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The Date rape Drug – Flunitrazepam - Electroanalytical Sensing Using Electrogenerated Chemiluminescence

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The electro-analytical sensing of Flunitrazepam is reported for the first time utilising $[Ru(bpy)_3]^{2+}$ as a Electrochemiluminescent reagent probe without any sample pre-treatment. The methodology is shown to be useful for quantifying low ng/mL Flunitrazepam in buffer. We have successfully demonstrated the detection of Flunitrazepam over a range of Flunitrazepam concentrations 2000 ng/mL to 50 ng/mL into a pH 4 buffer. A linear response is observed (*ECL emission/mV = 0.0094x+6.3204*, R^2 = 0.9937 and N = 5) with a detection limit of 39 ng/mL (based on 3-sigma).

Keywords: Flunitrazepam, Electrochemiluminescence and Electroanalytical Sensing.

1. INTRODUCTION

Flunitrazepam, a benzodiazepine is an anxiolytic and hypnotic drug known better under the name Rohypnol, the chemical structure of which is shown in Figure 1. It is normally administered as a short term treatment for sleeping disorders such as insomnia[1-2].

Flunitrazepam has been attracted a lot of attention since its illegal use as a "date rape drug" by spiking into alcoholic drinks above the recommended pharmacological dose of 0.5-1 mg in adults[1] to produce a prolonged and extreme intoxication. The sedative effect of the drug is increased by alcohol consumption which creates marked psychomotor impairment and causes the victim to suffer from a "blackout", a type of a short term amnesia that prevents the victim from recalling much if any of the attack. Its low dosage and high biotransformation makes its analysis very problematic because it is so rapidly cleared out from the body [3-4].

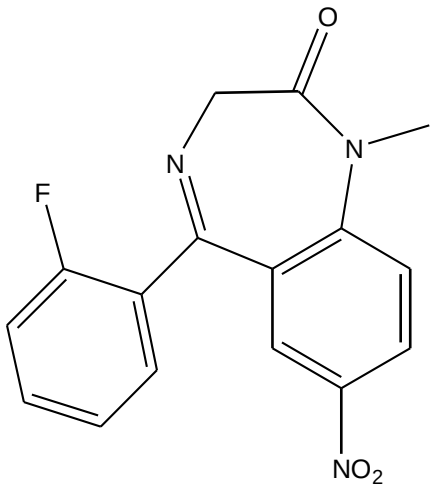
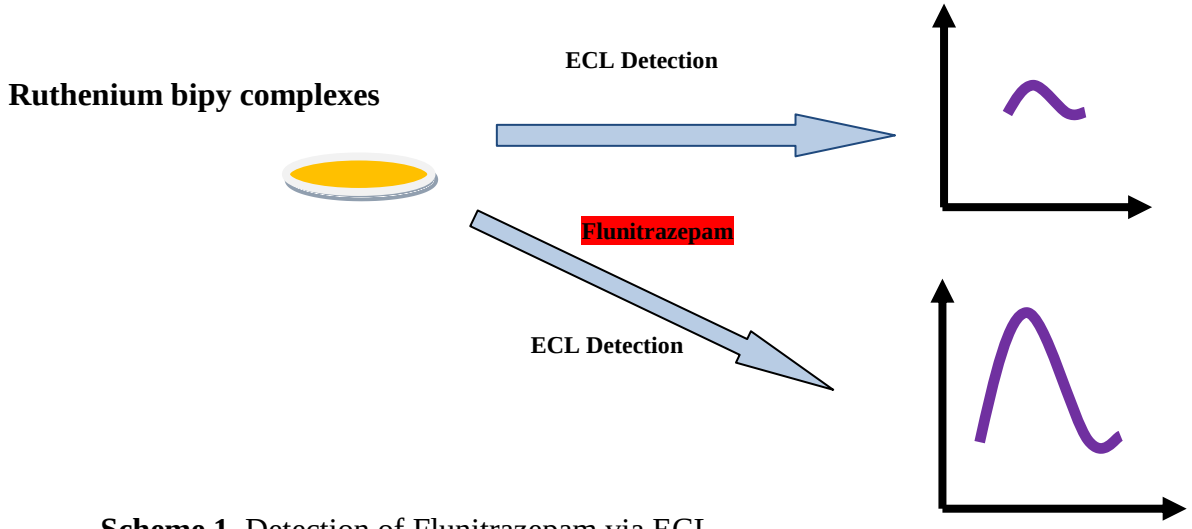


