Electroanalytical identification of 25I-NBOH and 2C-I via differential pulse voltammetry: a rapid and sensitive screening method to avoid misidentification.

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25I-NBOH is a new potent serotonin 5-HT_{2A} receptor agonist recently identified in blotter paper seizures. This compound is a thermolabile molecule that undergoes degradation under routine gas chromatography–mass spectrometry analysis leading to misidentification. In this paper, the voltammetric behavior of 25I-NBOH and 2C-I was investigated and their electroanalytical characteristics determined. A novel, fast and sensitive electrochemical method for detection of 25I-NBOH using SPCE is described and all method characteristics demonstrated the method to be analytically valuable. The method is selective and achieves full differentiation between 25I-NBOH, 2C-I and 25I-NBOMe.

Introduction

In recent years there has been an unprecedented rise in potentially dangerous psychoactive substances, usually offered as legal alternatives to substances under international control¹. The new psychoactive substances (NPS) market is in constant expansion with new compounds being available on a daily basis². A common strategy used by dealers to avoid legal restriction is to introduce slight changes in the molecular structure of already banned psychoactive products. Sometimes only a radical's position is changed, or an atom is added. The transient and often short-lived nature of some substances on the drug markets, the evolution of sequences of closely related compounds and the diversity of substances poses additional challenges for correct identification as limited databases are available³.

This is precisely the case of a new potent serotonin 5-HT_{2A} receptor agonist recently identified in blotter paper seizures in Brazil⁴. This compound named 25I-NBOH (2-([2-(4-iodo-2,5-dimethoxyphenyl)ethyl]amino)methyl)phenol) is derived from another phenethylamine, 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine) where a methoxy radical was substituted by a hydroxyl group in one of the benzyl rings (Figure 1). As the NBOMe compounds, NBOHs are N-benzylmethoxy derivatives of the 2C family presenting a substitution at position 4, often consisting of a halogen (i.e., chlorine, bromine, or iodine – 25X-NBOH), as a strategy to avoid prosecution.

25I-NBOH presents additional challenge for unequivocal identification as it is a thermo labile molecule that undergoes degradation when examined under gas chromatography–mass spectrometry (GC–MS). When subjected to high temperatures 25I-NBOH degrades into 2C-I (2-(4-iodo-2,5-dimethoxyphenyl)ethanamine), an already scheduled

amphetamine type stimulant, leading to misidentification⁵. The prevalence of this substance on the NPS market can be underestimated under GC–MS conditions, the most widely and routinely utilized analytical technique for drug sample analyses. It can misidentify 25I-NBOH due to its degradation into 2C-I (and corresponding 2C for the other members of the series)⁵.

Laboratory analyses found new compounds when 2C-I was identified on blotter papers and analysed by GC–MS demonstrating that drug concentrations in those samples were below the minimal dosage reported⁴. The usual form for 2C-I is in the form of tablets as this drug requires a significant quantity to produce any biological effects. Blotter papers have limited capacity to carry any compound and been commonly used with more potent drugs.

There are no prevalence studies regarding 25X-NBOHs but daily work in forensic laboratories in Brazil found these substances as NBOMe substitutes and occupies a section of the market in Brazil nowadays.

Despite many attempts in adjusting GC–MS conditions and even changing the extraction solvent, degradation cannot be avoided. The degradation takes place inside the GC–MS injector and appears to be caused by the high temperature inside the injector with the degradation products reacting with the alcohol used in the extraction procedure⁵. This is very alarming data as GC–MS is the most commonly used technique for identification/confirmation of the components in the test samples in forensic drug laboratories all over the world⁶ and may be subject to misidentification when analysing samples putatively containing NBOHs. Moreover misidentification of those compounds can lead to serious consequences for forensic laboratories as it can affect reliability and also have legal implications, as in some countries NBOH compounds are not scheduled when 2C-X are banned.

Figure 1. Chemical structure of 2C-I, 25I-NBOH and 25I-NBOMe.

Since 2017 some attempts were made to establish unequivocal identification of 25X-NBOHs compounds using other analytical methods. Morais *et al* (2017)⁷ used easy ambient sonic-spray mass spectrometry imaging (EASI-IMS) and ambient ionization MS method directly applied to the surface of the sample for 25I-NBOH screening. Earlier this year, Magalhães *et al.* (2019)⁸ applied handheld NIR spectrometer and chemometric methods to perform a rapid screening method to identify samples containing NBOHs drugs. Despite both papers claimed to fully identify NBOHs compounds using no destructive methods, no quantification studies were carried out.

