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ORIGINAL PAPER

Electrochemical oxidation of zopiclone

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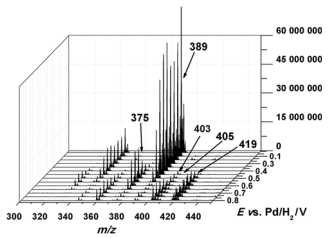
Abstract Electrochemical behaviour of zopiclone was investigated on glassy carbon electrode in static and rotation disc arrangement. Strong influence of kinetics and adsorption phenomena on the electrode processes was proved by voltammetric techniques. Controlled potential electrolysis in off-line and on-line combination with tandem mass spectrometry was employed for investigation of the products of electrochemical oxidation. *N*-Desmethyl zopiclone was identified and three other oxidation products formed by an introduction of one or two oxygen atom(s) to the molecule of zopiclone (including zopiclone *N*-oxide) were characterized. Based on mass spectrometric investigation of those products, piperazine moiety was proved as a target of electrochemical oxidation of zopiclone. Since *N*-desmethyl zopiclone and zopiclone *N*-oxide have been reported as products of enzymatic metabolism of the drug, the combination of electrochemistry with mass spectrometry may be considered as a reliable tool for simulation of some metabolic transformations.

Graphical abstract



Keywords Electrochemistry · Mass spectroscopy · Voltammetry · *N*-Desmethyl zopiclone

Introduction

Zopiclone (ZOP), chemically (*RS*)-6-(5-chloropyridin-2-yl)-7-oxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazine-5-yl 4-methylpiperazine-1-carboxylate (Fig. 1), is a hypnotic drug used for the treatment of insomnia. ZOP possesses a short duration of action with additional muscle relaxant and anticonvulsant properties. It was developed along with other so called Z-drugs, zaleplon, and zolpidem, as an alternative to the world-wide used benzodiazepines, which are controversial due to concerns about adverse psychological and physical effects, decreasing effectiveness, and physical dependence at their long-term usage.

ZOP is a chiral drug administered as a racemic mixture although the pharmacological activity is related to the (+)-(*S*)-ZOP known as eszopiclone. Both enantiomers are metabolised via cytochrome P450 enzyme system [1]. The main biotransformation products of

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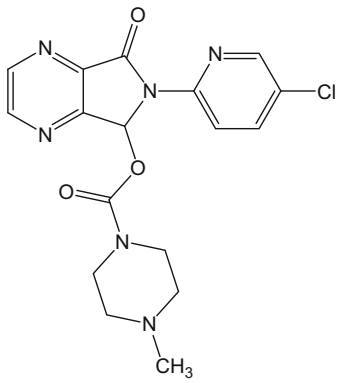

Fig. 1 Chemical structure of zopiclone

oxidative metabolism are *N*-desmethyl zopiclone and zopiclone *N*-oxide [1–4].

Chemical stability of ZOP was investigated in several studies. The drug was found to be stable under acidic conditions in aqueous-acetonitrile solutions [5]. However, rate of hydrolysis increased with pH and temperature in aqueous ethanolic media [6]. 2-Amino-5-chloropyridine was identified as the final degradation product of ZOP in alkaline solutions [7, 8].

Electrochemical behaviour of ZOP was investigated by several authors but with no attempt to identify the products of electrochemical reactions. Electrochemical reduction of ZOP was studied by Viré et al. [9] by different techniques. ZOP was reducible in two 2-electron steps in the pH range 0 to 12. The electrochemical behaviour was accompanied by strong adsorption in neutral and acidic solutions. Adsorption was employed for sensitive determination of ZOP by adsorptive stripping voltammetry [9]. Yilmaz et al. [10] have studied oxidation of ZOP at glassy carbon electrode (GCE) using adsorptive stripping voltammetry. ZOP oxidation was found to be an irreversible adsorption controlled process. Square wave voltammetry was used for determination of submicromolar concentrations of ZOP as well [10]. Number of other analytical methods has been used for ZOP determination starting from potentiometric titrimetry, prescribed by British Pharmacopeia [11] through the spectrophotometry [12] up to potentiometry with ion selective electrode [13]. However, chromatographic methods including gas [14] and liquid chromatography [15–17], often with mass selective detection, lead the dance in the field of drug analysis.