Figure 1. Structure of Flunitrazepam

Flunitrazepam is generally analysed by using immunoassays, Liquid Chromatography-Mass Spectroscopy (LC-MS)[5-7] or Gas Chromatography-Mass Spectroscopy (GC-MS)[8-11] methodologies in biological matrixes. Honeychurch and Hart[12] reported a technique for determining Flunitrazepam and Nitrazepam in beverage samples by Liquid Chromatography with dual electrode detection. Webb *et al* [13] described a method which involved the use of capillary zone electrophoresis without any sample pre-treatment, but the analysis time was 6.5 minutes per sample. The low dosage and high distribution is one of the problems with the analysis of Flunitrazepam. The drawbacks to these methods are that the times for the analysis are longer than 6 minutes per sample and a liquid–liquid extraction prior to analysis is also needed[2, 12-13].

Electrochemiluminescence (ECL) is an advantageous analytical tool, which is cost effective, portable and fast. $[Ru(bpy)_3]^{2+}$ is one the most important ECL reagents and the most widely used complexes for analytical investigations and applications owing to their excellent intrinsic characteristics[14-17]. It has been widely employed in biological matrixes[18], pharmaceutical[19] and some drugs containing tertiary amine functional group [18, 20-22] due to its continuance, sensitivity, reproducibility and selectivity towards many target analytes [18, 21, 23-24]. Generally, the amino group present in amine produces a secondary amine following by a dealkylation process in the presence of water. The radical cation of the amine is able to react with $[Ru(bpy)_3]^{2+}$ or $[Ru(bpy)_3]^{3+}$ to yield excited state $[Ru(bpy)_3^{+2}]^*$ and hence to produce light emission [25].

In this paper we investigate the ECL response and sensing of Flunitrazepam in aqueous solution (buffer). It is reported for the first time that the low ng/mL sensing of the target analytes such as Flunitrazepam are possible using ECL. The unique and novel benefits of this approach are that no-sample pre-treatment is required (such as dilution with a buffer). Furthermore this technique provides a highly reproducible and reliable platform for electrochemical measurement of the target analyte. The enhancement of the ECL emission is depicted in Scheme 1 when Flunitrazepam a tertiary amine reacts with $[Ru(bpy)_3]^{2+}$.



Scheme 1. Detection of Flunitrazepam via ECL.

2. EXPERIMENTAL SECTION

2.1 Reagents

All chemical reagents used to prepare solutions were purchased in their purest commercially available forms from Aldrich. All aqueous solutions were made up with water (of resistivity of not less than 18 MΩ cm) taken from an Elgastat filter system (Vivendi, Bucks., UK). All experiments were undertaken at 23± 2 ºC. Flunitrazepam and Tris-(2,2'-bipyridyl) Ruthenium(II) Dichloride Hexahydrate were obtained from Sigma Aldrich.

2.2 Apparatus

Electrochemical experiments were conducted in a conventional three-electrode borosilicate glass cell with a flat bottom, using a 3.0 mm diameter glassy carbon working electrode (Bioanalytical Systems, Inc., UK), a nickel wire counter electrode, and a saturated calomel electrode (SCE, Radiometer, Copenhagen, Denmark). All results reported in this paper are, however, corrected to the SCE. Electrochemical data were recorded using a commercially available computer-controlled potentiostat (Autolab PGSTAT30, Eco Chemie, Utrecht, Netherlands, or PalmSens Instruments device). The electrode was cleaned by rinsing with dichloromethane and polishing on a napped polishing cloth using 0.3 mm alumina slurry (Presi, France) immediately prior to experimentation.

For ECL experiments, the electrochemical cell was positioned on top of a miniaturised photomultiplier tube with maximal sensitivity close to the 610 nm photon (H5784-20, Hamamatsu Photonics, Enfield, UK, borosilicate window, range $300 \leq \lambda/\text{nm} \leq 920$, peak wavelength of 630 nm) protected by a shutter. The latter was opened for data recording after the whole apparatus had been placed in the dark, so as to minimise the background recording of daylight. The chemiluminescence signal was recorded using a chart recorder (Chessel Ltd., Worthing, Sussex); ECL experiments were only undertaken when the background signal had stabilised. The ECL data were not subjected to any

post factum correction for the absorbance of the borosilicate glass, nor for the distance between the electrode in solution and the bottom of the cell; small changes in the latter had little effect on the data reported herein.

