# Novel Reductive-Reductive Mode Electrochemical Detection of Rohypnol Following Liquid Chromatography and Its Determination in Coffee

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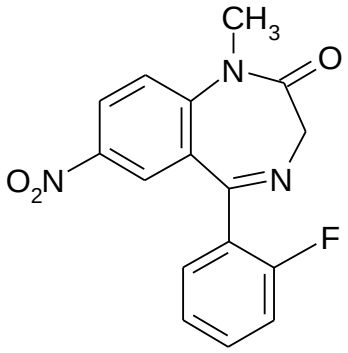
## Abstract

Rohypnol (flunitrazepam) has been determined by high performance liquid chromatography dual electrode detection (LC-DED), both in the redox mode and the dual reductive mode. Initial studies were performed to optimise the chromatographic conditions and these were found to be 50 % acetonitrile, 50 % 50 mM pH 2.0 phosphate buffer at a flow rate of 0.75 ml/min, employing a Hypersil $C_{18}$, 5 µm, 250 mm x 4.6 mm column. Cyclic voltammetric studies were made to ascertain the redox behaviour of Rohypnol at a glassy carbon electrode over the pH range 2-12. Hydrodynamic voltammetry was used to optimise the applied potential at the generator and detector cells; these were identified to be -2.4 V and +0.8 V for the redox mode and -2.4 V and -0.1 V for the dual reductive mode respectively. A linear range of 0.5 µg/ml to 100 µg/ml, with a detection limit of 20 ng/ml was obtained for the dual reductive mode. Further studies were then performed to identify the optimum conditions required for the LC-DED determination of Rohypnol in beverage samples. A convenient and rapid method for the determination of Rohypnol in beverage samples was developed using a simple sample pre-treatment procedure. A recovery of 95.5 % was achieved for a sample of white coffee fortified at 9.6 µg/ml Rohypnol.

**Keywords:** liquid chromatography dual electrode detection, drug facilitated assault, cyclic voltammetry, beverages, Rohypnol.

## 1. Introduction




(I)

Rohypnol (5-(2-Fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one, flunitrazepam) (I) has received a great deal of attention due its medical [1] and increased elicit usage [2-7]. It produces a variety of actions, including sedative effects, anxiolytic muscle-relaxation with a pronounced hypnotic effect and memory loss [8]. As a result, it has been employed in many medical applications such as in sleeping disorders, and as a pre-anaesthetic medication for procedures including colonoscopy. However, these effects are known to be heightened when taken with alcohol, and other drugs, consequently, it is often taken illicitly to enhance or elevate their effects [5]. The psychotropic sedative effects and memory loss exhibited by Rohypnol has led to it being increasingly reported in sexual assaults and increasingly involved in robberies, where the victim is rendered unable to resist the attacker and has an incomplete recollection of the attack. Spiking of the victim's drink by the assailant is a common *modus operandi* as it is relative

easy method to administer prior to such attacks [7] particularly as the amount of Rohypnol required is very low.

For the above reason, sensitive methods capable of determining Rohypnol in differing complex media are required to quantify and identify this drug. A number of methods have been developed, such as immunoassay [9], GC/MS [10] and HPLC [11,12]. However, there are relatively few reports on the determination of benzodiazepines in beverage samples [13-18]. One powerful technique, which has received little attention for the determination of benzodiazepines, is high performance liquid chromatography with dual electrode detection (LC-DED). To our knowledge, there are only a few reports on the use of this technique for the determination of any of the nitro substituted benzodiazepines [19,20]. LC-DED has been shown to be both highly sensitive and selective, and has been shown to give much improved results over LC single electrochemical detection [21,22]. DED utilises two electrochemical cells arranged in either series or in parallel located after the analytical column. In the series configuration the first upstream electrochemical cell can be used as a "generator" where an electroactive product can be formed which is then detected at the following downstream "detector" cell. This approach provides a number of advantages, the main one of these being that the electrochemically generated can be more easily oxidised or reduced than the parent compound. This allows for the selectivity of the system to be improved as the detector cell can be operated at a lower applied potential. Consequently, lower background currents, resulting in lower noise, are observed compared to those obtained at the higher working potentials required for direct electrochemical detection. Similarly, due to the

use of lower working potentials, the number of other compounds which can possible interfere is greatly reduced.