Electrochemistry coupled to mass spectrometry (EC/MS) can be used for simulation of some oxidative reactions involved in the metabolism of drugs and other xenobiotics [18]. The elucidation of oxidative metabolic reactions is a crucial point in the drug development. In general, the main route of drug elimination is oxidative biotransformation via CYP enzymes family and ZOP is not an exception as mentioned above. In vivo or in vitro experiments, commonly used for elucidation of the drug metabolism, are usually based on animal experiments which are tedious, ethically questionable, time consuming, and of limited reproducibility. Since oxidation reactions play a crucial role in the CYP-mediated biotransformation, it seems reasonable to use electrochemical oxidation as a possible instrumental tool for simulation of the oxidative degradation processes [19]. Electrochemistry coupled on-line or off-line to MS (especially to electrospray ionization mass spectrometry, ESI–MS) has been successfully used in many research areas including metabolic studies [20] as well as in peptide, protein, and DNA analysis [21] or quantification of biomolecules [22].

In this paper, electrochemical behaviour of zopiclone was studied using voltammetric methods on glassy carbon working electrode. Controlled potential electrolysis of ZOP solutions containing acetonitrile and aqueous buffer solutions of different pH followed by ESI–MS analysis of the reaction products as well as on-line coupling of EC flow-through cell with ESI–MS were used for generation and characterization of zopiclone oxidation products.

Results and discussion

Electrochemical behaviour of zopiclone

Cyclic voltammogram (Fig. 2a) of zopiclone in acetonitrile-aqueous buffer solution of pH 4.8 (1:1, v/v) recorded on glassy carbon electrode (GCE) shows one oxidation peak at potential $E_p = 1.02$ V (vs. saturated calomel reference electrode, SCE). No current response was observed in the reverse cathodic branch of voltammograms suggesting irreversibility of the electrode process. Similarly, differential pulse voltammogram (Fig. 2b) revealed one anodic peak at the potential $E_p = 1.04$ V.

The effect of the pH of BR buffer solutions on the oxidation signal of zopiclone was investigated using cyclic and linear sweep voltammetry (LSV) with GCE in static and rotating disc (RDE) arrangement, respectively. The anodic signal of zopiclone occurred in the pH range 2.8–9.5 in both arrangements. The oxidation signal was not observed in the more acidic buffer solutions (pH 1.9, 2.2, and 2.5). The limiting current measured on RDE increased with rising pH values up to pH about 6 and then remained almost constant up to pH 9.5 (Fig. 3). Oxidation in more alkaline solutions was not tested due to reported fast decomposition of ZOP under such conditions [6]. In acidic solutions with pH below 6, the oxidation of ZOP is a pH-dependent process. The half-wave potential shifts to lower values with increasing pH by −59 mV per pH unit (Fig. 3). This value indicates that the same number of protons and

