Analysis of tricyclic antidepressants using electrogenerated chemiluminescence

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A novel method has been investigated for the selective and sensitive determination of a range of tricyclic antidepressants including amitriptyline, doxepin, nortriptyline, promazine, chlorpromazine, imipramine, clomipramine, desipramine, protriptyline and trimipramine using electrogenerated chemiluminescence (ECL). The ECL mechanism is based on the reaction between tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] and the tertiary amino groups on the antidepressants. After selecting the best operating parameters calibration curves were obtained over three orders of magnitude for amitriptyline, doxepin, nortriptyline, promazine and chlorpromazine. Linear calibrations were used to obtain limits of detection in the range 0.09–0.24 μg ml^{-1} with relative standard deviations below 4% for five replicate samples. Rapid depression in the signal was observed with repeat analysis of imipramine, clomipramine, protriptyline, desipramine and trimipramine due to electrode fouling by the oxidation product of the reaction. Use of a lower concentration of the compound was found to alleviate the problem. Finally the concentration of doxepin was determined in a pharmaceutical preparation.

Introduction

The tricyclic antidepressants (TCA's) are a group of compounds that are used for the treatment of psychiatric patients suffering from clinical depression.[1] The chemical structures of these compounds are illustrated in Fig. 1. The monitoring of such compounds is important for quality assurance in preparations and for obtaining optimum therapeutic concentrations, while minimising the risk of toxicity. The therapeutic concentration range for most TCA's is approximately 100 to 300 μg l^{-1}, while toxic effects can occur when plasma concentrations exceed 500 μg l^{-1}.[2]

Trialkylamines and related compounds are difficult to detect as they are not easy to derivatise and they do not absorb very well in the UV-visible region, since they have low molar absorptivities. Also many methods require time-consuming sample preparation techniques, particularly gas chromatography.[3] All of the TCA's contain either a secondary or tertiary amine functional group that can be determined by utilising their electrogenerated chemiluminescence (ECL) reaction with tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] without prior derivatisation.[4] ECL detection is a technique in which chemiluminescence emission is produced directly or indirectly as a result of an electrochemical reaction. This detection technique is very sensitive and selective and the reactions involving $Ru(bpy)_3^{2+}$ can occur in aqueous solution in the presence of dissolved oxygen and other impurities.[5] The $Ru(bpy)_3^{2+}$ and the secondary or tertiary amine functional group on the tricyclic antidepressant are oxidised simultaneously by applying an appropriate voltage to the working electrode. The oxidation product of the amine undergoes deprotonation to form a radical. This reduces the $Ru(bpy)_3^{3+}$ to the excited state that subsequently emits light.[6] The emission intensity can therefore be related to the concentration of the TCA present. The proposed mechanism for the ECL reaction of doxepin is shown in Fig. 2.

In this work the ECL reaction described above has been investigated so as to develop a simple, but sensitive and selective method for the determination of TCA's. To illustrate the use of ECL for the analysis of 'real samples', the amount of doxepin was quantified in the commercial pharmaceutical preparation, Sinequan (Pfizer, Sandwich, Kent), and compared to the stated value. This product is used for the treatment of

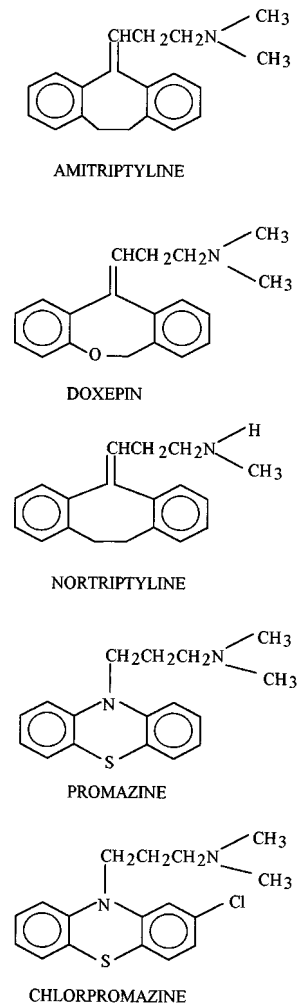

Fig. 1 Chemical structures of some of the TCA's.

psychiatric patients suffering from depression and is available in capsule formulations.