Previously [19,20], we have employed the LC-DED approach in what is referred to as the "redox mode". As the name implies compounds are first electrochemically reduced (most commonly) at the generator electrode and then oxidised at the subsequent detector electrode. Recently, however, we have reported on a variation of this system, which we described as the dual reductive mode [23]. For certain compounds such as *p*-nitrophenol, by selection of the correct experimental conditions, species can be formed at the generator electrode which can then be reduced further at the detector electrode at potentials close to 0 V. This adds a greater degree of selectivity as many of the common electrochemical interferences, such ascorbic acid, uric acid and phenolic derivatives will not undergo reduction at such small applied negative potentials.

In the present investigation we first investigated the voltammetric behaviour of Rohypnol at a glassy carbon electrode (GCE). We then optimised the chromatographic conditions. In the final section we investigate the possible advantages of both the redox mode the dual reduction mode for the determination of Rohypnol in beverage samples.

## 2. Experimental

### 2.1 Chemicals and Reagents

All chemicals were obtained from Fisher (Loughborough, UK), unless otherwise stated. Deionised water was obtained from a Purite RO200 - Stillplus HP System, fitted with a Pur-1-te ion-exchanger (Purite Oxon., UK). A 50 mM phosphate pH 2.0 buffer was prepared by titration of a solution of 50 mM disodium hydrogen phosphate, with 50 mM phosphoric acid. Primary

stock solutions of Rohypnol, (Sigma-Aldrich, Dorset, UK), were prepared by dissolving the required mass in acetonitrile or methanol to give a concentration of 10 mM. Working standards, for initial voltammetric studies, were prepared by dilution of the primary stock in sufficient acetonitrile, phosphate buffer, to give an overall concentration of 50 % acetonitrile, 50 % pH 2.0, 50 mM phosphate buffer. Standards for LC-DED analysis were made by dilution of the primary acetonitrile stock solution in mobile phase. Beverage samples were obtained from a local commercial outlet.

### 2.2 Apparatus and Instrumentation

#### 2.2.1 Cyclic voltammetry

Cyclic voltammetry (CV) was performed with an EG&G Princeton Applied Research (Princeton, NJ, USA) Model 263 potentiostat connected to a PC with EG&G Echem electrochemistry software. The voltammetric cell (Metrohm, Switzerland) contained a glass coated platinum wire auxiliary electrode, a saturated calomel electrode (SCE) (Russell, Fife, UK) and a glassy carbon electrode as the working electrode.

#### 2.2.2 High Performance Liquid Chromatography

HPLC studies were undertaken using a system consisting of an IsoChrom pump (Spectra Physics), with a 250 mm x 4.6 mm Hypersil Hypurity $C_{18}$, 5 μm column connected to a 7125 valve manual injector fitted with a 50 μl sample loop (Rheodyne, Cotati, USA). Sample extracts were determined using a mobile phase of 50 % acetonitrile, (Fischer, Far UV, HPLC grade) 50 % 50 mM pH 2.0 phosphate buffer, at a flow rate of 0.75 ml/min.

#### 2.2.3 Dual Electrode Detection

Both the generator and detector cells were as previously described [19,20]. The generator cell consisted of a two piece thin-layer cell, formed from an

upper Teflon block containing a GCE working electrode (3 mm diameter) and a bottom steel block serving as the pseudo-reference/counter electrode. The analytical detector cell consisted of a Teflon two-piece (top and base) thin-layer cell. The detector cell operated as a three-electrode configuration comprising a GCE (3 mm diameter), a stainless steel counter electrode and a Ag/AgCl reference. Teflon gaskets were purchased from BAS, Congleton, Cheshire, UK.

An EG&G Princeton Applied Research (Princeton, NJ, USA) Model 362 scanning potentiostat was used to control the potential at the generator cell at -2.40 V vs. the pseudoreference/counter steel electrode. The potential at the detector cell was controlled and the current monitored using a BAS LC-4B amperometric detector. Chromatograms were recorded using a Siemens Kompensograph X-T C1012 chart recorder.

