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ORIGINAL PAPER

Determination of flunitrazepam and nitrazepam in beverage samples by liquid chromatography with dual electrode detection using a carbon fibre veil electrode

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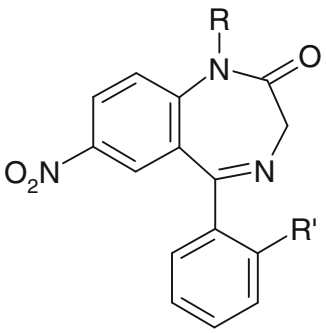
Abstract Both nitrazepam and flunitrazepam have been determined by high-performance liquid chromatography dual electrode detection (LC-DED) in the reductive–reductive mode, using a carbon fibre veil electrode (CFVE) in conjugation with a glassy carbon electrode. Initial studies were made to optimise the chromatographic conditions. These were found to be 45% acetonitrile-55% acetate buffer (50 mM, pH 4.1) at a flow rate of 1.0 ml/min, employing a Hypersil C18, 5 μm, 250 mm×4.6 mm column. Cyclic voltammetric studies performed to ascertain the redox behaviour of nitrazepam and flunitrazepam at a CFVE in the optimised mobile phase. Studies showed that similar voltammetric behaviour was obtained to that report at Hg or glassy carbon based electrodes. Further studies were then carried out to identify the optimum conditions required for the LC-DED determination of nitrazepam and flunitrazepam in beverage samples. Hydrodynamic voltammetry was used to optimise the applied potential at the generator and detector cells; these were identified to be −2.40 and −0.25 V, respectively. A linear range of 2.0 to 100 μg ml^{-1}, with a detection limit of 20 ng ml^{-1} was obtained. A convenient and rapid method for the determination of both nitrazepam and flunitrazepam in beverage sample was developed. Following a simple sample extraction procedure, extracts were examined using the optimised LC-DED procedure. An average percentage recovery of 95.5% (%CV=4.5%) for nitrazepam and 78.0% (%CV=8.8%) was achieved for a sample of "Pepsi Max" spiked at 1.0 μg ml^{-1} nitrazepam, 1.47 μg ml^{-1} flunitrazepam.

Keywords Carbon fibre veil · Liquid chromatography dual electrode detection · Drug facilitated assault · Cyclic voltammetry · Beverages · Benzodiazepines

Introduction



(I) R = R' = H

(II) R = CH_3, R' = F

A number of drugs originally developed for their medical use, such as the benzodiazepines nitrazepam (I) and flunitrazepam (II), have been receiving increasing attention in relation to their illicit use in assaults and robberies [1–3].

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Reports have highlighted that possibly 2,000 cases of drug facilitated robberies occur every year in UK alone [3], making such assaults an obvious problem. These generally go unreported as the victims are usually unwilling to discuss the crime with the police. More grievous, are the attacks of sexual predators, who use the sedative and the associated amnesia promoted by these drugs to incapacitate their victims [4–9], leaving them with an incomplete recollection of the attack. Many of the drugs utilised in such attacks require relatively small dosages to be effective and hence can be readily added to beverages without notable changes in taste or appearance [10].

A number of methods have been developed to determine these drugs, most focus on the application of gas chromatography-mass spectrometry (GC/MS), liquid chromatography-mass spectrometry (LC/MS) or immunoassay [11] for their determination in serum or biologically related samples. These require relatively extended extraction and derivatization; in the case of GC/MS, the use of deuterated standards [12]. Recently, we have reported on the determination of nitrazepam in bovine and human serum using liquid chromatography dual electrode detection (LC-DED) in the redox mode [13]. LC-DED has been shown to be both highly sensitive and selective and has been shown to give much improved results over LC with single electrochemical detection [14, 15]. In this mode, two electrochemical cells are arranged in series after the analytical column; the upstream electrochemical cell, often being referred to as the "generator" cell, as it is used to generate an electroactive product 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 product is more easily oxidised or reduced than the parent compound. Therefore, the selectivity of the system is improved as the detector cell can be operated at a lower applied potential. Consequently, lower background currents are observed compared to those obtained at the higher working potentials leading to an enhancement in sensitivity. In addition, due to the use of lower working potentials, the number of other compounds which can possibly interfere is greatly reduced.

