atropine, homatropine and cocaine in standard solutions. Solid-phase extraction was used to separate the drugs from their matrix and the method was applied to the determination of spiked urine samples. The limits of quantitation for pethidine, atropine, homatropine and cocaine in urine were \(1.0 \times 10^{-6}, 2.0 \times 10^{-6}\) and \(4.0 \times 10^{-6}\) mol l\(^{-1}\), respectively, with recoveries of between 90 and 110%.

Introduction

Pethidine, atropine, homatropine and cocaine (Fig. 1) belong to the alkaloid family and have various pharmaceutical applications. They are used as anticholinergic, antispasmodic and preanaesthetic agents. Cocaine has a long history of human use and abuse and its effects on the central nervous system are well known. The determination of these compounds in biological fluids has always been of great importance for the evaluation of their effects. There are several analytical techniques available to determine these compounds. Chromatographic methods are the most extensively used,\(^{1-4}\) because they have high sensitivity and good selectivity, but the procedures are lengthy and often involve derivatisation methods to convert the analytes to detectable forms or to improve the column efficiency and peak shapes. During recent years, some alternative, more rapid, methods have been developed for screening purposes. Cherkaoui et al.\(^{5}\) reported a capillary zone electrophoresis method for the determination of atropine, homatropine and scopalamine in ophthalmic solutions and a limit of quantitation value of \(1.0 \times 10^{-5}\) mol l\(^{-1}\) for each drug was achieved. An atropine selective electrode with a PVC membrane has also been reported\(^{6}\) which has good selectivity but the lower limit of linear response is relatively high at \(1.2 \times 10^{-5}\) mol l\(^{-1}\). An ion-selective piezoelectric sensor has also been reported, which was demonstrated to be sensitive as well as selective for the determination of the atropine sulfate although a relatively long wash time was needed to recover the sensor after a series of measurements.\(^{7}\) A spectrophotometric method\(^{8}\) has been reported for the determination of atropine with good selectivity but poor sensitivity (the lower limit of linear range being \(3.3 \times 10^{-7}\) mol l\(^{-1}\)).

Electrogenerated chemiluminescence (ECL) methodology provides a very sensitive means of detection for the analysis of drugs. Its applications have been extensively reviewed.\(^{9,10}\) In work previously reported by the authors, a number of analytical methods were established for the analysis of various drugs,\(^{11-13}\) but they were not applied to real biological fluids. The effect of complex matrices remains one of the major obstacles for chemiluminescence methods. He et al.\(^{14}\) described a chemiluminescence method for the determination of oxalic acid and tartaric acid in urine, but in fact synthetic urine was used and a 500-fold dilution had to be made before satisfactory results were obtained. It is obvious that direct determination of drugs in a urine sample would not be possible without prior separation of the analytes.

Solid phase extraction (SPE) is an increasingly useful sample preparation technique. With SPE, many of the problems associated with liquid–liquid extraction can be prevented, such as incomplete phase separation, less-than-quantitative recoveries and disposal of large quantities of organic solvents. SPE has been extensively used to isolate the drugs from their matrices prior to analysis, as described for the chromatographic analysis of pethidine, cocaine and their metabolites.\(^{1,2}\) In this paper a simple, rapid screening method with ECL has been developed for the determination of drugs in urine matrices using SPE cartridges for sample clean up.

Experimental

Reagents

Pethidine hydrochloride, atropine sulfate salt, DL-homatropine hydrobromide and cocaine hydrochloride were purchased from Sigma (Poole, Dorset, UK). A stock standard solution of each

Fig. 1 Chemical structures of pethidine, atropine, homatropine and cocaine.

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compound was prepared by directly dissolving the reagents in purified water (produced by reverse osmosis followed by ion exchange, Elgastat UHQ PSII, Elga Ltd., High Wycombe, Buckinghamshire, UK). Tris-(2,2'-bipyridine)ruthenium(II) hexahydrate (Pract., 90–95%) was obtained from Fluka (Gillingham, Dorset, UK). All the stock solutions were stored in the dark at 2 °C and were stable for at least 1 month. The buffers used in this experiment contained either sodium dihydrogenorthophosphate (AnalaR, 99–102%), sodium borate, sodium carbonate anhydrous (AnalaR, 99.9%) or sodium acetate anhydrous (AnalaR, 99%), all from Merck (Poole, Dorset, UK). The pH was adjusted with analytical reagent grade sodium hydroxide, glacial acetic acid or hydrochloric acid.

Instrumentation

The ECL instrumentation was the same as has been described previously with the exception of the flow cell. The thin layer flow cell was constructed from two layers of Perspex separated by a 0.3 mm thick PTFE spacer. A channel, 4 mm in width and 30 mm long, was milled into the spacer to provide the flow path. One of the Perspex layers contained the inlet and outlet, the working electrode, counter electrode and reference electrode, while the other sheet was polished to optical quality. The electrodes consisted of an aluminium disc working electrode (30 mm²), platinum-wire counter electrode and silver pseudo-reference electrode and potentials were applied to the electrodes using a three-electrode potentiostat built in-house. The photomultiplier (PMT) was located in front of the polished face of the flow cell. All connections in the flow system were constructed from 0.8 mm internal diameter PTFE tubing obtained from Anachem (Luton, Bedfordshire, UK). Solutions were moved through the system with a peristaltic pump (Gilson Minipuls 3, Anachem) with 1.42 mm id flexible PVC tubing. The light was detected using a photomultiplier tube (Thorn EMI, 9789QB, Ruislip, Middlesex, UK) and the signals were amplified and recorded using a chart recorder (Chessel, Worthing, Sussex, UK). The high voltage power supply for the PMT was a Model 3000R from Thorn EMI.