**2.3 Cyclic voltammetric studies**

Cyclic voltammograms were initially recorded in plain solutions of 10 % methanol, 90 % phosphate buffer and then in the same solution containing 1.0 mM Rohypnol. Degassing was achieved by purging with oxygen free nitrogen (BOC, Guildford, UK) for 5 minutes to eliminate oxygen reduction waves. A starting potential of 0.0 V was used, with an initial switching potential of -1.5 V and a second switching potential of +0.8 V, with a final potential of -0.5 V. The effect of scan rate was studied over the range 20 mV/s to 200 mV/s.

**2.4 Hydrodynamic Voltammetry**

Hydrodynamic voltammetry (HDV) was performed by injecting fixed volumes of a standard solution of Rohypnol and varying the applied potential between -1.9 V and -3.1 V (*vs.* Stainless steel) for the downstream generator electrode, and between -0.6 V and +1.1 V (*vs.* Ag/AgCl) for the upstream detector cell.

HDVs were constructed by plotting the recorded peak current against the applied potential. The optimum potential was determined from the position of the plateau on the hydrodynamic wave.

### 2.5 Sample Pre-treatment Procedure

A Cappuccino style white milk coffee was purchased from a local commercial outlet and a 250 µl aliquot was taken and added to a glass vial followed by 500 µl of acetone. The resulting mixture was then passed through a 2 µm PTFE syringe filter (Gelman Laboratory, Acrodisc CR 13 mm syringe filter) and the filtrate collected in a glass vial. The filter was then washed with 200 µl of acetone and the resulting solution combined with the original filtrate in the same glass vial. The combined filtrates were then blown down to dryness under nitrogen. The residue was then reconstituted in mobile phase (50% acetonitrile, 50% 50 mM pH 2.0 phosphate buffer), and syringe filtered again. Aliquots of the filtrate were then examined by LC-DED using a generator potential of -2.4 V and a detector potential of -0.1 V.

## 3. Results and discussion

### 3.1. Cyclic voltammetric investigations

Initial cyclic voltammetric studies were performed with a 1 mM solution of Rohypnol, dissolved in 0.1 M phosphate buffer pH 5, in the presence of 10 % methanol (10 ml). Figure 1 shows the CV obtained at a GCE using a scan rate ($\upsilon$) of 50 mV/s. This shows two reduction peaks, R1 and R2 on the first negative scan. R1 was concluded to be due to the reduction of the nitro group to hydroxylamine (eq. 1).

$$R\text{-}NO_2 + 4e^- + 4H^+ \rightarrow R\text{-}HNOH + H_2O \qquad (1)$$

The second reduction peak, R2 we believe results from the irreversible reduction of the 4,5-azomethine group (eq. 2).

$$R'\text{-}C{=}N\text{-}R'' + 2e^- + 2H^+ \rightarrow R'\text{-}CH\text{-}NH\text{-}R'' \quad (2)$$

R1 is oxidised on the return anodic scan to a nitrosamine species (eq. 3), to give the oxidation peak O1.

$$R\text{-}HNOH \rightleftharpoons R\text{-}NO + 2e^- + 2H^+ \quad (3)$$

On subsequent scans, a further reduction peak, R3 is seen. We believe that this is a result of the reduction of the nitrosamine, back to a hydroxylamine (eq. 3). The ΔEp were calculated at pH 5 to be 490 mV, demonstrating the quasi-reversible nature of the redox pair.

This behaviour is similar to that observed for nitrazepam in our previous study [19]. However, this differs notably from that reported at Hg electrodes [24]. In our present study using a GCE, the $4e^-$, $4H^+$ reduction peak of the nitro group (R1) is twice the magnitude of the reduction process R2, the latter we conclude is the $2e^-$, $2H^+$ irreversible reduction of the 4,5-azomethine group. However, at Hg electrodes two reduction waves of equal magnitude are reported [24], the first resulting from the $4e^-$, $4H^+$ reduction of the nitro group, as reported here, but at Hg this is followed by a second reduction wave of equal magnitude, resulting form the combined $2e^-$, $2H^+$ reduction of the 4,5-azomethine bond, together with the $2e^-$, $2H^+$ reduction of the hydroxylamine to the corresponding amine. We deduced that, in our present study, R2 is the result of the reduction of the 4,5-azomethine group, and not the reduction of the hydroxylamine to an amine, as we would not observe the

resulting redox couple (O1/R3) if the amine had been formed during the process producing R2.