Experimental

Reagents

Tris(2,2′-bipyridyl)ruthenium(II) hexahydrate (Pract., 90–95%) was obtained from Fluka (Gillingham, UK). The buffers used contained sodium dihydrogenorthophosphate (AnalaR, 99–102%) from Merck (Poole, UK), sodium acetate (AnalaR, 99%) from BDH (Poole, UK) or sodium carbonate (AnalaR, 99%) from Beecroft and Partners (Rotherham, UK). The pH was adjusted with either sodium hydroxide (analytical-reagent grade, 98%) from Rhône Poulenc (Manchester, UK) or glacial acetic acid (analytical-reagent grade, 99%) from Koch-Light (Haverhill, UK). A selection of tricyclic antidepressants was obtained from Sigma (Poole, UK) and Sinequan (a pharmaceutical preparation containing doxepin) was obtained from Pfizer (Sandwich, Kent). All solutions were made up in water prepared by reverse osmosis followed by ion exchange (Elgastat UHQ, PSII Elga Ltd., UK) and no further purification of reagents was required.

Instrumentation

The instrumentation used has been described in detail previously.[7] A 100 μl sample loop was used and all connections in the flow injection system were constructed from 0.8 mm internal diameter PTFE tubing obtained from Anachem (Luton, Bedfordshire, UK). The flow cell was built from solid PTFE and housed in a light-tight aluminium box. Potentials were applied to the electrodes using a three-electrode potentiostat. The electrodes consisted of a platinum disc-working electrode and a silver pseudo-reference electrode, both housed within the flow cell. A platinum-wired counter electrode was also incorporated downstream of the flow cell. The light was detected using a photomultiplier tube (Thorn EMI, 9789QB, Ruislip, UK) and the signals were amplified and recorded using a chart recorder (Chessel, Worthing, Sussex, UK).

Experimental procedure

Optimisation. A flow rate of 2.0 ml min^{-1} and a $Ru(bpy)_3^{2+}$ concentration of 1 mmol l^{-1} were used to give a sufficiently sensitive ECL signal but also to minimise reagent consumption. These parameters have been previously optimised for the application of the $Ru(bpy)_3^{2+}$ system to the detection of amines.[8] A voltage pulse of 2.5 s was used in the buffer concentration optimisation experiments. This pulse length was sufficiently long for the maximum ECL intensity to be achieved.

The pH and the voltage were optimised simultaneously using a multivariate approach, since these two parameters interact. To obtain ECL the potential must be greater than the oxidation potential of the amine and the pH must be high enough to allow deprotonation of the TCA. A pH range of 3.5–11.5 was examined for each compound by using a selection of buffers. The TCA (1×10^{-4} mol l^{-1}) and $Ru(bpy)_3^{2+}$ (1×10^{-3} mol l^{-1}) were pre-mixed with the buffer and pumped continuously through the flow cell while a linear voltage ramp from +0.51 to +2.01 V was applied to the working electrode. The photomultiplier tube (PMT) was held at 1250 V throughout the experiment. The pH/buffer combination, which gave the greatest signal to blank ratio and the voltage at which the peak maximum occurred at this pH, were observed.

The effect of buffer concentration was investigated over the range 0.01–0.5 mol l^{-1}. Previous reports have shown that a minimum buffer concentration of 0.01 mol l^{-1} is required for sufficient conductivity to occur between the electrodes but at high buffer strengths the ECL reaction is inhibited.[9] Experiments were carried out by pre-mixing the TCA (1×10^{-4} mol l^{-1}) and $Ru(bpy)_3^{2+}$ (1×10^{-3} mol l^{-1}) with the buffer and pumping continuously through the flow cell, while applying 2.50 s voltage pulses at the optimum voltage for the particular TCA. The PMT was held at 1250 V over the course of the experiment.

Calibration. A calibration for each compound was carried out utilising the optimised conditions. A concentration range of 1–400 μmol l^{-1} was analysed for each sample. Each standard also contained 1×10^{-3} mol l^{-1} $Ru(bpy)_3^{2+}$. 100 μl of each standard were injected into a buffer carrier stream, and this was transported over the working electrode that was held constant at the optimum voltage for the TCA being analysed. The PMT was held at 1250 V. Five replicate injections were made for each calibration standard.