Analytical procedure

A continuous flow system was used in this experiment. Standard solutions of drugs or sample solutions were pre-mixed with tris-(2,2'-bipyridine)ruthenium(II) and buffer solutions. These solutions were then separately pumped through the flow cell where a 20 s pulsed voltage cycle was applied to the solutions. The voltage cycle consisted of 10 s at the selected voltage and 10 s to take the electrode to ground and to re-set the voltage. The cycle was used to prevent deterioration of the electrode surface with repeated analysis. The light produced at the electrode was detected by the photomultiplier and the electrode surface with repeated analysis. The light was detected using a photomultiplier tube (Thorn EMI, 9789QB, Ruislip, Middlesex, UK) and the signals were amplified and recorded using a chart recorder.

Sample preparation

In this experiment, solid phase cartridges with a reverse phase packing (3 ml ISOLUTE C₁₈ columns (Jones Chromatography, Hengoed, Mid-Glamorgan, UK) were chosen to separate the drugs from their matrix. The procedure employed was a modified version of the manufacturer’s procedure. The cartridge was conditioned by rinsing it with a 3 ml aliquot of methanol followed by a 3 ml aliquot of water. The retention and quantitative elution of the analytes from the cartridge was evaluated by recovery studies. The initial evaluation was for standard solutions of analyte. A 1 ml aliquot of 1 × 10⁻⁴ mol⁻¹ of the analyte of interest was added to the cartridge and then rinsed with a 3 ml aliquot of water, a 2 ml aliquot of 10% methanol and 1 ml aliquot of methanol. The analyte was then eluted from the cartridge by a 5 ml aliquot of acetone. The eluent was collected, and evaporated to dryness under nitrogen. Buffer and Ru(bpy)₃²⁺ were directly added to the residue and the amount of analyte in the residue was then determined by the optimised ECL method.

Urine samples were collected from volunteers and filtered through a 0.45 µm filter. Different known quantities of the analytes were then added to the urine and the samples were analysed using the proven solid phase extraction clean up method followed by ECL detection.

Results and discussion

Optimisation of system

The important variables in ECL are the applied voltage and buffer conditions. The change in ECL emission intensity with applied voltage was investigated between 0–2 V. Each solution was continuously pumped through the flow cell whilst the voltage applied to the working electrode was increased and the highest ECL emission intensity was found to be 1.3 V for all four analytes. This remained the optimum voltage even when different buffers and pH values were investigated. The effect of changing the pH value of the buffer was then investigated in relation to the ECL emission intensity. These studies were carried out with a set of standard solutions for each compound, covering the pH range 6–11, whilst the voltage at the working electrode was set at 1.3 V. It was found that the ECL intensities of the blank solution and sample solutions increased as the pH value of the buffer increased and hence the S/N (signal to blank ratio) was used to find the optimum pH. This increase in emission intensity with increasing pH value has also been seen by Barnett et al. in CL systems. When the buffer pH exceeded 10, the chemiluminescence response still increased as the pH increased, but the blank response also increased dramatically and the ratio of signal to noise began to decrease. The four drugs were found to give high ECL responses under similar conditions, i.e., at 1.3 V in a weakly alkaline medium. An example of these results can be seen in Fig. 2 for pethidine, in which the effect of different buffers and pH on the ECL response can be seen. Sodium carbonate buffer, which has an enhancement effect on the ECL signal for morphine, was observed to quench the ECL of tris-(2,2'-bipyridine)ruthen-
ium(II) in this experiment. When compared with sodium carbonate buffer, sodium borate buffer gave a higher ECL intensity. Sodium borate buffer was also more useful than phosphate buffer because it could buffer more alkaline solutions. The concentration of buffer was shown to have an insignificant effect on the ECL intensity, so a relatively lower concentration, 0.05 mol l\(^{-1}\), was chosen to ensure low blank values. The same trends as were observed for pethidine were also seen for atropine, homatropine and cocaine but at different electrogenerated CL intensities. In Fig.1 it can be seen that atropine, homatropine and cocaine have similar chemical structures. Atropine gives the strongest ECL intensity and cocaine the lowest. This can be explained by the fact that cocaine has one more electron-withdrawing substituent on the ring structure and this will destabilise the radical intermediate.\(^{11}\)

The effects of flow rate on ECL intensity were also investigated. The results showed that an increase in flow rate led to an increase in ECL intensity, as has been shown in previous work.\(^{17}\) A 5 ml min\(^{-1}\) flow rate was chosen in order to get good sensitivity without consuming excess reagents. To get a fast flow over the electrode surface, a thin (0.3 mm) PTFE spacer was used to construct the flow cell, and this was shown to be very effective in improving the ECL emission intensity.