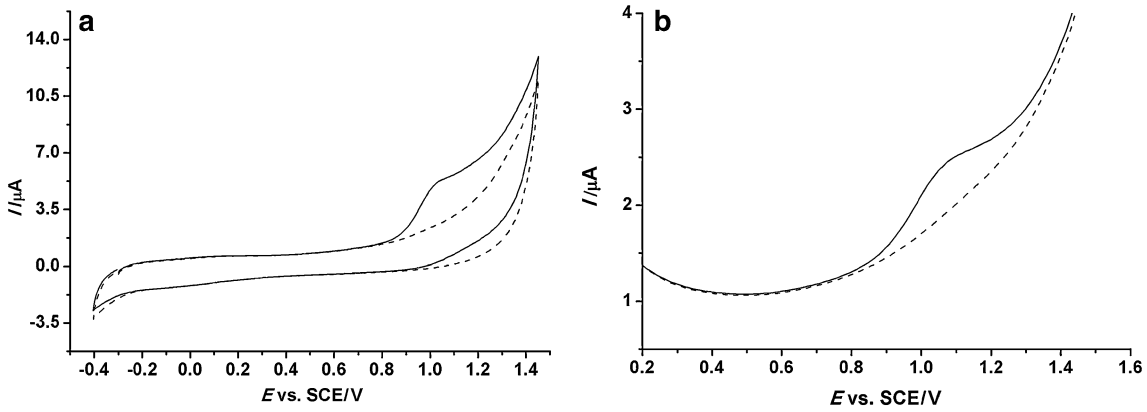

Fig. 2 Voltammograms of 5×10^{-4} mol dm^{-3} ZOP (*solid line*) in supporting electrolyte (*dashed line*) containing BR buffer solution pH 4.8 and acetonitrile (1:1, v/v) recorded by cyclic voltammetry (**a**), scan rate 50 mV s^{-1} and differential pulse voltammetry (**b**) scan rate 20 mV s^{-1}, modulation amplitude 25 mV, pulse width 500 ms

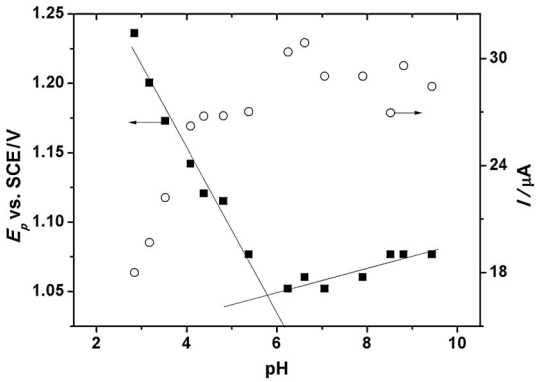

Fig. 3 Dependence of half-wave potential (*filled square*) and limiting current (*open circle*) of zopiclone ($c = 5 \times 10^{-4}$ mol dm^{-3}) on pH measured on RDE, angular rotation speed: 157 rad s^{-1}, scan rate 0.4 V s^{-1}

electrons are involved in the electrode reaction. At pH 6–9.5, the half-wave potential shift was negligible. The intersection point of two regression straight lines at pH 5.9 can be related to change in the protolytic forms of ZOP (p$K_A = 6.79$ [23]).

The effect of scan rate (v) on zopiclone oxidation was investigated by LSV on GCE in BR buffer pH 4.8 with acetonitrile (1:1, v/v). The anodic peak of ZOP increased with increasing scan rates within the range 0.01–0.9 V s^{-1}. The dependence of log I_p—log v revealed two linear segments with the slopes 0.71 (in the scan range 0.01–0.2 V s^{-1}) and 0.89 (in the range of 0.3–0.9 V s^{-1}) (see Supplementary Material, Fig. S1). The values of the slopes are higher than the theoretical value 0.5 for purely diffusion controlled process indicating the influence of adsorption on the electrochemical reaction. The dependence of the peak potential E_p on log v resulted in two straight lines. Peak potential did not change within the slow scan rates interval up to 0.2 V s^{-1}. However, a significant shift in peak potential by 41 mV per log unit was observed when scan rate exceeded 0.3 V s^{-1}. The fact indicates the influence of other processes, next to diffusion, in the rate determining step at higher scan rates. Kinetics of the electron transfer reaction, kinetics of the chemical reaction preceding the electron transfer (deprotonation seems to be the most likely in this case; see pH dependence of limiting current on Fig. 3) and adsorption on the electrode surface are the most probable reasons.

The adsorption of zopiclone on electrode surface was investigated by experiment in which electrode was immersed into the stock solution of zopiclone for 300 s, and then the electrode surface was washed with deionized water. The electrode was placed into electrolyte and CV voltammogram was recorded (see Supplementary Material, Fig. S2). A small current signal at 1 V corresponding to ZOP adsorbed on the electrode can be distinctly recognized in comparison to the blank measured with clean, mechanically polished, electrode. The signal proved the tendency of the drug to adsorb on the electrode surface. Also the sign of the nonlinearity on the calibration dependence of the peak current on the concentration of ZOP can be ascribed in large extent to the adsorption on the GCE (Supplementary Material, Fig. S3).