The effect of scan rate was studied at pH 2, 4, 6, 8 and 10, over the scan rate 10 - 200 mV/s. For all the peaks studied, peak current ($i_p$) values were found to be proportional to the square of scan rate, demonstrating diffusion controlled processes. The Ep of all peaks identified was found to be pH dependent shifting to more negative potentials with decreasing pH.

### 3.2 Hydrodynamic Voltammetry

Figure 2a shows the cathodic HDV obtained with the generator cell over the potential range -1.9 to -3.1 V. The HDV exhibits a single wave which reaches a maximum at -2.4 V. Figure 2b shows the HDV obtained when the detector cell was changed from -0.2 V to +1.2 V, while keeping the generator cell at a potential of -2.4 V. The current response observed with the latter cell was found to exhibit a reduction maxima at *ca*. -0.1 V and an oxidation maxima at +1.0 V. Consequently, further studies were undertaken using an applied potential of -2.4 V at the generator cell, and +0.8 V at the detector cell for the redox mode detection; then the same generator cell potential and -0.1 V for the detector cell for dual reductive mode investigations respectively.

### 3.3 Effect of Flow Rate

Figure 3 shows the relationship of flow rate with peak area (coulombs) for the dual reductive mode. As we have observed in our previous study on *p*-nitrophenol [23], flow rate has a marked effect on the behaviour of compounds with our LC-DED system. The peak area was found to increase with decreasing flow rate, a phenomena that might be expected as the amperometric degree of electrolysis of the two electrodes approaches coulometric conditions with slower flow rates. The advantage of increased

sensitivity that this parameter offers is however, offset by the associated increase in retention time and band broadening, consequently, a flow rate of 0.75 ml/min was chosen for future studies as a compromise between sensitivity and assay time.

### 3.4. Proposed mechanism for the electrochemical behaviour of Rohypnol

We believe that scheme 1 shows the possible mechanism occurring for the redox and dual reductive detection mode. Rohypnol (I) is first reduced to give the nitroso species (II), via a $2e^-$, $2H^+$ reduction with the associated loss of $H_2O$. This species can then undergo a further $2e^-$, $2H^+$ reduction at the "detector" electrode in the case of the redox dual electrode system to give the hydroxylamine species (III).

This is unlike that seen by cyclic voltammetry undertaken in quiescent conditions as the residence time in the generator cell is relative short, a substantial portion of this intermediate could still be present. It should be mentioned that single cell LCEC in the reductive mode is not feasible for low concentrations of Rohypnol due to the presence of species which produce large interfering peaks [19].

### 3.5 Calibration Study

A linear range of 0.5 μg/ml to 100 μg/ml was obtained with an associated detection limit of 20 ng/ml, based on a signal to noise ratio of three.

### 3.6 Analytical Application

To assess the performance of the LC-DED, six replicate determinations of Rohypnol in spiked and unspiked beverage samples, was undertaken. Aliquots of the sample were prepared using the procedure described in section 2.6, and quantification was achieved by external calibration. In order to

demonstrate the application of the LC-DED assay to forensic "drink spiking" cases we fortified a sample of white Cappuccino style coffee at a level of 9.6 μg/ml Rohypnol. We were partially interested in investigating this type of beverage, containing a large portion of possible interferences such as fats and phenolic compounds as many other beverages can be readily assayed by simply diluting the sample with mobile phase or supporting electrolyte [17]. Figure 4 shows the LC-DED chromatograms for extracts of coffee spiked with 9.6 μg/ml (i) and (ii) for unspiked coffee (iii). Figure 4(i) shows that in the redox mode from a retention time of 11 minutes onwards the chromatogram is totally obscured by a large off-scale unresolved peak, completely masking the area where Rohypnol elutes. However, as shown in figure 4(ii), in the dual reductive mode, a well-defined peak for Rohypnol is recorded. As can be readily seen the method gives reliable data at the concentrations investigated relevant to cases of drink spiking.