Results and discussion

Effect of pH and applied voltage

The ECL signal for the blank samples [containing $Ru(bpy)_3^{2+}$ dissolved in buffer only] increased gradually with increasing pH up to 8.5 when the increase became rapid. This is because a background emission occurs between $Ru(bpy)_3^{2+}$ and OH^- ions and therefore it is better to work at lower pH values so that the blank signal is minimised.

Fig. 3 illustrates the ECL signal intensity variation with pH for the compounds tested in acetate and phosphate buffer, only doxepin was soluble in carbonate buffer. The optimum pH for each compound was selected as the one that gave the highest sample signal-to-blank signal ratio. The optimum applied voltage was measured as the voltage where the peak maximum occurred for the pH which gave the best response. Table 1 summarises the optimised parameters for the TCA's.

Nortriptyline had a higher optimum pH than the other TCA's because in order to deprotonate the nitrogen in the secondary amine functional group, a more basic solution was needed. This is because there are fewer alkyl groups on the secondary amine functional group than in tertiary amine groups found in the other TCA's which are electron releasing and make deprotonation of the compound easier and increase the stabilisation of the resulting radical. The optimum voltage for this compound was

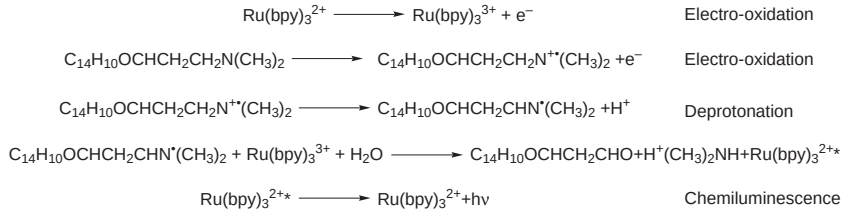

Fig. 2 Proposed mechanism for ECL reaction involving $Ru(bpy)_3^{2+}$ and doxepin.

also quite high relative to the other TCA's, since a higher potential is needed to oxidise the compound in the electro-oxidation step. Amitriptyline and doxepin had lower pH and optimum voltage values than nortriptyline, since they contain a tertiary amine functional group that makes the compound easier to protonate and oxidise.

Chlorpromazine and promazine also had low optimum pH values since they contain tertiary amine groups. These compounds also contain an additional tertiary amine group adjacent to the aromatic rings, which may be more readily oxidised. This means that the relatively low optimum pH values obtained for chlorpromazine and promazine are partially due to the reactivity of this group. The electro-oxidation step for promazine occurred at a lower potential than for chlorpromazine since chlorpromazine contains highly electronegative substituted chlorine that will stabilise the compound and make oxidation more difficult. The variation of signal intensity with buffer concentration for the range of compounds was then tested. The optimum buffer concentration (shown in Table 1) was chosen as the concentration that gave the highest signal-to-blank ratio. This was the concentration that provided adequate conductivity but did not significantly inhibit the ECL response.

TCA compounds with degrading ECL signals

Some of the tricyclic antidepressants that were analysed gave ECL signals that decreased significantly over generation of a few peaks only (10–12 mV). These compounds were imipramine, clomipramine, protriptyline, desipramine and trimipramine. Experiments to investigate the cause of this signal decrease were carried out using clomipramine, as this compound gave one of the most badly degrading signals. It was thought that the decrease could be either due to the solution degrading with age or because of electrode fouling.

A solution containing 1×10^{-3} mol l^{-1} $Ru(bpy)_3^{2+}$ and 1×10^{-4} mol l^{-1} clomipramine was prepared in 0.05 mol l^{-1} phosphate buffer of pH 6.5. This was pumped continuously through the flow cell at a rate of 2.0 ml min^{-1}, and a voltage ramp from 0.51 to 2.01 V was applied. The PMT was held at 1250 V. Analysing a solution, then repeating the analysis after 20 min tested the effect of the age of the solution, but the intensity of a sample analysed after 20 min was the same as the initial intensity and therefore this was not the problem.

A cyclic voltage from +2.5 to −2.5 V was then applied to clean the electrode, however the signal continued to degrade significantly (by 10 mV). Buffer and methanol/buffer (70:30) washes were then carried out to wash the flow cell through and also to remove any deposits on the electrode and this time the signal only degraded by 2.25, 1.50 and 1.50 mV for 3, 4 and 5 min washes, respectively. Therefore, it was decided to carry out a pH/voltage optimisation for clomipramine with a 4 min methanol/buffer wash between each sample analysed. This technique proved successful since only one measurement was taken for each pH in each buffer, so in total 13 sample solutions were analysed with a 4 min wash between each one. (No wash was needed between the blank solutions). An optimum pH of 5.5 in acetate buffer was detected, together with an optimum applied voltage of 1.32 V.