In our recent investigations [13] on the determination of nitrazepam in serum, we utilised a LC-DED system that employed 3 mm diameter glassy carbon electrodes as the working electrodes in both the generator and detector cells. The current efficiency of such amperometric thin-layer cells tends to be notably lower than that seen for coulometric cells [16]. In the present study, we have sought to improve the efficiency of our detection system by substituting a carbon fibre veil electrode (CFVE) for the GCE in the generator cell. Recent studies by Ieropoulos et al. [17, 18] have shown the superior electrochemical properties that can be attained using these CFVEs. This material is known to have a very large surface area coupled to a low resistivity. The much larger surface area of the CFVE, we believe, would allow for a higher percentage of the target analytes being converted in the generator cell and hence increase the sensitivity of the method.

Cyclic voltammetric investigations were performed to ascertain the behaviour of nitrazepam and flunitrazepam at these CFVEs. The chromatographic and electrochemical conditions were then optimised, and the LC-DED assay was applied to the determination of nitrazepam and flunitrazepam in a spiked soft drink.

Experimental

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 acetate pH 4.1 buffer was made by titration of a solution of 50 mM sodium acetate, with a 50 mM acetic acid solution. Separate primary stock solutions of nitrazepam and flunitrazepam (both Sigma-Aldrich, Dorset, UK) were prepared by dissolving the required mass in acetonitrile to give a concentration of 10 mM. Working standards for initial voltammetric studies were prepared by dilution of the primary stock in sufficient acetonitrile, acetate buffer, to give an overall concentration of 45% acetonitrile–55% acetate buffer (pH 4.1, 50 mM). Standards for LC-DED analysis were made by dilution of the primary acetonitrile stock solution in mobile phase. Paracetamol, ascorbic acid, caffeine, Martius Yellow, Orange II and Orange G were obtained from Sigma-Aldrich (Dorset, UK). The 2-amino-5-nitrobenzophenone was obtained from Acros Organics (Geel, Belgium). The soft drink (Pepsi Max) was obtained from a local commercial outlet.

Apparatus and instrumentation

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 either a carbon fibre veil electrode (CFVE; 20 m^2 g^{-1}, 5 Ωm in the machine direction and 9 Ωm in the cross direction; PRF Composite Materials Poole, Dorset, UK) or a glassy carbon electrode (6 mm

diameter) as the working electrode. The SEM of this material (Fig. 1) shows that the open woven structure of the CFVE can be readily seen. The fibres were found to have an average diameter of ca. 8 μm.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) studies were undertaken using a system consisting of an IsoChrom pump (Spectra Physics), with a 250 mm×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 45% acetonitrile, (Fischer, Far UV, HPLC grade) 55% acetate buffer (50 mM, pH 4.1), at a flow rate of 1.0 ml min^{-1}. Initial HPLC investigations were undertaken using an Agilent 1100 HPLC system with UV detection at 254 nm.

Dual electrode detection

Both the generator and detector cells were similar in construction to that previously described by ourselves [13, 19]. The generator cell consisted of a two-piece thin-layer cell, formed from an upper Teflon block containing the CFVE and a bottom steel block serving as the pseudo-reference/counter electrode. A strip of carbon veil was cut with scissors so that the dimensions were 15 mm×5 mm. This strip was then positioned between the two halves of the thin-layer cell, and electrical connection was achieved by a glassy carbon contact in the lower half of the cell. 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 electrode. Teflon gaskets were purchased from BAS, Congleton, Cheshire, UK.

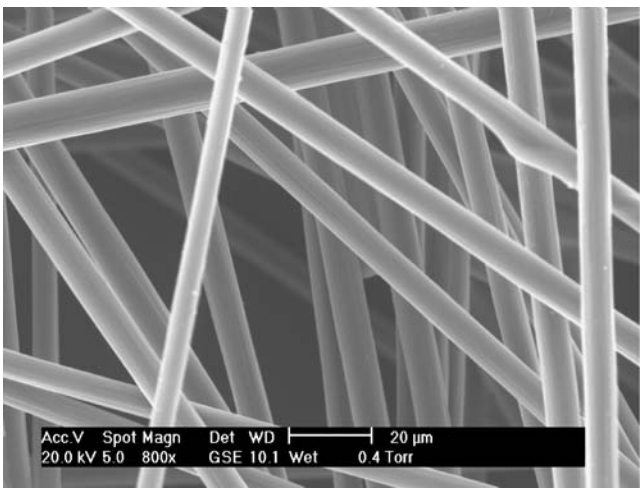

Fig. 1 Scanning electron micrograph of carbon fibre veil electrode

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 held at −0.25 V vs. Ag/AgCl and the current monitored using a BAS LC-4B amperometric detector. Chromatograms were recorded using a Siemens Kompensograph X-T C1012 chart recorder.