3. RESULTS AND DISCUSSION

3.1 Optimisation of the ECL protocol

We first study the selection of the correct concentration of tris-(2,2'-bipyridyl) ruthenium(II) dichloride hexahydrate. The amount of $[Ru(bpy)_3]^{2+}$ used should be sufficient to produce a good and stable luminescent signal without excessive self-quenching occurring (*i.e.,* external conversion) but it should not be excessive due to reagent cost. Examining the level of $[Ru(bpy)_3]^{2+}$ typically used in similar studies by others groups[26-31] it was found that the common concentration of $[Ru(bpy)_3]^{2+}$ used in these studies was 1 mM. Obviously a greater concentration of $[Ru(bpy)_3]^{2+}$ will give a stronger ECL signal until self-quenching becomes an issue therefore it was decided to use a $[Ru(bpy)_3]^{2+}$ concentration of 1 mM in this work.

Next we study the effect of pH on the ECL emission of the $[Ru(bpy)_3]^{2+}$. A 2000 ng/mL of Flunitrazepam standard solution and tris(2,2'-bipyridyl)ruthenium(II) chloride hexahydrate 1 mM was prepared in acetate buffer 1 mM and used to optimise the required pH for the reaction. Figure 2 shows the effect of pH on the ECL signal for the reaction between 2000 ng/mL of Flunitrazepam, 1 mM $[Ru(bpy)_3]^{2+}$ in 1 mM acetate buffer.

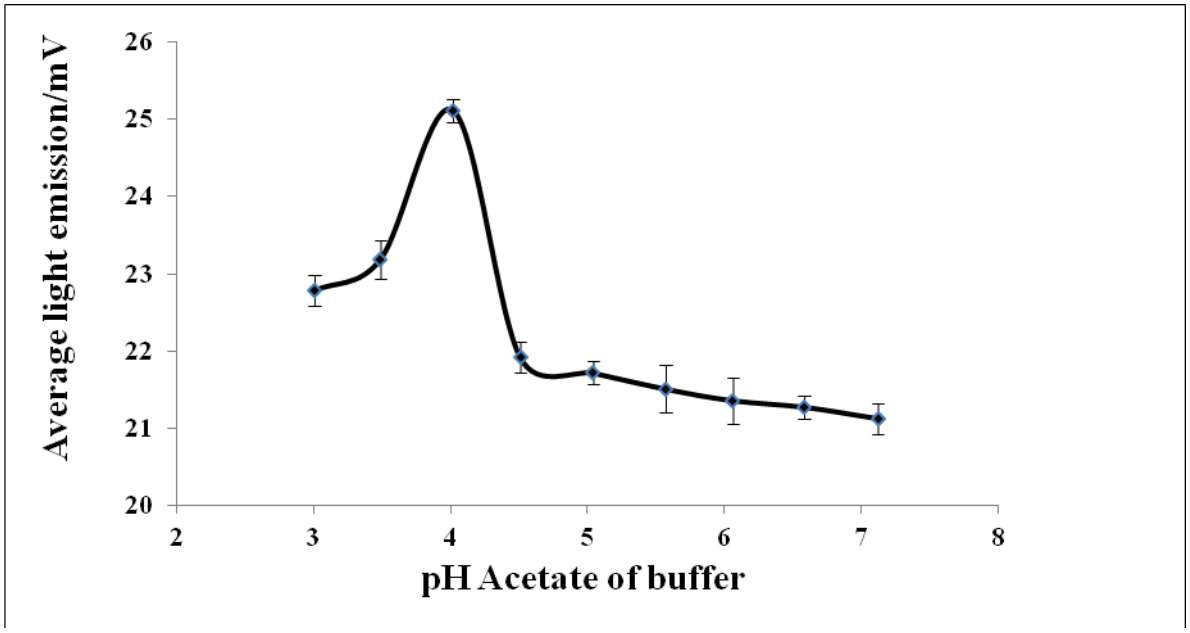


Figure 2. pH effect on ECL.

The experiment results showed that ECL responses of Flunitrazepam are highly dependent on the pH solution as illustrated in Figure 2. Strong chemiluminescence responses were observed in the pH range from 3.0 to 4.0. Very weak ECL responses were obtained after the pH of the solution reached 4.0.

The change in ECL emission as a function of pH with three replicates, average of ECL emission, standard deviation and the percentage of the relative standard deviation are illustrated in Table 1.