An optimisation of the buffer concentration and calibration could not be carried out since a 4 min wash between each sample would result in experiments which were impractical in terms of duration. It was also shown that although the addition of methanol to the sample solutions improved the degradation it also had serious detrimental effects on the intensity of the ECL signal. A reduced concentration of clomipramine (1×10^{-5} mol l^{-1}) in the solutions resulted in a steady ECL signal that did not degrade.

It is presumed that the degradation in signal is due to electrode fouling, caused by an oxidation product, which is produced from the compound. This product seems to be insoluble in buffer but soluble in methanol solutions. Using a lower concentration of the compound in the analysis alleviates this problem.

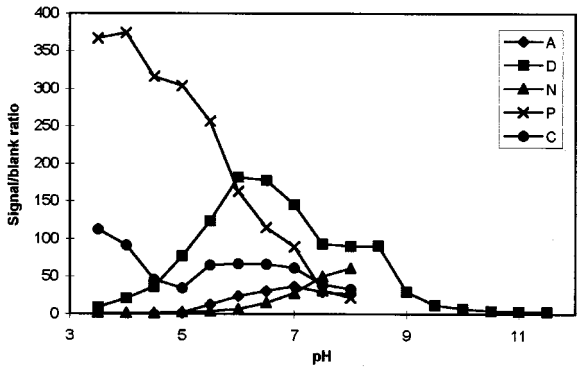

Fig. 3 Variation of ECL response with pH; ◆-amitriptyline, ■-doxepin, ▲-nortriptyline, ×-promazine, ●-chlorpromazine.

Table 1 Optimised conditions for ECL

Compound	Voltage/V	Buffer type	pH	Buffer concentration/mol l^{-1}
Amitriptyline	1.26	Phosphate	7.0	0.050
Doxepin	1.32	Phosphate	6.0	0.060
Nortriptyline	1.35	Phosphate	8.0	0.025
Promazine	1.26	Acetate	4.0	0.025
Chlorpromazine	1.40	Acetate	3.5	0.050

Calibration

Using the conditions given in Table 1 and following the procedure described in the experimental section linear log–log calibration plots were obtained for amitriptyline, doxepin, nortriptyline, promazine and chlorpromazine over three decades of concentration, *i.e.* 1–400 μmol l^{-1} (see Fig. 4). Each point on each graph is the mean of five replicate injections. The limit of detection (LOD) was determined by plotting the last six points of the calibration on a linear scale; this was over the concentration range 1–200 μmol l^{-1} for each TCA compound. This graph was used to assign the LOD as the concentration of the TCA compound producing a signal equal to that produced by the blank plus three times the standard deviation of the blank (Table 2). The calibration graphs exhibit good linearity, which is indicated by the values for the regression coefficients. The slopes of the calibration graphs illustrate the relative sensitivities of the TCA's, with amitriptyline being the most sensitive and chlorpromazine the least sensitive. The reproducibility (Table 2) was investigated at each concentration level used for the calibrations and the mean RSD values (calculated from five replicates at each concentration level) illustrate that the results

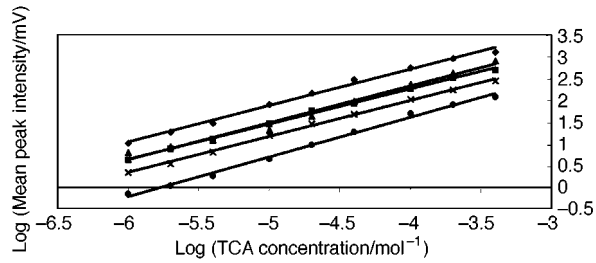

Fig. 4 Log–log calibration plots obtained for the TCA compounds; ◆-amitriptyline, ■-doxepin, ▲-nortriptyline, ×-promazine, ●-chlorpromazine.

were highly reproducible. The limits of detection were sufficiently low as to be valuable for detecting these compounds at therapeutic and toxic levels and are comparable with other techniques. For example a recent paper by Negrusz *et al.*[10] used gas chromatography-mass spectrometry to measure doxepin levels in hair. The limit of detection they obtained was 0.25 ng mg^{-1} doxepin in hair (which was a concentration of 0.31 μg ml^{-1} in the solution injected into the instrument). The retention time of the doxepin on the gas chromatography column was 11.48 min.