Cyclic voltammetric studies

Cyclic voltammograms were initially recorded in plain solutions of 45% acetonitrile–55% acetate buffer (pH 4.1, 50 mM) and then in the same solution containing 0.1 mM nitrazepam or flunitrazepam. Degassing was achieved by purging with oxygen-free nitrogen (BOC, Guildford, UK) for 5 min to eliminate oxygen reduction waves. A starting potential of 0.0 V was used, with an initial switching potential of −1.4 V and a second switching potential of +0.6 V, with a final end potential of −0.5 V. The effect of scan rate was studied over the range 5 to 200 mVs^{-1}.

Hydrodynamic voltammetry

Hydrodynamic voltammetry (HDV) was preformed by injecting fixed volumes of a combined standard solution of nitrazepam and flunitrazepam and varying the applied potential between −2.0 and −2.6 V for the down stream generator electrode, and between 0.0 and −0.4 V 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.

Scanning electron microscopy

Scanning electron microscopy was carried out using a Philips XL 30 ESEM.

Sample extraction procedure

A 50-ml volume of beverage was adjusted to pH 8.0 with 1.0 M NaOH and was transferred to a 100 ml separating funnel. Ten milliliters of dichloromethane was then added, and the resulting mixture was shaken by hand for 1 min. The two phases were then allowed to separate, and the lower organic phase was transferred to a glass vial and dried with anhydrous $CaSO_4$. The dried extract was then transferred to a separate glass vial and blown down to dryness under a stream of nitrogen. The resulting residue was then taken-up in 0.5 ml of mobile phase, via the aid of

sonication. Aliquots of the resulting solution were then examined by LC-DED.

Results and discussion

Reversed phase liquid chromatography of nitrazepam and flunitrazepam

Previously, we have investigated the LC-DED determination of nitrazepam using a mobile phase of 60% methanol 40% pH 4.1 acetate buffer [13]. However, using this system, we were unable to resolve nitrazepam from flunitrazepam. In this present study, we have undertaken further studies to optimise the mobile phase to resolve these two compounds.

Figure 2a shows the effect of changing the percentage of methanol in the mobile phase between 40% and 70% on the resulting peak resolution (Rs) of nitrazepam and flunitrazepam. Only at a methanol concentration of <50% were we able to partially resolve the two compounds. However, using such a mobile phase produced excessive long retention times for both nitrazepam and flunitrazepam. Consequently, we investigated other possible alternative mobile phases. Figure 2b shows the results of using acetonitrile, instead of methanol. With the former, we were able to resolve nitrazepam from flunitrazepam over the acetonitrile concentration range of 40% to 70%. Resolution of the two peaks was found for all the acetonitrile concentrations studied. The optimum balance between assay time and Rs was found using 45% acetonitrile–55% acetate buffer (pH 4.1, 50 mM) at a flow rate of 1.0 ml/min and consequently was used in further investigations.

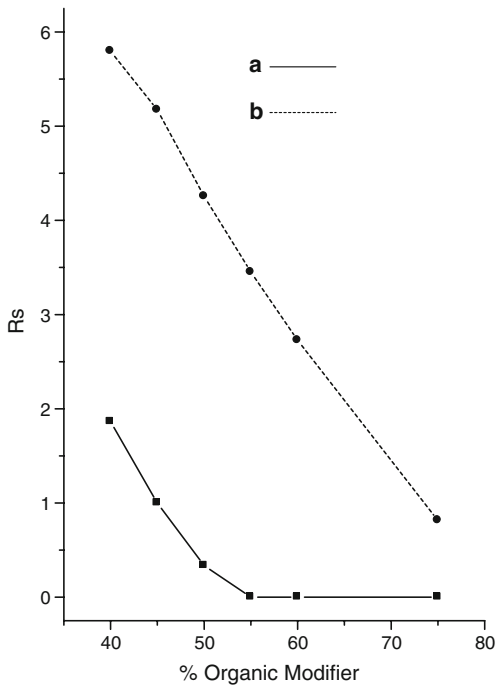

Fig. 2 The effect of *a* methanol and *b* acetonitrile percentage on the liquid chromatographic resolution (*Rs*) of nitrazepam and flunitrazepam

Cyclic voltammetric investigations of nitrazepam and flunitrazepam

The electrochemical behaviour of nitrazepam and flunitrazepam was examined by cyclic voltammetry in the optimised mobile phase containing either a 0.1 mM solution, in the case of the CFVE, or 1 mM solution for GCE investigations of each of the two benzodiazepines.