## 4. Conclusions

An assay involving LC-DED, in both the dual reductive mode and in the redox mode has been successfully developed for the determination of trace levels of Rohypnol in beverage samples. The chromatographic separation is achieved using a $C_{18}$ reversed phase column in conjunction with acetonitrile-phosphate buffer as mobile phase. A convenient and simple sample pre-treatment procedure could be employed, which merely involved the addition of acetone to the sample followed by filtration. Therefore, this assay should be readily applicable to emergency and forensic examinations.

## 5. Acknowledgements

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## Figure and Table Legends

**Figure 1.** Typical cyclic voltammogram, obtained at a scan rate of 150 mV/s, for a 1 mM solution of Rohypnol in 10% methanol, buffered with 0.1 M phosphate at pH 5.2. Starting potential 0.0 V, initial switching potential -1.5 V, and second switching potential +0.8 V.

**Figure 2.** (a) Cathodic hydrodynamic voltammogram for 1.4 μg injections of Rohypnol. (b) Anodic hydrodynamic voltammogram for 1.4 μg injections of Rohypnol. Generator cell held at -2.4 V.

**Figure 3.** Effect of flow rate on Rohypnol peak area (charge in nQ).

**Figure 4.** Representative chromatograms of "Cappuccino" style white coffee samples obtained by LC-DED in the redox mode for (i) fortified at 9.6 μg/ml (ii) LC-DED dual reductive mode, fortified at 9.6 μg/ml and (iii) unadulterated. R = Rohypnol.