Dependence of limiting current on square root of rotation velocity of RDE was measured by LSV in solution of pH 4.8, pH 7.1, and pH 9.4. In neutral and alkaline solutions the limiting current increases with increasing square root of the rotation speed. The course of the dependence is typical for the mechanism influenced by both diffusion of the depolarizer and kinetics of the electron transfer (see Supplementary Material, Fig. S4). On the contrary, limiting current measured in acidic solution is almost independent on square root of rotation velocity. The course is usually observed in systems controlled by the kinetics of the electron transfer or the kinetics of the chemical reaction

preceding the electron transfer (i.e., systems where diffusion is not the rate determining step). The interpretation of the main course of the dependence fits together with conclusion resulting from the dependence of the peak potential on the scan rate during LSV mentioned above.

MS analysis of oxidation products of zopiclone

For more detailed characterization of the oxidation products two experimental arrangements were used. First arrangement consisted of an off-line controlled potential electrolysis of zopiclone on the large surface Pt electrode combined with a tandem mass spectrometric measurement of the electrolysed zopiclone solutions. Second one involved on-line coupling of electrochemical flow-through cell containing the working porous graphite electrode with mass spectrometer (EC/MS). In both types of experiment, electrolysis was performed in acetonitrile/ammonium acetate buffer solution pH 3.5, 6.8, and 9.5 (1:1, v/v).

For off-line experiment the controlled potential values suitable for exhaustive electrolysis were chosen according to courses of cyclic voltammograms of zopiclone recorded in different media. To eliminate the influence of nonelectrolytic reaction of zopiclone, the mass spectra of electrolysed solutions were compared to those of control samples. The control samples were obtained by electrolysis of zopiclone solutions at potentials where no electrochemical reaction occurs.

Figure 4a shows mass spectrum of ZOP standard in which the pseudomolecular ion $[M + H]^+$ at $m/z = 389$ is evident. MS/MS spectrum (Fig. 4b) revealed the main fragment ion at $m/z = 345$ corresponding to a loss of CO_2. This unusual scission of CO_2 from the molecule, giving evidence of rather strong association of the piperazine ring with another heterocyclic part of the ZOP structure, has already been reported [24]. Formation of fragments at $m/z = 245$ and $m/z = 263$ is described in the inset of Fig. 5b. The minor fragment at $m/z = 217$ corresponding to loss of CO from cyclopyrrolon moiety of the ion with $m/z = 245$ is also characteristic for ZOP fragmentation. Fragmentation pattern corresponds with the already published results [24]. The differences in intensities of fragments found in this work and those published in the above mentioned paper are due to different setup of collision cells. It is worth noting that ion with $m/z = 263$ has been reported as the product of zopiclone hydrolysis [8].

Figure 5a shows MS spectrum of electrolysed ZOP solution, containing the mixture of oxidation products and the rest of ZOP, which was recorded in off-line setup. Four main signals of proposed oxidation products can be observed. First oxidation product (designated as P1, $m/z = 375$) corresponds to *N*-desmethyl zopiclone (difference 14 Da from the m/z of ZOP). In MS/MS spectrum the loss of CO ($m/z = 347$) and CO_2 ($m/z = 331$), respectively, are observed (Fig. 5b). Besides, intensive signals at $m/z = 263$ and $m/z = 245$ confirm that both pyridine and the pyrrolo-pyrazine rings remain unchanged and thus, the oxidative demethylation occurs on piperazine skeleton. The fragmentation pattern of the product P1 corresponds to those reported for *N*-demethylated metabolite of zopiclone [24].