The differences in ECL activity between this series of compounds can be explained by considering their structures. Compounds containing tertiary amine groups are more ECL active than those containing secondary amine groups. This is because the electron deficient radical intermediate in the reaction will be stabilised by the presence of alkyl chains on the amine nitrogen atom, due to an inductive effect, and thus the more stable the radical intermediate, the more efficient the ECL is. Promazine and chlorpromazine both contain an additional tertiary amine group directly attached to the two aromatic rings which will lower the ECL intensity of these compounds, since this group may be preferentially oxidised in a competing reaction. This explains why promazine and chlorpromazine are less sensitive than the other TCA's analysed. Chlorpromazine is less sensitive than promazine, probably due to the presence of substituted chlorine having electron-withdrawing properties that will destabilise the amine radical and make oxidation more difficult. Nortriptyline contains a secondary amine functional group only and this explains why it is not as sensitive as compounds containing tertiary amine groups. Amitriptyline and doxepin will be the most ECL-active compounds since they only contain a tertiary amine functional group. However, doxepin is less sensitive than amitriptyline, possibly due to the presence of the electronegative oxygen atom, which will destabilise the amine radical slightly.

The problem of matrix effects on the ECL signal was investigated in the pharmaceutical preparation Sinequan, produced by Pfizer. This preparation contains doxepin (117.2 mg g^{-1}) and is potentially dangerous because toxicity to doxepin occurs when the plasma level is only three times the upper therapeutic range and the drug is prescribed to people suffering from depression who are prone to suicide.[10] Doxepin is the only active ingredient in Sinequin capsules which also contain cornstarch, magnesium stearate and sodium lauryl sulfate.

The preparation was determined using the standard additions method. All the solutions were prepared in 0.06 mol l^{-1} phosphate buffer of pH 6, as previously optimised for doxepin. A calibration graph in the concentration range 1–20 μmol was used. The equation of the regression line was calculated to be: $y = 4.0 \times 10^5 x + 5.2$, where y = mean peak intensity (in mV) and x = quantity of doxepin added (in mol^{-1}). The gradient of the calibration was about an order of magnitude lower than that obtained with standard solutions and this was most likely due to the presence of the surfactant in the matrix. The regression coefficient of the calibration line was 0.9975. The mean RSD value was calculated to be 4.03 and shows that the results were reproducible over the range tested. The experimentally determined concentration of doxepin in the Sinequan present in the solution was 3.65±0.47 μg ml^{-1} which agrees with the value of 3.21 μg ml^{-1} that would be expected in the solution using the value stated by the manufacturers.

Table 2 Calibration data for five tricyclic antidepressants

Compound	Calibration equation	r (n = 6)	Mean RSD (%) (n = 5)	LOD/ μg ml^{-1}
Amitriptyline	$y = 7.0 \times 10^6 x + 1.76$	0.999	2.82	0.09
Doxepin	$y = 3.0 \times 10^6 x + 1.53$	0.999	3.09	0.10
Nortriptyline	$y = 2.0 \times 10^6 x + 4.50$	0.995	4.34	0.31
Promazine	$y = 1.0 \times 10^6 x + 0.89$	0.999	3.12	0.16
Chlorpromazine	$y = 0.7 \times 10^6 x + 0.56$	0.991	3.88	0.24

Conclusions and future work

Electrogenerated chemluminescence has proved to be a suitable analytical method for the determination of a range of TCA's including amitriptyline, doxepin, nortriptyline, promazine and chlorpromazine. Severe signal degradation was however observed for imipramine, clomipramine, protriptyline, desipramine and trimipramine and this was shown to be due to electrode fouling caused by an oxidation product of the reaction. Use of a lower concentration of the compound in the analysis alleviated the problem.

The method has now been shown to be effective for the analysis of a TCA in a pharmaceutical product and the next step will be to determine TCA's in biological fluids. To successfully achieve this, sample clean up will be needed prior to analysis and a solid-phase extraction (SPE) procedure is currently being developed. It will be necessary to overcome matrix effects. Care will need to be taken in selecting the solvents used in the SPE step however as, for example, methanol is known to degrade the ECL signal.

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