Table 1. The change in ECL emission as a function of pH for a single electrode replicated three times.

pH of Acetate Buffer	Replicate 1 ECL emission (mV)	Replicate 2 ECL emission (mV)	Replicate 3 ECL emission (mV)	Average ECL emission (mV)	Standard deviation	%Relative standard deviation
3.01	22.75	22.89	22.72	22.75	0.090738	0.000907
3.49	23.23	23.13	23.21	23.23	0.052915	0.000529
4.02	25.15	25.1	25.09	25.15	0.032146	0.000321
4.51	21.96	21.88	21.91	21.96	0.040415	0.000404
5.04	21.76	21.69	21.71	21.76	0.036056	0.000361
5.57	21.52	21.45	21.57	21.52	0.060277	0.000603
6.06	21.35	21.31	21.43	21.35	0.061101	0.000611
6.58	21.24	21.31	21.29	21.24	0.036056	0.000361
7.12	21.12	21.09	21.18	21.12	0.045826	0.000458

Following we explored the effect of $[Ru(bpy)_3]^{2+}$ with and without Flunitrazepam. Depicted in Figure 3 are illustrated the voltammograms of: a) 1 mM $[Ru(bpy)_3]^{2+}$ in 1 mM acetate buffer at pH 4 without Flunitrazepam and b) 2000 ng/mL of Rohyonol in 1 mM $[Ru(bpy)_3]^{2+}$ with 1 mM acetate buffer at pH 4 both acquired at 100 mV/s.

Figure 3a shows a voltammogram with a pair of redox waves with an oxidation peak at 1.10V and a reduction peak at 1.02 V, which was attributed to the one-electron redox reaction of $[Ru(bpy)_3]^{2+}$. In the presence of Flunitrazepam (Figure 3b), the oxidation peak current increased dramatically, and the oxidation peak potential hardly shifted this is due to the oxidation of Flunitrazepam. The addition of Flunitrazepam also increased the oxidation rate kinetics of $[Ru(bpy)_3]^{2+}$ resulting in the increase of ECL intensity which agrees with the mechanism proposed in Scheme 3.

Next, after determining that the optimum experimental pH for the ECL generation with Flunitrazepam and $[Ru(bpy)_3]^{2+}$ using glassy carbon electrode was pH 4, the effect of varying the scan rate was studied as depicted in Figure 4. In order to investigate the reaction kinetics, the effect of scan rate on the redox of $[Ru(bpy)_3]^{2+}$ and Flunitrazepam was investigated and a superimposed voltammogram was shown in Figure 4.

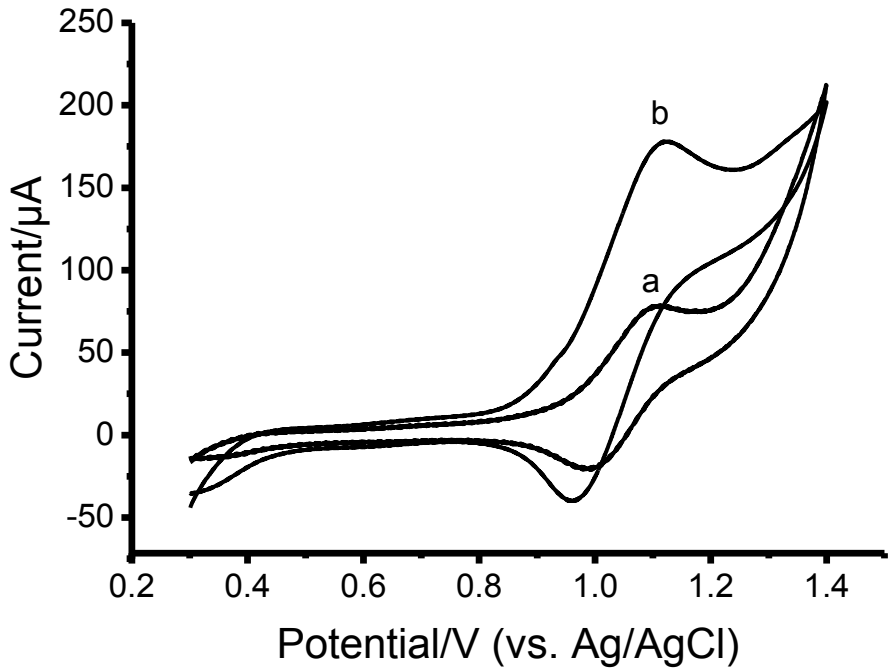


Figure 3. Cyclic voltammograms of the glassy carbon electrode.(a) 1 mM $[Ru(bpy)_3]^{2+}$ in acetate buffer pH 4; (b) 2000ng/mL of Flunitrazepam in 1 mM $[Ru(bpy)_3]^{2+}$ with 1 mM acetate buffer at pH 4 both acquired at scan rate100 mV/s.