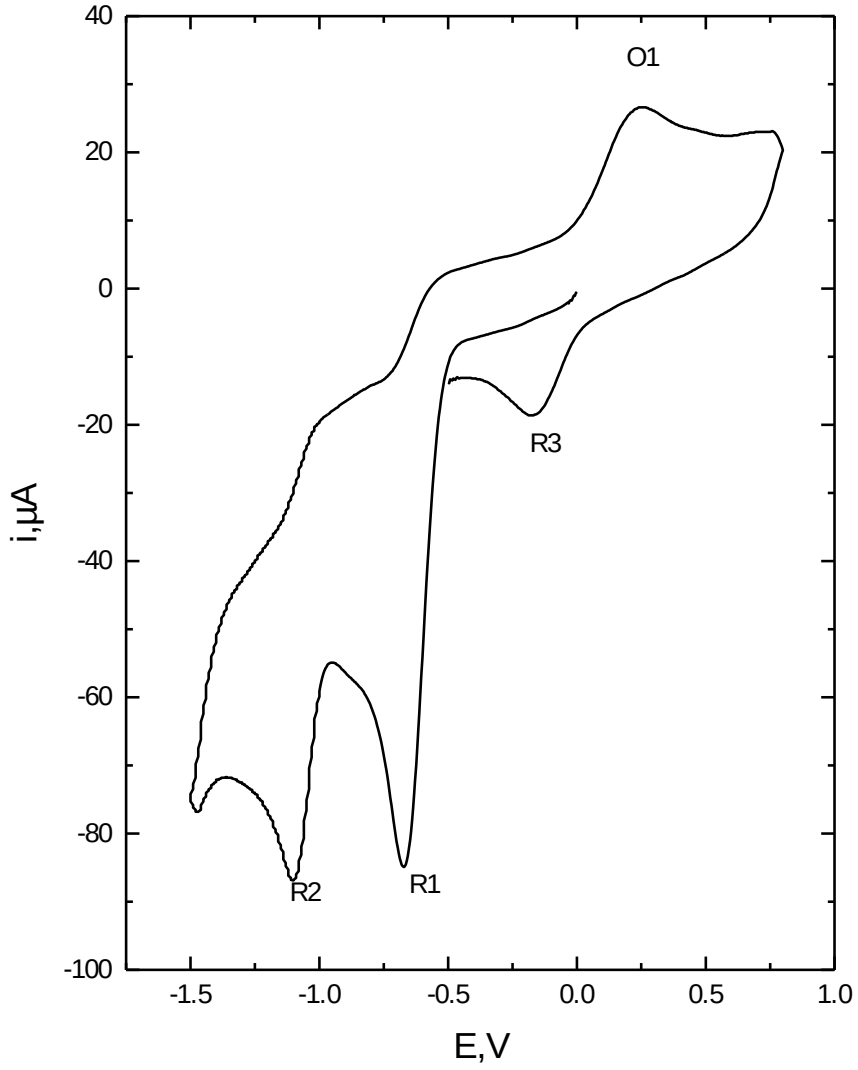



**Figure 1**

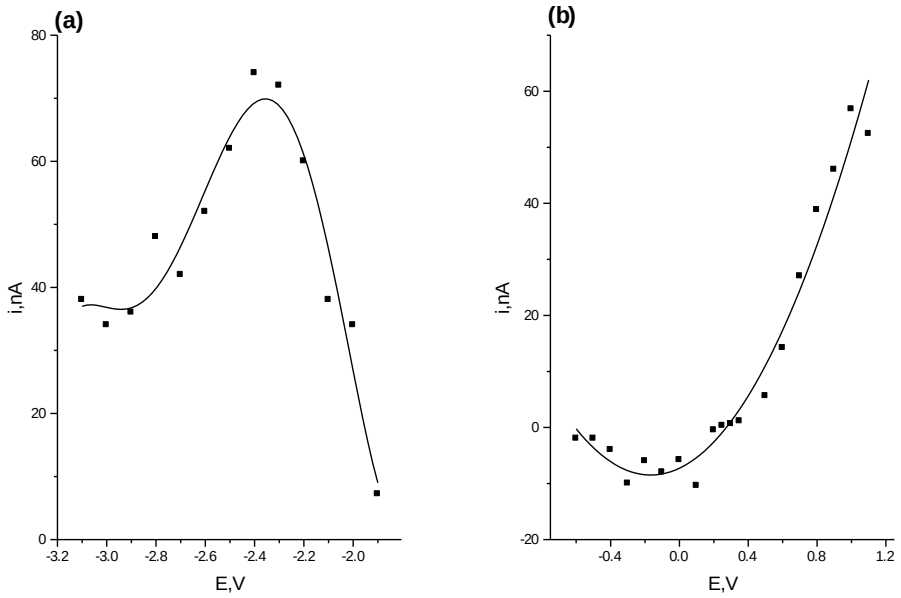

(a)
80
60
40
20
0
i,nA
-3.2
-3.0
-2.8
-2.6
-2.4
-2.2
-2.0
E,V
(b)
60
40
20
0
-20
i,nA
-0.4
0.0
0.4
0.8
1.2
E,V