Second product (P2, $m/z = 405$) corresponds with the addition of one oxygen atom to the ZOP molecule. Presence of fragments at $m/z = 263$ and $m/z = 245$ suggests that the oxidation takes place on the piperazine ring as well (Fig. 5c). Formation of *N*-oxide related to oxidative metabolism of ZOP has been already described in literature [1]. Electrochemical formation of *N*-oxide(s) has also been reported for another drug possessing tertiary amine group [25]. Oxidation of one of nitrogen atoms in the piperazine ring is thus a reasonable possibility explaining this type of ZOP oxidation. However, oxidation of *N*-methyl group or the carbons of the piperazine skeleton accompanied by piperazine ring opening cannot be excluded in this case.

Third product (P3, Fig. 5d, $m/z = 403$) corresponds with a gain of one oxygen and loss of two hydrogen atoms. The formation of fragments at $m/z = 263$ and $m/z = 245$ suggests that pyridine and pyrrolo-pyrazine rings remain

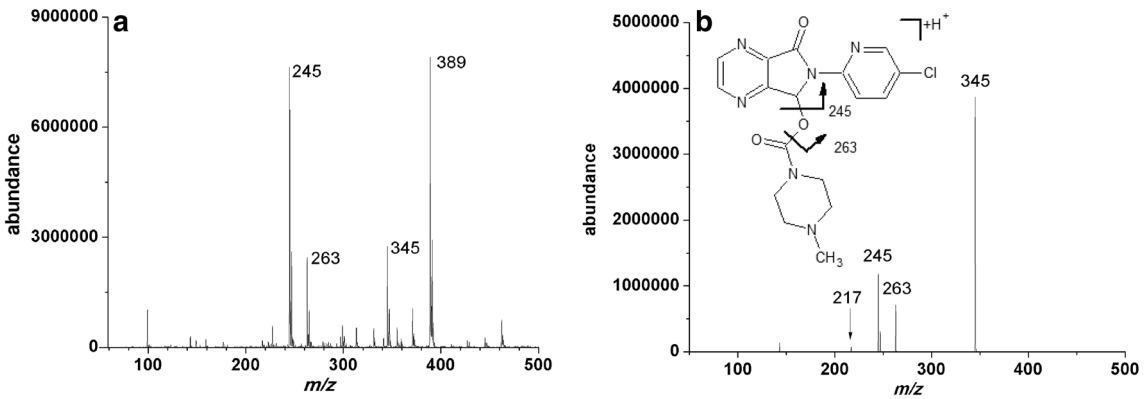

Fig. 4 MS (**a**) and MS/MS spectrum (**b**) of zopiclone (5×10^{-4} mol dm^{-3}) in water/CH_3CN (1:1, v/v)