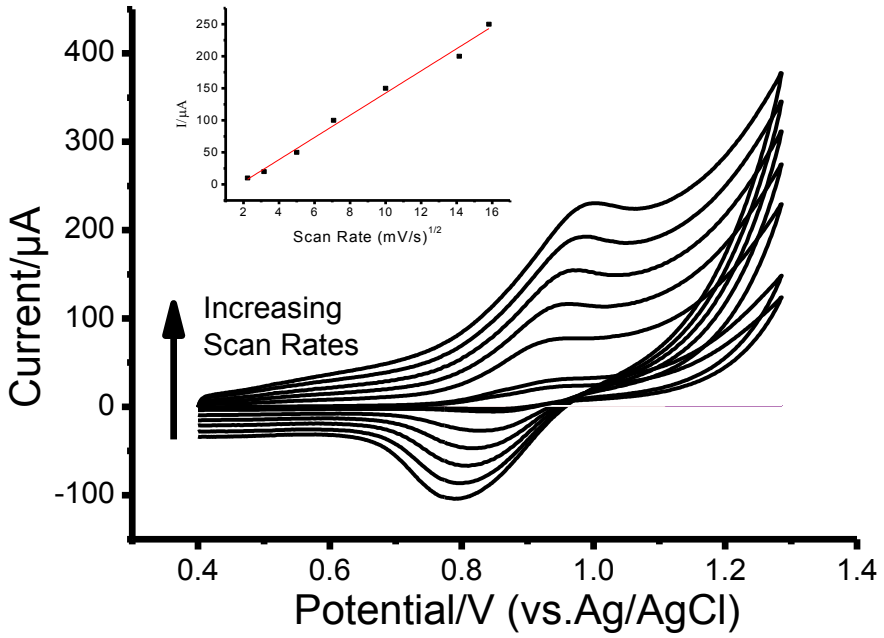
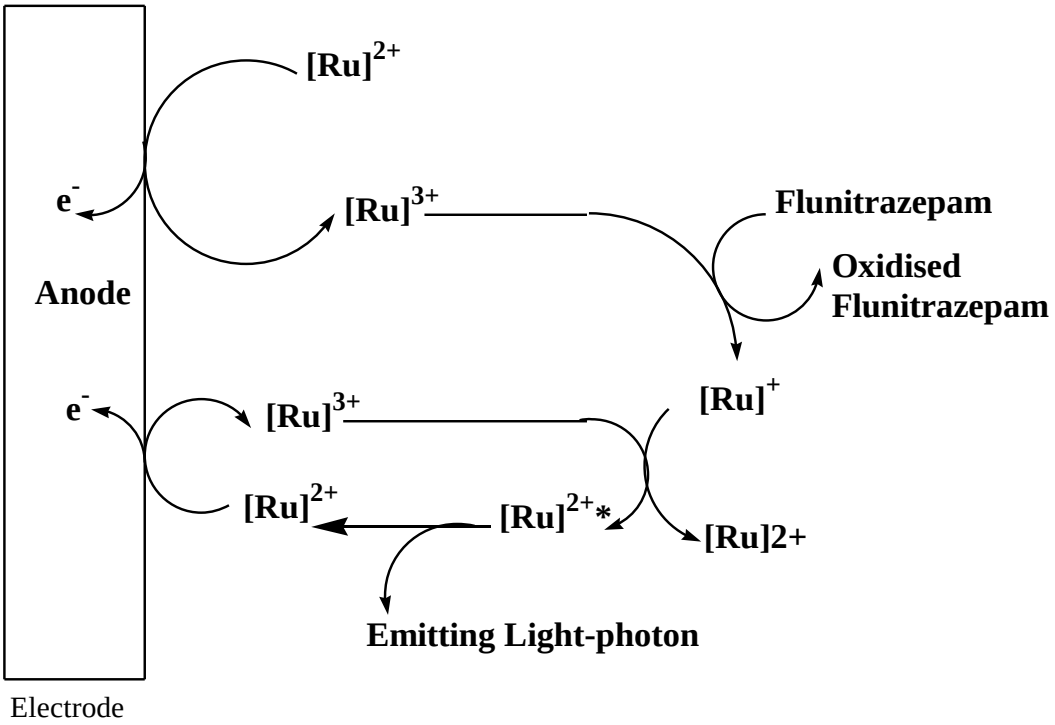


Figure 4. Voltammograms for scan rates between 5 and 250 V/s in a solution of 2000 ng/mL Flunitrazepam, 1 mM $[Ru(bpy)_3]^{2+}$ with 1 mM acetate buffer at pH 4. The cross-lines indicate the point of origin and the arrow shows the direction of the initial potential sweep.