Figure 3a (I) shows the cyclic voltammogram of a plain solution of 45% acetonitrile–55% acetate buffer (pH 4.1, 50 mM), obtained at a 0.5 cm × 1.0 cm CFVE. A small peak at ca. −1.0 V was seen, which was thought to result from the reduction of oxygen. However, this was still present after 15 min of purging with nitrogen; therefore, it may be due to the functional group on the CFVE. Figure 3a (II) shows the cyclic voltammogram obtained for 0.1 mM nitrazepam at the CFVE in 45% acetonitrile–55% acetate buffer (pH 4.1, 50 mM). As can be seen, a number of redox processes are readily observable. On the negative-going scan, two reduction signals are seen. We believe that the first peak (R1) is the result of the $4e^-$, $4H^+$ reduction of the 7-nitro group to a hydroxylamine (Eq. 1). The second reduction peak, R2 being the result of the irreversible $2e^-$, $2H^+$ reduction of the 4,5-azomethine group (Eq. 2). On the return positive-going scan, a single oxidation peak O1 is seen, resulting from the $2e^-$, $2H^+$ oxidation of the hydroxylamine, (generated at R1) to a nitroso species (Eq. 3). If the scan is then reversed after this peak, a further reduction peak, R3 is then seen, exhibiting a ΔEp value of 420 mV, demonstrating the quasireversible nature of the couple at the CFVE. We believe R3 is a result of the reduction of the nitroso species, formed at O1 back to the hydroxylamine in Eq. 3.

$$R{-}NO_2 + 4e^- + 4H^+ \rightarrow R{-}NHOH + H_2O \quad (1)$$

$$R - C = N - R' + 2e^- + 2H^+ \rightarrow R - HC - NH - R' \quad (2)$$

$$R - NHOH \leftrightharpoons R - N = O + 2e^- + 2H^+ \quad (3)$$

Figure 3b (II) shows the cyclic voltammogram gained for a 0.1 mM solution of flunitrazepam obtained under the same conditions as above for nitrazepam. Flunitrazepam was found to exhibit similar behaviour to nitrazepam under