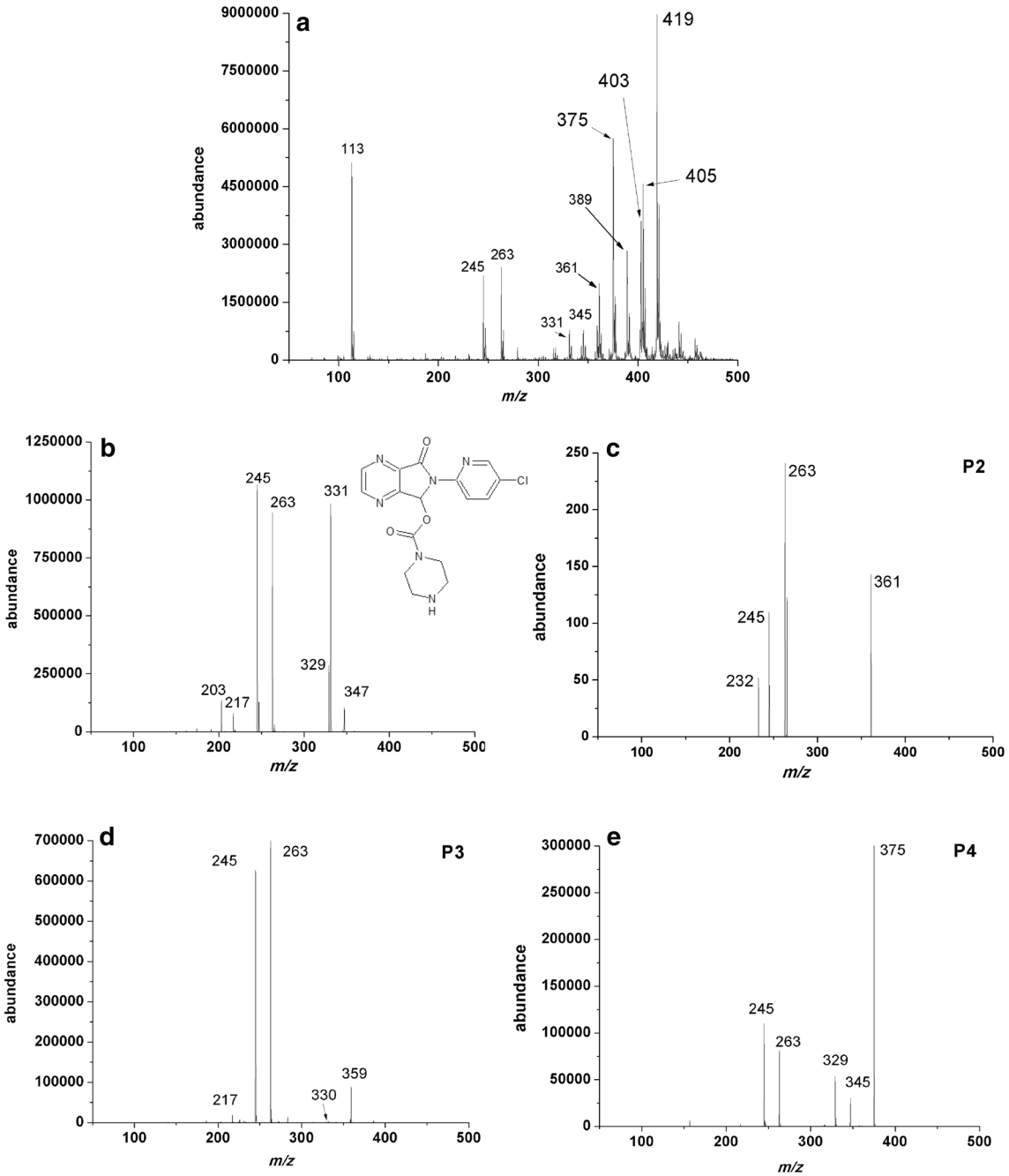

Fig. 5 MS spectrum of electrolysed ZOP (**a**), containing a mixture of oxidation products and the rest of ZOP, and MS/MS spectra of main oxidation products: P1 at $m/z = 375$ (**b**), P2 at $m/z = 405$ (**c**), P3 at $m/z = 403$ (**d**), and P4 at $m/z = 419$ (**e**) acquired in solution of zopiclone ($c = 5 \times 10^{-4}$ mol dm^{-3}) electrolysed in acetonitrile/ammonium acetate buffer solution pH 6.8 (1:1, v/v) 30 min on Pt gauze electrode at $E = 1.2$ V (vs. SCE)

intact as in the case of the previously discussed oxidation products P1 and P2. The fragment at $m/z = 359$ corresponds with loss of CO_2 typical for ZOP derivatives. Minor fragment at $m/z = 330$ corresponds probably with a cleavage of formyl radical indicating the presence of aldehyde group preferentially linked to one of the piperazine nitrogen atoms.

Fourth product (P4, $m/z = 419$) can be explained by the addition of two oxygen and loss of two hydrogen atoms. MS/MS spectrum of P4 (Fig. 5e) shows intensive signal corresponding with the loss of CO_2 typical for ZOP derivatives (dominant fragment at $m/z = 375$). As mentioned above, fragments at $m/z = 263$ and $m/z = 245$, respectively, suggest oxidative process occurring at the