As it can be seen, an oxidation peak and reduction peak were observed in all scan rates, indicating that the electrochemical response of $[Ru(bpy)_3]^{2+}$ and Flunitrazepam is a reversible electron process. Scan rates over the range 5 to 250 mV/s were explored which revealed a linear response described by the following equation (IP/μA = 17.10 μA/(V s^{-1})$^{1/2}$ +30.07 μA, R^2 = 0.99) of the peak current (IP) as a function of the square root of the scan rate (ν). Indicating diffusion controlled electrochemical process. 100mV/s scan rate was chosen to allow multiple scans at lower concentrations without the signal reducing effects of analyte diffusion.

3.2 ECL Detection of Flunitrazepam

Next, after exploring and optimizing the ECL conditions, was to detect Flunitrazepam using $[Ru(bpy)_3]^{2+}$ as a ECL reagent. Since the ECL emission of the system arose from the energetic electron-transfer reduction between the electrogenerated $[Ru(bpy)_3]^{3+}$ and the electro-oxidation production of Flunitrazepam. The ECL reaction of $[Ru(bpy)_3]^{2+}$ with Flunitrazepam can be expressed as follows in Scheme 2.



Scheme 2. Emission of light with Flunitrazepam via ECL.

Following we show an typical response of ECL signal mV for 2000 ng/mL of Rohyonol in 1mM $[Ru(bpy)_3]^{2+}$ with 1 mM acetate buffer at pH 4.