For many years electrochemical techniques have been used to correctly identify many illicit substances^{9–18} including novel psychoactive substances^{19–21}. Recently some studies described the analytical determination of phenethylamines derivatives namely the compounds of the NBOMe group via cyclic and differential pulse voltammetry and square wave voltammetry^{22–25}. Observing the fact that some electrochemical techniques proved to be a powerful tool for analytical determination of phenethylamine derivatives, and that the improvement of a specific and appropriate analytical method to rapidly detect and quantify 25I-NBOH is urgent, an electrochemical method was developed to accurately identify NBOH compounds. In this work, for the first time, the voltammetric behaviour of 25I-NBOH is presented. A very sensitive and specific method to identify 25X-NBOH avoiding misidentification as 2C-X or 25I-NBOMe is disclosed. The method is cost effective, portable and exhibits high sensitivity and selectivity towards the target analytes. It can also avoid misidentification of NBOHs compounds.

Experimental

Materials

All reagents used were analytical grade from Sigma Aldrich (Gillingham, UK). Deionised water (resistivity > 16 Ω cm) was used throughout all the experiments. 0.2 M PBS buffer was used as supporting electrolyte. Certified standard of 25I-NBOH was purchased from Chiron (Trondheim, Norway). 25I-NBOMe and 2C-I certified standards were purchased from Sigma-Aldrich (Cerilliant). Blotter papers were seized by the Federal District Civil Police in Brazil between the years 2016 and 2017.

Instruments

All electrochemistry experiments and measurements were performed using a portable bipotentiostat/galvanostat μ stat 400 (Dropsense, Oviedo, Spain) connected to a personal computer (Dell Vostro, Windows 7, Dell, TX, USA) using Dropview 8400 software (Dropsense). Experiments were performed using screen-printed carbon electrodes (DRP-150 - WE carbon, CE platinum, RE silver) with 3mm working area purchased from Dropsense. GC-MS analysis were performed using a Perkin Elmer® Clarus®500 GC interfaced to Clarus®500 GC mass spectrometer using a quadrupole mass analyzer. The system was controlled with PerkinElmer® TurboMass™ GC-MS Software version 5.4.2.1617.

Methods

Street samples extraction was carried out with no pre-treatment and at room temperature as follow. One blotter paper was placed in an Eppendorf tube and 1mL of PBS was added. The tube was then gently vortexed to extract the NBOH. The DPV analysis was carried immediately by adding 50 μ L of the extract to the electrode. Cyclic Voltammetry (CV) was performed using a potential range from -0.2 V to +1.4 V, step potential 0.01 V and scan rate 0.1 V/s. Differential Pulse Voltammetry (DPV) was performed using a potential range from -0.1 V to +1.4 V, step potential 0.01 V, pulse potential 0.02 V, scan rate 0.1 V/s and pulse time 10 ms. For GC-MS analyses an Agilent SLB-5ms capillary column (30 m x 0.20mm i.d., 0.20 μ m film thickness) was used. Sample injection volume was 1 μ L with a 25:1 split ratio. Helium was used as carrier gas, with a constant flow rate of 1 mL min⁻¹. Injector temperature was set to 280 °C. The oven program started at 150 °C held for 1.5 min, ramped up at 30 °C min⁻¹ to reach 250 °C and held for 1 min to be ramped up at 50 °C min⁻¹ to 300 °C and held for 3 min. The transfer line temperature was set at 300 °C. The solvent delay was set to 1.5 min. Mass scan range was m/z 35–550.

Results and discussion

Electrochemical characterization of 25I NBOH and 2C-I.

The investigation of the electrochemical behaviour by cyclic voltammetry (CV) using a carbon screen-printed electrode showed two oxidative processes observed at +0.74 V (peak I, figure 2) and +1.09 V (peak II, figure 2) for 25I-NBOH and one single oxidative process at +1.20 V for 2C-I (peak V, figure 2). A single reduction process was also observed at -0.05 V for 25I-NBOH and +0.05V for 2C-I (peak III and VI respectively, figure 2) and it was dependent of peak II/V occurrence. Both compounds also presented an oxidation peak at lower potential, E_p =+0.25 V (peak IV, figure 2) for 25I-NBOH and E_p =+0.24 V (peak VII, figure 2) for 2C-I if a second scan was performed in the same electrode. This finding agrees with the literature as 25I-NBOMe, 25B-NBOMe and 25C-NBOMe also present similar behaviour^{22,23}.