**Figure 2**

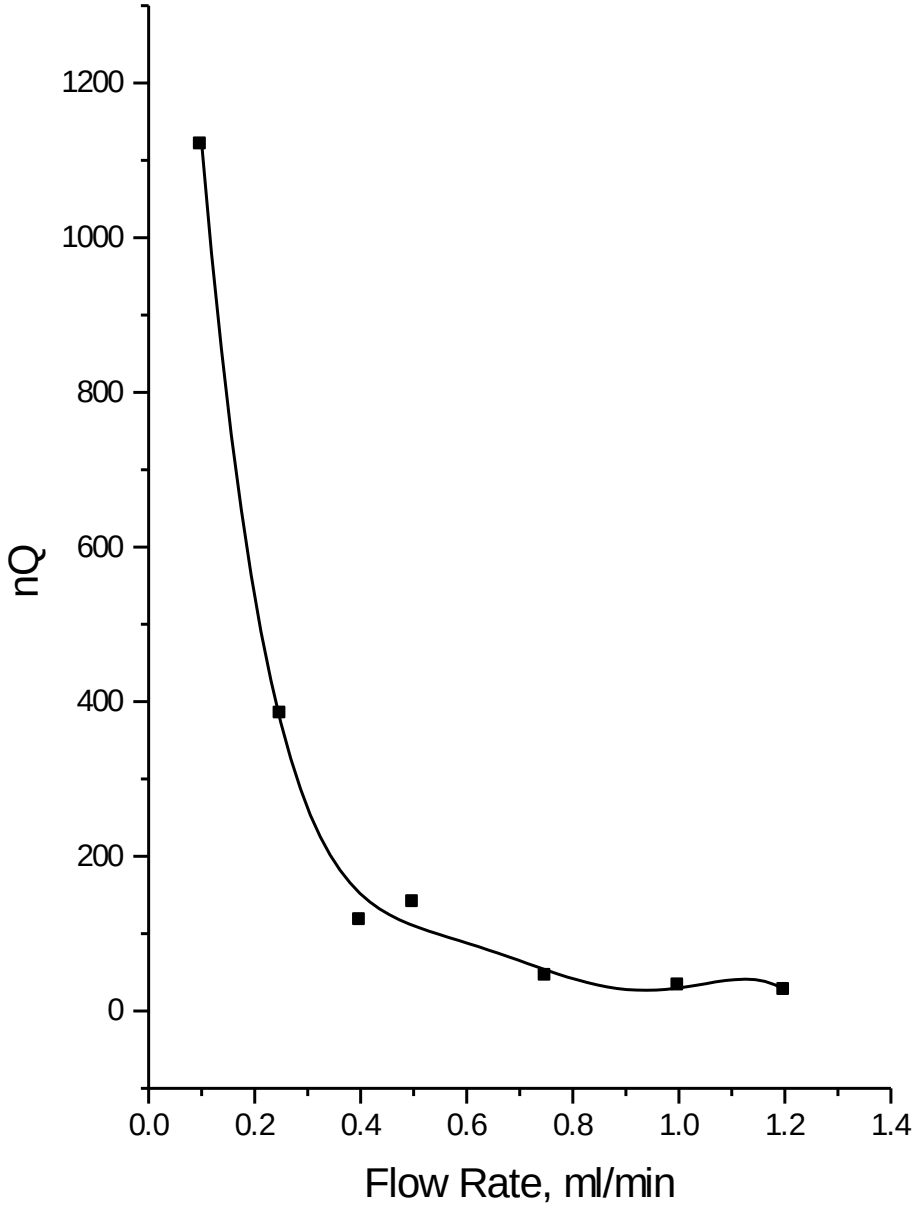

nQ
1200
1000
800
600
400
200
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Flow Rate, ml/min