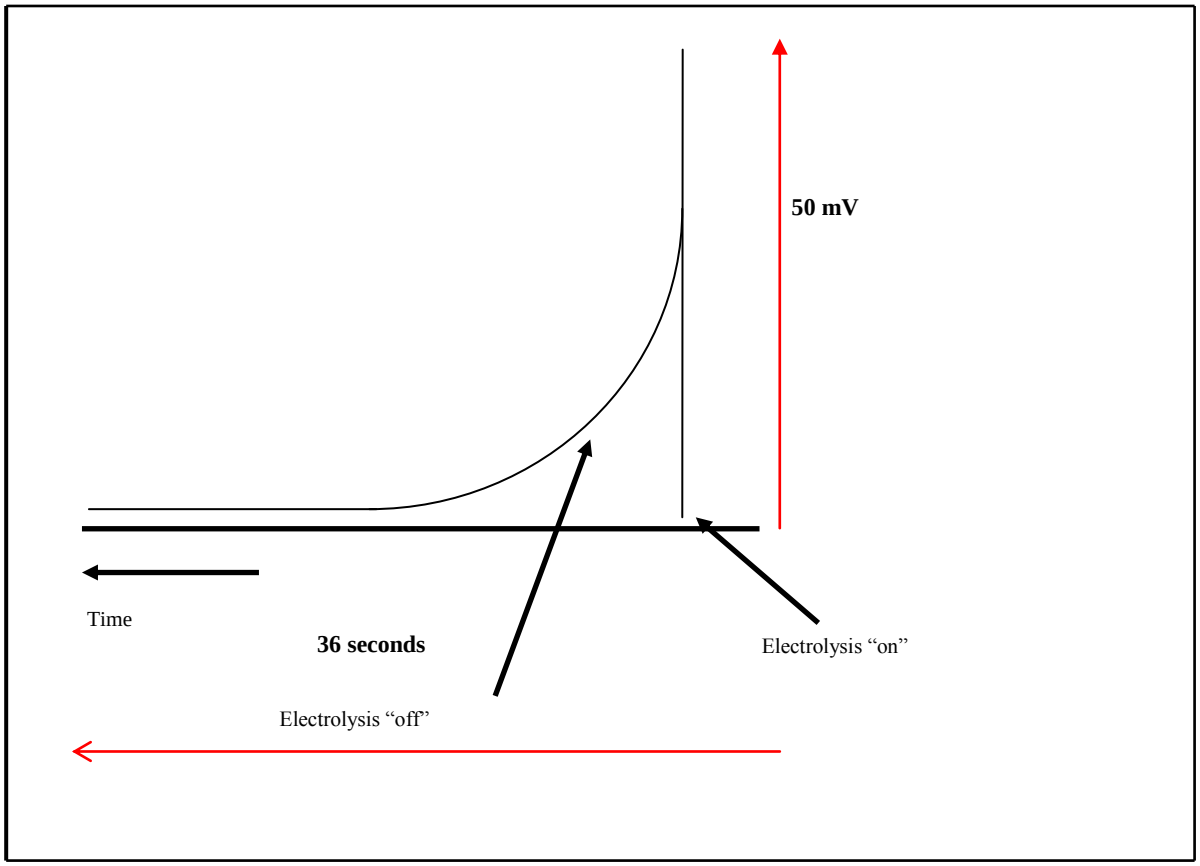


Figure 5. Typical response of ECL transient (signal vs. time) obtained under a single stepped potential electrolysis for 2000 ng/mL of Flunitrazepam in 1mM $[Ru(bpy)_3]^{2+}$ with 1 mM acetate buffer at pH 4.

The value of the ECL emission was 25.10 mV for 2000 ng/mL of Rohyonol in 1mM $[Ru(bpy)_3]^{2+}$ with 1 mM acetate buffer at pH 4 and the time employed to record the whole spectrum was 36 seconds. When the voltage was applied from t = 0 to 19 seconds there was no ECL emission. Between 19 seconds and 20 seconds a sharp peak was formed due to the ECL emission. After 20 seconds the ECL emission decayed exponentially due to a catalytic response until reaches the end 36 seconds. This behaviour can be explained in terms of producing ECL from a redox reaction of $[Ru(bpy)_3]^{2+}$ and activation of Flunitrazepam as observed previously for amines in water (Scheme 3) [26].

$$[Ru(bpy)_3]^{2+} \longrightarrow [Ru(bpy)_3]^{3+} + e^-$$
$$[Ru(bpy)_3]^{3+} + R_2NCH_2R \longrightarrow [Ru(bpy)_3]^{2+} + R_2N^{+\cdot}CH_2R$$
$$[Ru(bpy)_3]^{2+} + R_2N^{+\cdot}CH_2R + H_2O \longrightarrow R_2NH + OCHR + [Ru(bpy)_3]^{+} + 2H^{+}$$
$$[Ru(bpy)_3]^{3+} + [Ru(bpy)_3]^{+} \longrightarrow [Ru(bpy)_3]^{2+} + [Ru(bpy)_3^{2+}]^{*}$$
$$[Ru(bpy)_3^{2+}]^{*} \longrightarrow [Ru(bpy)_3]^{2+} + h\nu \ (\lambda_{max}=610nm)$$

Scheme 3. ECL reaction processes of amine and $[Ru(bpy)_3]^{2+}$.

The shape of the ECL transients illustrated in Figure 5 verifies that there is a solution pH-dependent response in the cessation of ECL when the electrochemical perturbation is removed: in acidic solutions there is a slow, first-order decay of the ECL signal.

The ECL intensities of Flunitrazepam were measured as shown in Table 2.

Table 2. The change in ECL emission as a function of the Flunitrazepam concentration for a single electrode replicated three times.

Flunitrazepam (ng/mL)	Replicate 1 ECL emission (mV)	Replicate 2 ECL emission (mV)	Replicate 3 ECL emission (mV)	Average ECL emission (mV)	Standard deviation	%Relative standard deviation
2000	25.1	25.2	25.13	25.15	0.051316	0.000513
1000	16.2	16.16	16.11	16.16	0.045092	0.000451
500	10.18	10.21	10.09	10.13	0.06245	0.000624
250	9.21	9.07	9.14	9.12	0.07	0.0007
50	7.28	7.19	7.45	7.32	0.132035	0.00132
Blank	6.03	5.95	5.83	5.9	0.100664	0.001007