**Calibration**

Standards were prepared for each compound by pre-mixing the stock solution with Ru(bpy)\(_3\)\(^{2+}\) and buffer. Using the optimised experimental conditions, calibrations for pethidine, atropine, homatropine and cocaine were obtained and the results are shown in Table 1. Good linearity was seen for all the compounds with pethidine having the highest sensitivity, followed by atropine, homatropine and cocaine for the reasons explained previously. The limits of detection are shown in Table 1. They were calculated as 3\(\times\) standard deviation of the blank signal + blank signal. They were good for all the compounds but the limits of detection for pethidine were a factor of 10 times better than for cocaine. These limits of detection were excellent compared with the other screening methods discussed in the introduction,\(^{5-8}\) being well below the 1 \(\times\) 10\(^{-5}\) mol l\(^{-1}\) levels reported for those methods. The RSD values were acceptable at all concentration ranges measured, as was the sample throughput at 180 samples h\(^{-1}\).

**Interference study**

To develop a method for the determination of drugs in urine, a number of chemical reagents that were most likely to be present in urine were selected for the interference study. As is shown in Table 2, most commonly existing chemical species do not interfere with ECL. Oxalic acid, ascorbic acid, uric acid and CO\(_2\) do, however, give significant interference effects. These interferences therefore had to be separated from the drugs by solid phase extraction.

**Analysis of drugs in a spiked urine matrix**

The solid phase extraction step was first evaluated using standard solutions of the analyte. Following the procedure described in the Experimental section the recoveries of the drugs in standard solutions were evaluated. The recoveries (n = 3) for pethidine, cocaine, atropine and homatropine were 98.6, 100.3, 102.1 and 99.5% at the 2 \(\times\) 10\(^{-6}\) mol l\(^{-1}\) level, respectively. These results showed that the drugs could be quantitatively recovered using the sample preparation procedure.

The procedure was then evaluated for urine samples using the procedure described in the Experimental section. It was apparent that there was still a small chemiluminescence emission for the urine blank even after passing the sample through the SPE cartridge. The interfering species was not considered to be the polar species that had been investigated as known interferents. For example, when a 1 ml aliquot of 1 \(\times\) 10\(^{-5}\) mol l\(^{-1}\) of the strongly interfering oxalic acid was introduced to the SPE cartridge, and the sample procedure was followed, the oxalic acid was washed straight through with methanol and water and was not trapped with the analytes on the cartridge. The presence of this blank emission meant that the limits of detection achievable in standard solutions were not achievable in the real samples. Table 3 shows the lowest concentrations of spiked drugs in urine that can be achieved.

### Table 1 Calibration characteristics and detection limits

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range/mol l(^{-1})</th>
<th>Calibration equation(^{a})</th>
<th>Correlation coefficient (n = 6)</th>
<th>LOD/mol l(^{-1})</th>
<th>RSD range (%) (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pethidine</td>
<td>4 (\times) 10(^{-7})-1.3 (\times) 10(^{-4})</td>
<td>(y = 2.31 \times 10^{6} x + 0.293)</td>
<td>(R^2 = 0.9997)</td>
<td>6.8 (\times) 10(^{-4})</td>
<td>1.7-4.2</td>
</tr>
<tr>
<td>Atropine</td>
<td>1 (\times) 10(^{-6})-2.0 (\times) 10(^{-4})</td>
<td>(y = 2.06 \times 10^{6} x + 1.10)</td>
<td>(R^2 = 0.9991)</td>
<td>2.2 (\times) 10(^{-7})</td>
<td>2.9-5.1</td>
</tr>
<tr>
<td>Homatropine</td>
<td>1 (\times) 10(^{-6})-2.0 (\times) 10(^{-4})</td>
<td>(y = 9.46 \times 10^{6} x + 0.447)</td>
<td>(R^2 = 0.9993)</td>
<td>3.2 (\times) 10(^{-7})</td>
<td>2.3-4.4</td>
</tr>
<tr>
<td>Cocaine</td>
<td>2 (\times) 10(^{-6})-2.3 (\times) 10(^{-4})</td>
<td>(y = 2.34 \times 10^{6} x + 0.737)</td>
<td>(R^2 = 0.9997)</td>
<td>6.5 (\times) 10(^{-7})</td>
<td>2.3-4.7</td>
</tr>
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</table>

\(^{a}\) Where y is relative chemiluminescence emission in mV and x is concentration of analyte in mol l\(^{-1}\).
with satisfactory recoveries (i.e. recoveries between 90–110%). Work is proceeding to identify the interfering species in the urine samples.

Conclusions

In this work, a simple and rapid assay involving sample clean up by solid phase extraction, followed by ECL detection, has been developed for the determination of a range of alkaloid type drugs in urine. The detection limits achieved in standard solutions were well below the $1 \times 10^{-6}$ mol l$^{-1}$ level. Good recoveries were obtained from spiked urine samples following the solid phase extraction procedure. The ECL properties of the metabolites of these drugs are currently being investigated further.

References

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