Figure 2. Cyclic voltammetry profiles were obtained using a screen-printed carbon electrode in 0.05 mg/mL 25I-NBOH (A) and 2C-I (B) aqueous solutions at pH 7 in 0.2 M PBS and a scan rate 0.1 V/s. First scan is represented as a solid line, second scan as a dashed line.

The CV analysis of 25I-NBOH demonstrated that peak I (figure 2) is compatible with a monoelectronic electron transfer, obtained by measuring the difference between peak potential E_p (mV) and the potential at half peak height $E_p/2$ (mV) of the first anodic peak, rendering a value of 48 mV. It is reasonable to conclude that this peak is the result of the electrochemical oxidation of the secondary amine present in the NBOH as the oxidation potentials observed are similar to those previously described in the literature for other similar compounds containing secondary amines^{9,26,27}, including NBOMes compounds²². This fact is also corroborated by the absence of a peak on this potential range in the 2C-I. This last compound

presents a primary amine, instead of a secondary amine as in 25I-NBOH, which oxidases at much higher potentials (not explored in our experiments) explaining the presence of just one oxidation peak.

The electrochemical analysis and literature review indicated that peak II and peak V (figure 2) are due to the halogen oxidation to a hydroxyl group due to nucleophilic attack via SN2 type displacement and subsequent oxidation to a ketone (quinone/catechol equilibrium) as also observed for 2C-B, 25B-NBOMe and 25I-NBOMe²². The occurrence of peaks IV and VII, only present if a second scan is performed in the same electrode, resembles typical quinone/catechol equilibrium (figure 2). All oxidations and reductions mechanisms are explained in figure 2.

Electrochemical grafting is also believed to happen in the electrochemical oxidation of 25I-NBOH as, if further scans are performed in the voltammetric analysis, a decrease in the amine oxidation peak (I) and a progressive increase in the current corresponding to redox couple (III/IV) can be noticed. This behaviour was already described in the literature for NBOMes compounds²².

The effect of the scan rate (ν) on the peak current (i_p) and the peak potential (E_p) upon the electrochemical oxidation of 25I-NBOH and 2C-I were also examined in a 0.05 mg/mL solution at pH 7. A linear relationship was observed between $\log i_p$ and $\log \nu$ corresponding to the equations: $\log i_p$ (I) = $0.58 \log \nu + 1.80$ for 2C-I; $\log i_p$ (I) = $0.52 \log \nu + 1.67$ for 25I-NBOH peak I and $\log i_p$ (II) = $0.53 \log \nu + 1.75$ for 25I-NBOH peak II, where i_p is in mVs^{-1} . The slopes obtained at 0.58, 0.52 and 0.53 respectively, are close enough to the theoretically expected value of 0.5 for a purely diffusion-controlled current²⁸. It is reasonable to assume that peaks I, II and V (figure 2) follow an EC scheme with a fast chemical reaction as only small changes in the peak potential for all three peaks and no reduction peak in the cathodic direction were present at higher scan rate. The same behaviour is observed in the NBOMes compounds²².

Differential Pulse Voltammetry was also used to study the relationship of E_p vs pH. For values of pH between 4.0 and 10.0, peak II for 25I-NBOH and peak V for 2C-I showed to be pH independent. Peak I for 25I-NBOH showed a linear dependence with pH and a slope of -64mV/pH suggesting an irreversible reaction mechanism involving the same number of protons and electrons²¹ (figure 3). This result is in the order of magnitude expected for a monoelectronic/monoprotonic reaction (59.2 mV/pH at 25°C)²⁹.

Figure 3. Influence of pH on the potential of a 0.05 mg/mL 2C-I solution ($\blacktriangle$), 25I-NBOH peak I ($\bullet$) and peak II ($\blacklozenge$) solution dissolved in PBS analysed by DPV. Scan rate 0.1 V/s.

Electroanalytical determination of 25I-NBOH and 2C-I.

To achieve unmistakable electrochemical identification of 25I-NBOH two other compounds must be taken into consideration: 2C-I, as at GC-MS conditions it is a product of 25I-NBOH degradation and cannot be differentiated from it; and 25I-NBOMe, which is a very similar compound to 25I-NBOH and could produce oxidation/reduction peaks in very similar potentials.

Differential pulse voltammetry was used to achieve the electroanalytical determination of 25I-NBOH, 25I-NBOMe and 2C-I. 25I-NBOH, 25I-NBOMe presented two oxidation processes and 2C-I one oxidation process, in accordance with the oxidation observed in CV (figure 4). The analytical differentiation of 2C-I is due to the absence of the early oxidation peak since the oxidation of the primary amine present in this substance occurs at much higher potentials than the potential range applied in our experiments.