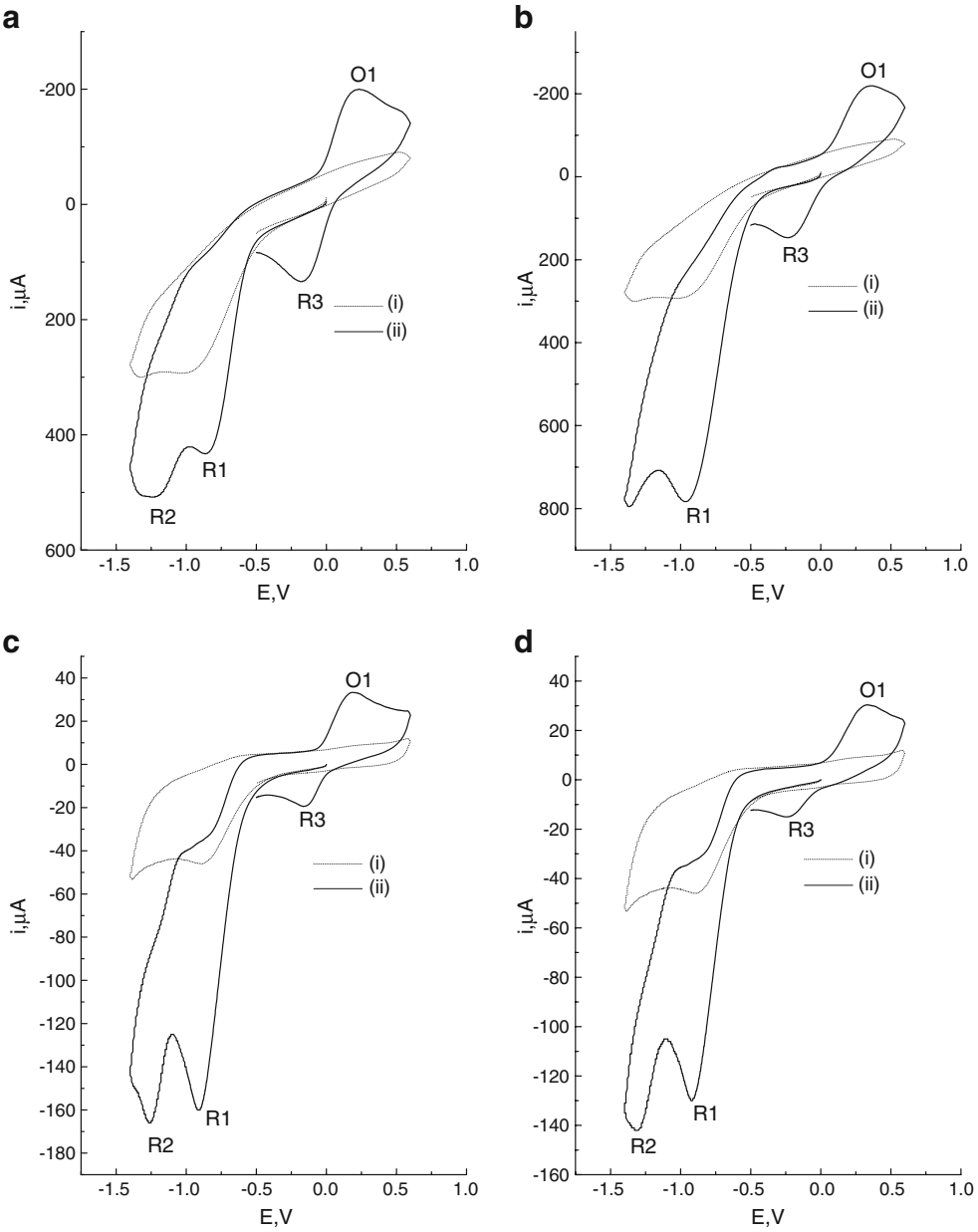

Fig. 3 Cyclic voltammograms, for **a** CFVE 0.1 mM nitrazepam, **b** CFVE 0.1 mM flunitrazepam **c** GCE 1.0 mM nitrazepam and **d** GCE 1.0 mM flunitrazepam. *i* denotes the absence of and *ii* the presence of the benzodiazepine. Starting potential 0.0 V, initial switching potential −1.5 V, second switching potential +0.6 V at a scan rate of 100 mV s^{-1}, in 45% acetonitrile–55% acetate buffer (50 mM, pH 4.1)

these conditions. However, there is no evidence for the separate reduction of the 4,5-azomethine group, when using a switching potential of −1.4 V. Therefore, the reduction of the azomethine group may occur at similar potentials to that of the nitro reduction. This is substantiated by a comparison of the magnitude of the i_p of R1 for flunitrazepam and nitrazepam; the former is 350 μA and the latter is 700 μA. The subsequent oxidation of the hydroxylamine to a nitroso species is seen at O1 (Eq. 3), and again, as for nitrazepam, a further reduction signal R3 is seen if the scan direction is switched after the oxidation process O1; this being the reduction of the nitroso group back to a hydroxylamine (Eq. 3), giving a ΔEp value of 600 mV.

The effect of scan rate on the resulting voltammetric peak current (i_p) of nitrazepam and flunitrazepam was studied over the range 5 to 200 mV s^{-1}. The i_p of all the identified peaks were found to be linearly related to the square root of scan rate, demonstrating the process to be diffusion controlled.

The cyclic voltammetric investigations show that both nitrazepam and flunitrazepam exhibited similar electrochemical redox reactions at the CFVE to that observed with other more traditional materials such as Hg [20] and GCE [13]. This can be readily seen from the cyclic voltammograms obtained at a GCE for nitrazepam (Fig. 3c) and flunitrazepam (Fig. 3d) with the only notable difference being the much larger current densities obtained at the CFVEs. As a result of these findings, we believed that it was possible to use the CFVE for the LC-DED determination of these compounds.