piperazine ring. The introduction of two oxygen atoms into the piperazine skeleton can proceed by several ways leading to different products. One of the theoretical possibilities involves a formation of an *N*-carboxylic group. Since the loss of CO_2 from the fragment at $m/z = 375$ is missing in the MS/MS spectrum, this possibility is not likely. A formation of *N*-oxide and aldehyde group either on terminal methyl group or on piperazine carbon skeleton (accompanied by a ring opening) can be considered as another possibility. A fragment at $m/z = 345$ corresponds with a loss of formaldehyde from fragment at $m/z = 375$ suggesting the presence of aldehyde group preferentially linked to one of the nitrogen atoms from piperazine part. Fragment at $m/z = 329$ can be explained by consequent loss of oxygen atom from the fragment at $m/z = 345$. Similarly to previously discussed oxidation product P2, *N*-oxide formed on one of the piperazine nitrogen atoms is the most probable option. Thus, the oxidation product P4 presumably contains one aldehyde group and one oxygen atom linked to piperazine nitrogen atoms.

In off-line experiments the highest relative intensities (intensity of particular oxidation product divided by the sum of intensities of all signals in given spectrum) are observed in acidic conditions for all oxidation products. When compared the signals of particular oxidation products in acidic conditions, the signal of P1 reaches the highest value. This oxidation product exhibits the highest decrease of relative intensity with increasing pH as well. This fact points out different chemical property of P1 compared to the rest of detected oxidation products (e.g., p*K* value and proton-affinity in gas phase).

On-line connection of electrochemical cell directly to mass spectrometry interface was performed and compared with the results from the above discussed off-line experiments. Zopiclone solution in 1:1 (v/v) mixture of acetonitrile and ammonium acetate buffer of pH 3.5, pH 6.8, and pH 9.5 was continuously pumped through flow cell containing porous graphite electrode. Constant potential within the range from 0 to 0.8 V (vs. Pd/H_2 reference electrode) in 100 mV (0–0.4 V) and 50 mV (0.4–0.8 V) increments, respectively, was applied on the electrode and appropriate mass spectra were collected. The electrode potential of 0 V vs. Pd/H_2 reference electrode corresponded to 0.234 V vs. SCE at pH 7.0. Oxidation products were detected within 4 min after their formation in the cell at the flow rate 4 mm^3 min^{-1}. Resulting "mass voltammograms" are shown in Fig. 6. For better lucidity, the mass voltammograms are displayed in the m/z range from