**Figure 3**

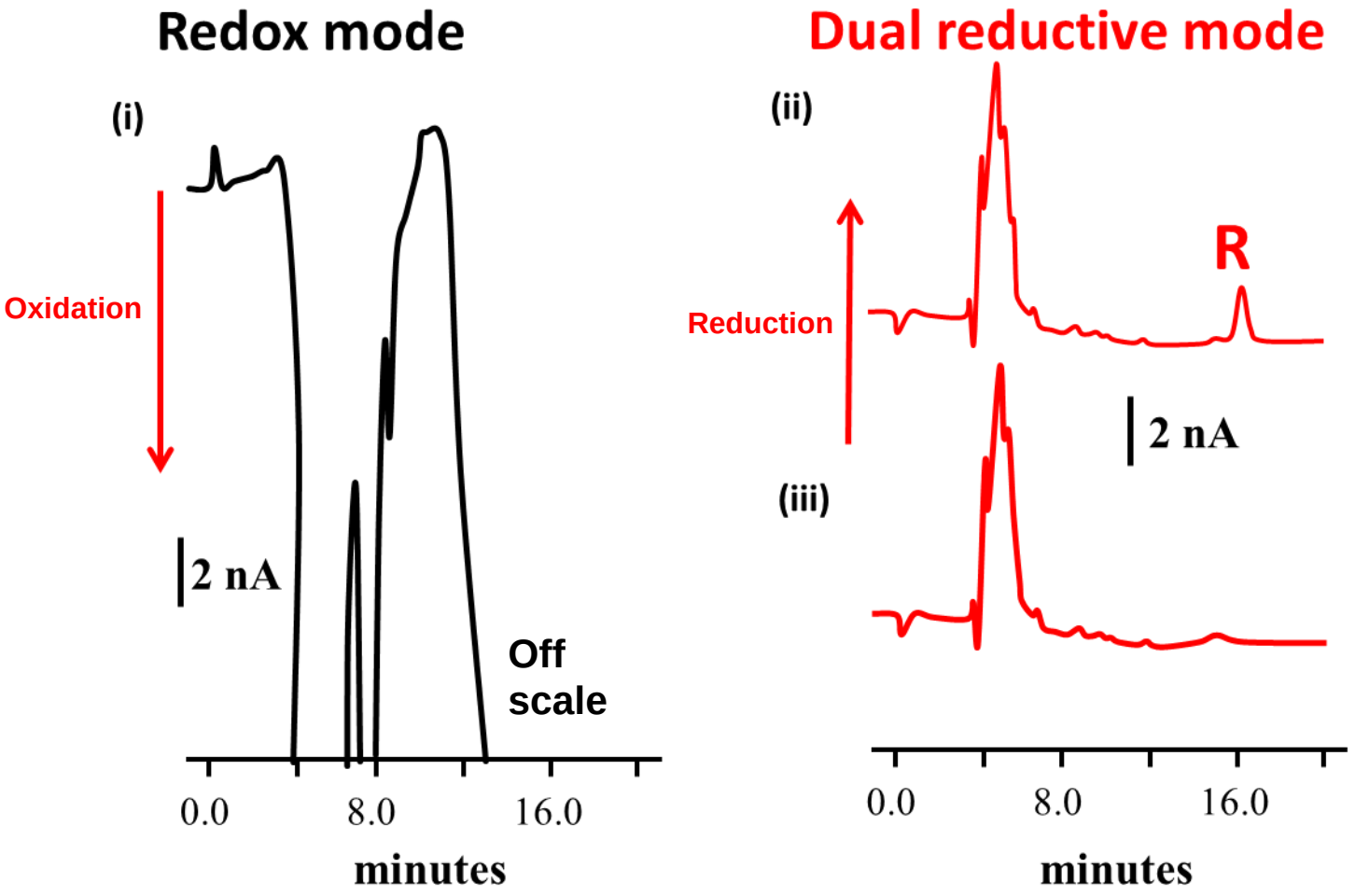
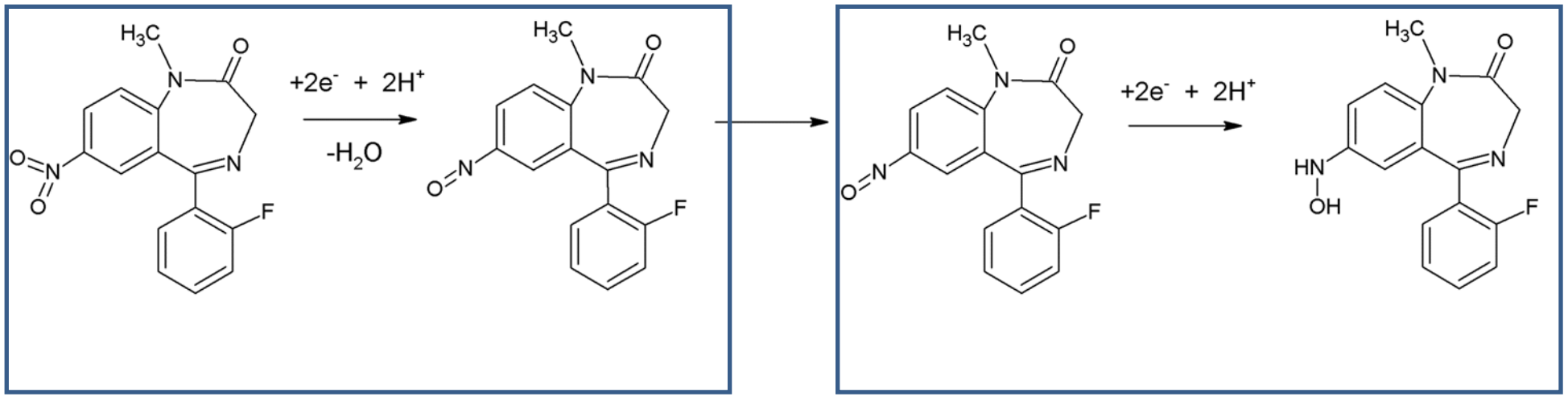

Redox mode
(i)
Oxidation
2 nA
Off
scale
0.0
8.0
16.0
minutes
Dual reductive mode
(ii)
R
Reduction
2 nA
(iii)
0.0
8.0
16.0
minutes

**Figure 4**




**Generator** → **Detector**

**Scheme 1**

## 6. References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