For 25I-NBOH and 25I-NBOMe, the analytical identification was performed using the second oxidation process. This oxidation represents the extraction of the halogen atom from the compound structure and proved to be very specific despite the similarity between the analysed substances demonstrating the powerfulness of the technique. The first oxidation process was important in the quantification analysis as this peak current presents a strong relationship with the compound concentration.

Figure 4. Differential pulse voltammetric profiles obtained using a screen-printed carbon electrode in a solution with 0.05 mg/mL of 25I-NBOH (solid line), 25I-NBOMe (dashed line) and 2C-I (dotted line) in a pH7 PBS buffer. Scan rate 0.1 V/s.

The optimum instrumental parameters used in the DPV were chosen by studying the variation in step potential, pulse potential, number of scans and pulse time. The method was optimized to enhance peak height and reproducibility. Under the experimental parameters described an eight-point calibration graph ($0.01\text{mg/mL} - 0.08\text{ mg/mL}$, $n=5$), was used to verify method linearity and sensitivity. The peak current was found to be directly proportional to the drug concentration in the concentration range analysed. Electroanalytical data were studied in terms of limit of detection (LOD) and limit of quantification (LOQ) values and the statistical evaluation of the regression lines and the analytical characteristics of the method are summarized in Table 1.

Table 1. Analytical parameters for the quantitative determination of 2C-I, 25I NBOH and 25I NBOMe using DPV. x = concentration (mg/mL), y = peak current (V).

Reports in drug users' forums describe that light effects can be felt when as little as $200\mu\text{g}$ of 25I-NBOH is administrated. Usual dosage per blotter paper for this drug ranges from $500\mu\text{g}$ to $900\mu\text{g}$. The LOD and LOQ found in our method is 0.01mg/mL and 0.02mg/mL respectively, demonstrating that the reported method has analytical value for forensic purposes. The precision of the method was studied for repeatability and reproducibility. Repeatability was evaluated with five repeated electrochemical measurements using a 0.05mg/mL solution under optimal conditions. The mean measured potential was $+1.110 \pm 0.001\text{ V}$ for 2C-I, $+0.73 \pm 0.010\text{ V}$ (peak I) and $1.070 \pm 0.001\text{ V}$ (peak II) for 25I-NBOH and $+1.020 \pm 0.010\text{ V}$ (peak III) and $+1.210 \pm 0.010\text{ V}$ (peak IV) for 25INBOMe (Figure 3). Intraday and Inter-day precision were evaluated by repeatedly ($n=5$) measuring the target drug at three levels of concentration (0.025 , 0.042 and 0.072 mg/mL) within a day and over three consecutive days. Precision was

expressed as the relative standard deviation (%RSD) in table 1. Trueness (accuracy) was expressed as a percentage relative error (bias %) between the calculated mean concentrations and experimental concentrations values.

The robustness of the method was tested introducing controlled changes in the pH of the analysed solutions (pH 7.0 – 7.3) and varying the concentration of the supporting electrolyte (0.20 – 0.18M) and no statistically significant changes were found neither in peak current or peak potential. A Friedman test was used for statistical comparison and did not detect any differences across multiple test attempts ($p=0.135 > p=0.05$).

Cross validation

GC-MS was used to cross validate the electrochemical method as it is the golden method for drug analysis. The experimental value observed by the voltammetric method was compared against the reference method in a nine point concentration set (0.015 to 0.075 mg/mL) in triplicate and a linear regression based on peak height demonstrated a good correlation between the two methods ($R^2 = 0.98$; $y = 0.9446x + 0.0038$) (figure 5). The average error was 1.60% for 25I NBOH, 1.93% for 2C-I and was 0.07% for 25I NBOMe. Uncertainty, measured as 3 times the standard deviation, was 0.08% for low concentration points, 0.17% for mid concentration points and 0.71% for upper concentration.

Figure 5. Comparison of concentration values obtained in the experimental set with GC-MS and DPV ($n=3$ for each concentration).