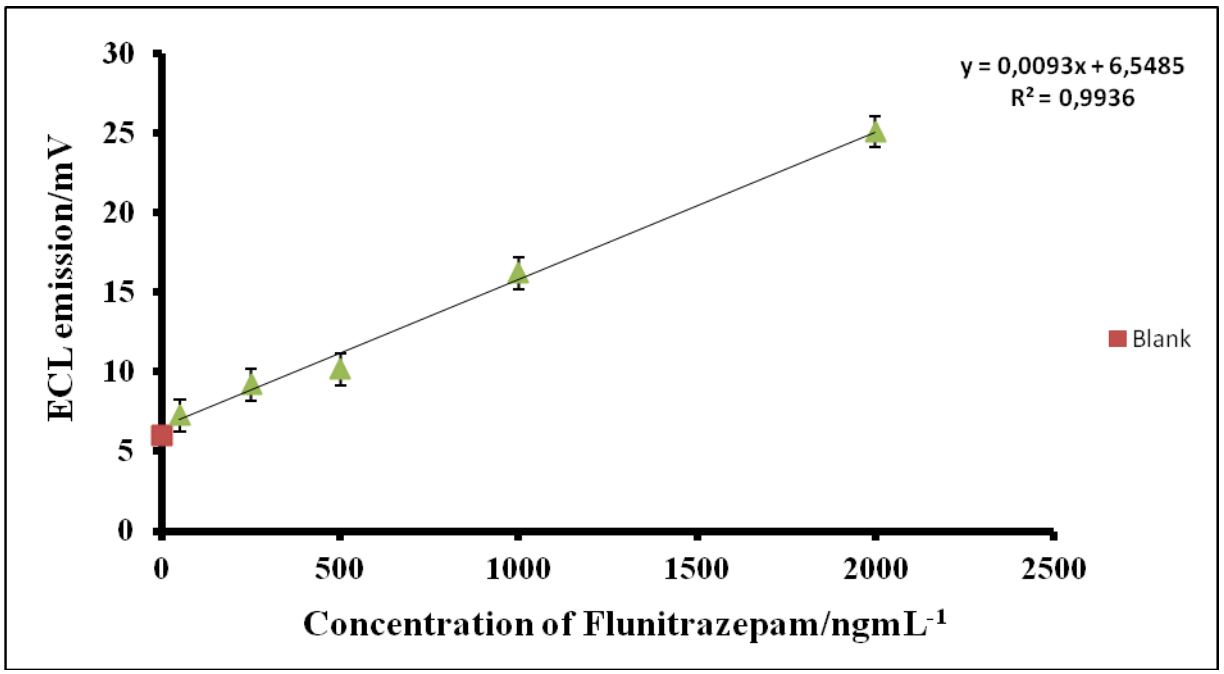


Figure 6. The effects of Flunitrazepam concentration on ECL emission.

This table illustrates the ECL emission of Flunitrazepam, with a range of different concentrations of Flunitrazepam from 2000 to 0 ng/mL. The ECL response depends on the Flunitrazepam concentration.

The blank also presents an ECL emission value, this is due to the presence of chloride ions coming from tris-(2,2'-bipyridyl) ruthenium(II) dichloride hexahydrate in aqueous medium. This reflects the kinetics of the ion-coupled electron transfer process which agrees with previous work by Bard regarding the distinct effect of halides on ECL intensity, potentially due to ionic stabilisation of the transition state[32-33].

The experiment results depicted in Figure 6 showed that the ECL intensity increased with the concentration of Flunitrazepam. The ECL emission intensities in mV were measured and plotted versus the Flunitrazepam concentration in ng/mL.

The ECL intensity increased linearly with the concentration of Flunitrazepam over the concentration range from 50 to 2000 ng/mL with an *ECL emission/mV = 0.0093x+6.5485*, R^2 = 0.9936 and N = 5) with a detection limit of 39ng/mL (based on 3-sigma). As shown in Figure 6, ECL intensities displayed a good reproducibility with a good precision for repetitive measurements at different concentrations of Flunitrazepam including the blank.

4. CONCLUSIONS

We have successfully provided for the first time an electrochemically sensing methodology via ECL with $[Ru(bpy)_3]^{2+}$ for detecting Flunitrazepam, a drug reported to be used extensively in case of "date-rape". Flunitrazepam is a tertiary amine and is extremely difficult to derivatise but we have successfully determined Flunitrazepam by ECL reaction with $[Ru(bpy)_3]^{2+}$, without prior derivatisation. The Flunitrazepam electroanalytical protocol utilises for the first time ECL allowing Flunitrazepam to be readily determined in without the requirement of any pH modification or further sample preparation. The ECL detection of Flunitrazepam showed a high sensitivity and a reproducible linear response. A linear response is observed (*ECL emission/mV = 0.0094 x +6.3204*, R^2 = 0.9937 and N = 5) over the range 2000 ng/mL to 50 ng/mL with a detection limit of 39ng/mL (based on 3-sigma).

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