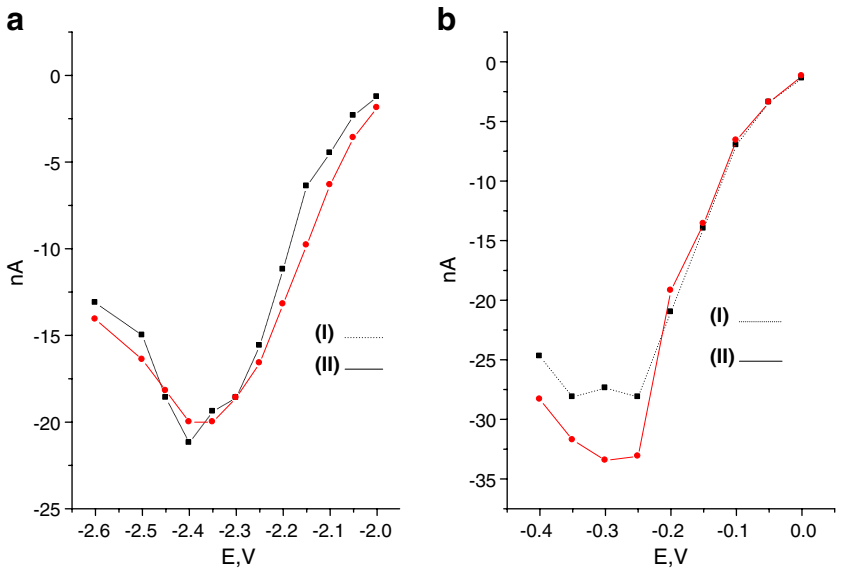

Fig. 4 Hydrodynamic voltammogram for 50 μl injections of a 0.5 mM mixed standard of *I* nitrazepam and *II* flunitrazepam, **a** generator varied, detector held at −0.20 V, and **b** generator held at −2.40 V, detector varied

Hydrodynamic voltammetry

Hydrodynamic voltammetric (HDV) investigations were undertaken to identify the optimum potential required at the generator cell and the detector cell for the determination of nitrazepam and flunitrazepam. The effect of changing the applied potential at the generator cell containing a CFVE was monitored at the detector cell using a fixed applied potential of −0.20 V. It should be mentioned that studies using both CFVE as the generator and detector proved to give a poor signal to noise ratio. Therefore, further studies were carried out using a CFVE-based generator and a glassy carbon-based detector. Figure 4a shows the resulting HDV obtained at the generator cell studied over the range −2.0 to −2.60 V. The HDV was found to exhibit a plateau between −2.30 and −2.45 V, with the maximum current being seen at −2.40 V. However, this was found to be a cathodic current resulting from a reduction and not the oxidation of the hydroxylamine to nitroso, as would expected from our cyclic voltammetric studies. We investigated the possibility that this reduction process might be the result of the direct reduction of nitrazepam or flunitrazepam at the detector cell. However, with the generator cell switched off, no signal was seen for either of the two compounds when using an applied potential of −0.20 V at the detector cell. Therefore, the mechanism responsible for the analytical response appears to be a similar reaction sequence to that observed in our earlier studies using LC-DED for *p*-nitrophenol [19].

Further HDV investigations were made to identify the optimal applied potential for the detector cell. Figure 4b shows the resulting HDV obtained with an applied potential of −2.40 V at the generator cell. The current response at the downstream detector cell was found to increase with increasing potential, until −0.25 V, where it formed a plateau until −0.35 V. Beyond this value, the current response was seen to decrease with increasing negative potential. However, at potentials more negative than −0.25 V, the background current was found to increase. Further analytical studies were hence undertaken using an

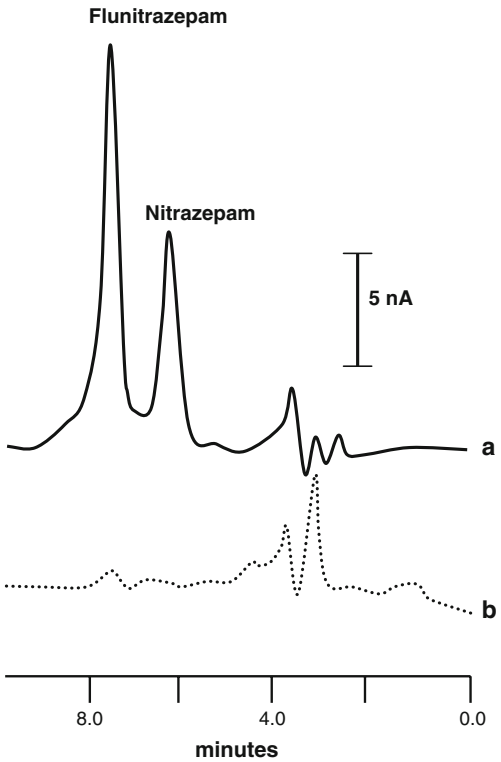

Fig. 5 Representative chromatograms of a beverage sample (Pepsi Max) obtained by LC-DED for *a* fortified at 1.0 μg ml^{-1} of nitrazepam and 1.47 μg ml^{-1} flunitrazepam and *b* unadulterated

applied potential of −2.40 V at the generator cell and −0.25 V at the detector cell.