Street samples analysis

To prove our method's ability to discriminate between 25I-NBOH and 25I-NBOMe in real samples and for further validation, 15 blotter papers seized from 2016 and 2017 by the Federal District Civil Police in Brazil were tested. Ten samples contained 25I-NBOH ($n=10$) and seven holding 25I-NBOMe ($n=7$) were analysed using the voltammetric method described in this study and the results were compared with GC-MS data. Semi-quantitative analysis demonstrated that for street samples individual 25I-NBOH doses can vary from 300 μ g to 1000 μ g. The correct differentiation between 25I-NBOH and 25I-NBOMe was achieved in 73% (13 samples) of the analysed material. An example of street samples analysis can be found on figure 6. An average calculated error and relative standard deviation (RSD%) were also calculated for both methods and reported to be 0.007 mg/mL (%RSD=7.09) for 25I-NBOH and 0.006 mg/mL (%RSD=6.52) for 25I-NBOMe, respectively.

Figure 6. Differential pulse voltammetric profiles obtained using a screen-printed carbon electrode in street samples containing 25I-NBOH (continuous line) and 25I-NBOMe (dashed line). Scan rate 0.1 V/s.

Misidentification and error could occur in voltammetric analysis, as in some cases 2C-I and 25I-NBOMe were also identified in 25I-NBOH samples (LC-QTOF analysis – data not published). In general, results obtained by Brazilian laboratories show that the percentage of blotter papers containing mixtures in street samples are low and it is not clear

if the presence of 2C-I is due to incomplete chemical reaction for 25X-NBOH production or whether this could be related to 25X-NBOH degradation products due to labile nature of 25X-NBOH. These data corroborate the usefulness of the suggested method as a screening technique to securely differentiate both compounds when the analysis of blotter papers is required.

Conclusions

25I-NBOH is a thermolabile compound that undergoes degradation in routine GC-MS analysis leading to misidentification. In this paper, the voltammetric behavior 25I-NBOH and 2C-I was investigated for the first time and the mechanism and analytical characteristics determined. A rapid and sensitive electroanalytical method for unequivocal 25I-NBOH identification is presented. The technique demonstrated to be extremely powerful as it can differentiate between very similar compounds (25I-NBOH and 25I-NBOMe) and can avoid misidentification that is typical when 25I-NBOH is submitted to GC-MS analyses. The method also proved to be quick, cost effective and was developed in a portable platform. All method characteristics demonstrated the method to be analytically valuable illustrating how paramount an electrochemical method can be even when other analytical techniques failed.

Conflicts of interest

There are no conflicts to declare.

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Figure 1. Chemical structure of 2C-I, 25I-NBOH and
25I-NBOMe

Figure 2. Cyclic voltammetry profiles obtained using a screen-printed carbon electrode in 0.05 mg/mL 25I-NBOH (A) and 2C-I (B) aqueous solution at pH 7 in 0.2 M PBS. Scan rate 0.1 V/s. First scan is represented as a solid line, second scan as a dashed line.

Figure 3. Influence of pH on the potential of a 0.05 mg/mL 2C-I solution (▲), 25I-NBOH peak I (●) and peak II (◆) solution dissolved in PBS analysed by DPV. Scan rate 0.1 V/s.

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Figure 5. Comparison of concentration values obtained in the experimental set with GC-MS and DPV (n=3 for each concentration).

Figure 6. Differential pulse voltammetry profiles obtained using a screen-printed carbon electrode in street samples containing 25I-NBOH (continuous line) and 25I-NBOMe (dashed line). Scan rate 0.1 V/s.

	2C-I	25I NBOH		25I NBOMe	
regression equation a	$y = 88.65x + 1.63$	$y = 59.50x + 1.31$		$y = 126.09x + 1.22$	
correlation coefficient (R ²)	0.98	0.98		0.98	
standard error of the slope	4.19	3.27		5.6	
standard error of the intercept	0.21	0.16		0.28	
linearity range (mg/ml)	0.01 - 0.08	0.01 - 0.08		0.01 - 0.08	
Calibration graph (n=5)					
LOD (mg/ml)	0.012	0.011		0.004	
LOQ (mg/ml)	0.021	0.023		0.012	
Accuracy	0.74%	1.20%		0.66%	
		peak I	peak II	peak I	peak II
Intraday precision (n=5)					
peak potential (V)	0.0%	0.41%	1.14%	1.00%	0.93%
peak current (mA)	3.28%	2.82%	4.39%	3.53%	1.81%
Intreday precision (n=5)					
peak potential (V)	1,78%	1.24%	1.93%	1.30%	1.04%
peak current (mA)	3.37%	2.82%	6.79%	3.58%	2.34%

Table 1. Analytical parameters for the quantitative determination of 2C-I, 25I NBOH and 25I NBOMe using DPV. x = concentration (mg/mL), y = peak current (V).