Studies of possible interferences and related compounds

Some potentially interfering compounds that could be present in various beverages or biological samples were subjected to the proposed method. Paracetamol, caffeine, aspirin, ascorbic acid and the azo dye, Orange G, each at 1 mM were found not to give any response using the optimised conditions. The nitro aromatic compounds; 3,5-dinitrobenzoic acid, 2,6-dinitrotoluene, 2-amino-5-nitrobenzophenone (chemical degradation product of nitrazepam) and Martius Yellow were all found to give well-defined peaks, but with retention times removed from that of nitrazepam and flunitrazepam. Interestingly, the azo dye Orange II gave a well-defined response, but was again resolved from our two target compounds. Both hydroquinone and *p*-aminophenol gave detectable signals, but were seen as part of the unretained fraction.

Calibration plot, limit of detection and precision

Combined standard solutions of nitrazepam and flunitrazepam in the concentration range 0.0–1,000 ppm (equivalent to 0.0–50 μg on column) were prepared in mobile phase and measured by the optimised LC-DED procedure. The calibration plot was found to be linear from 140 ng to 16.7 μg for nitrazepam, with a slope of 2.318 nA μg^{-1}, with an associated R^2 value of 0.9920. Flunitrazepam was found to be linear between 184 ng and 24.5 μg, with a slope of 2.504 nA μg^{-1} and an R^2 value of 0.9917. The coefficient of variation was determined on five replicate measurements of 11.0 ng and was calculated to be less than 5% for both compounds. The limit of detection was calculated by making replicate current measurements at the retention time of nitrazepam and flunitrazepam (n=5) on a blank solution; the detection limits based on three times the mean of these measurements gave a value of 5.0 ng for nitrazepam and 5.0 ng for flunitrazepam on column.

Analytical application

Due to the association between flunitrazepam and drug-facilitated assault, Roche, the manufactures of Rohypnol, one of the more common formulations of flunitrazepam, have made the tablets easier to detect by adding a dye to the formulation. Olsen et al. [10] have shown that this dye was easily observable in such drinks as water and beer but did not alter the appearance of dark-coloured drinks, such as Coca Cola. They also noted no alteration in taste with beverages fortified with flunitrazepam. In light of this, we have focused our initial studies on a similar coke-based drink, Pepsi Max.

Six replicate determinations of nitrazepam and flunitrazepam in spiked and unspiked beverage samples were undertaken. Aliquots of the beverage sample were extracted as described in the "Sample extraction procedure" section; quantification being achieved by external calibration. Figure 5 shows a representative chromatogram for beverage extracts, for (a) fortified with 1.0 μg ml^{-1} of nitrazepam and 1.47 μg ml^{-1} flunitrazepam, and (b) unadulterated beverage. Mean recoveries of 95.5% (n=6) and 78.0% (n=6) were obtained, with associated percentage coefficients of variation of 4.5% and 8.8% for nitrazepam and flunitrazepam, respectively. The data demonstrates that the method shows promise for such beverage samples containing such concentrations.

Conclusions

We have developed an LC-DED assay for the simultaneous determination of nitrazepam and flunitrazepam using the reductive–reductive mode of detection conditions which were optimised for their determination in a beverage sample; the detection limits were 5.0 ng on column.

The developed assay was shown to be able to successfully determine nitrazepam and flunitrazepam in a sample of Pepsi Max, giving a percentage coefficient of variation of 4.5% for nitrazepam at 1.0 μg ml^{-1} and 8.8% for flunitrazepam at 1.47 μg ml^{-1}; mean recoveries of 95.5% and 78.0%, respectively, were obtained.

This is, to our knowledge, the first report on the electrochemical behaviour of nitrazepam or flunitrazepam at carbon fibre veil electrodes and its exploitation using LC-DED. We intend to explore the possibility of applying this approach to the analysis of other drug substances of forensic and biomedical importance.

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