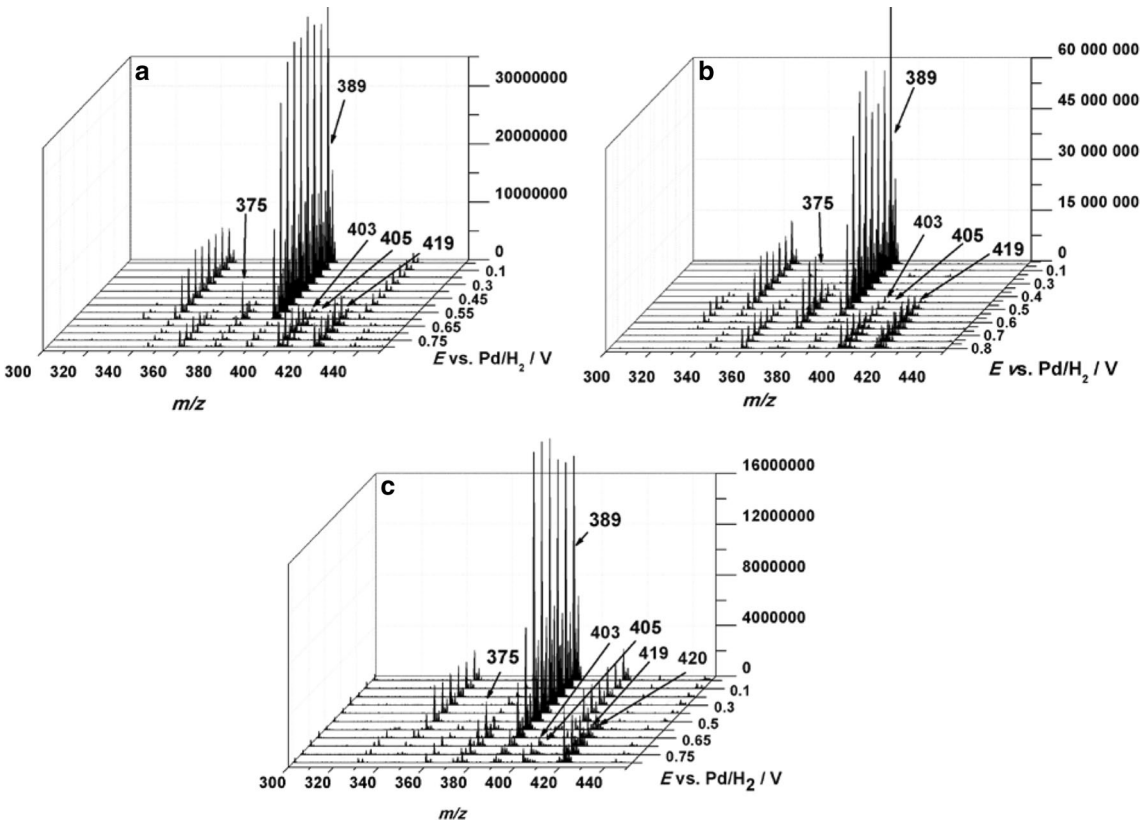

Fig. 6 Mass voltammograms of zopiclone ($c = 5 \times 10^{-4}$ mol dm^{-3}), 1:1 (v/v) acetonitrile/ammonium acetate buffer solution of pH 3.5 (**a**), pH 6.8 (**b**), and pH 9.5 (**c**) acquired in on-line EC/MS

300 to 450, in which the changes in the intensities of the signals of ZOP and its oxidation products were observed. Fragment ions with $m/z = 245$ and $m/z = 263$, common to ZOP and its oxidation products, were observed on the voltammograms at all pH and potentials (data not shown). Oxidation of zopiclone evaluated as a decrease of ZOP signal in acetate buffer solution of pH 3.5 and 6.8 starts at potential 0.5 V vs. Pd/H_2 reference electrode, in acetate buffer solution of pH 9.5 the oxidation starts at lower potential. Unlike acidic and neutral media, in alkaline solution of pH 9.5 on-line experiments reveal one more oxidation product at $m/z = 420$ (designated as P5) beside the above mentioned products P1–P4 (Fig. 6c). Even m/z value suggests the presence of odd number of nitrogen atoms. In the case of the discussed product P5 of ZOP oxidation it corresponds to incorporation of one nitrogen atom into the structure. Collision spectrum (MS^2, Supplementary Material, Fig. S5) of P5 provided three dominant fragments at $m/z = 402$, 390, and 358. The fragment at $m/z = 402$ corresponds to a loss of water suggesting the presence of one extra –OH group. Subsequent loss of CO_2, typical for ZOP derivatives, leads to the most abundant fragment at $m/z = 358$. The fragment at $m/z = 390$ can be ascribed to a loss of CH_2O, NO or CH_2-NH_2. Besides, a minor fragment at $m/z = 403$ was also observed during fragmentation of P5. Tentatively, this fragment can be explained as a loss of ammonia from parent ion. The structure of the P5 product was not revealed from the acquired MS data. Note that P5 was observed in on-line EC/MS experiments conducted in alkaline ammonium buffer. We suggest that P5 arises from a side reaction of ZOP with ammonia under oxidative conditions. When sodium ions were used as buffer constituent instead of ammonium ones, P5 product was not observed. This observation supports the proposed introduction of one nitrogen atom from ammonia to ZOP structure.

In on-line experiments the relatively sharp decrease of ZOP intensity and increase of oxidation products is observed (Supplementary Material, Fig. S6–S8). The processes occurring in acidic, neutral, and alkaline conditions reveal similar main features. The increase of P1 response starts at lower potentials compared to other oxidation products (P2–P4), intensity of signal reaches its maximum faster and at higher potentials (>0.6 V) decreases fast. The formation of the other products occurs at higher potentials and changes in intensities are less dependent on potentials compared to P1. Therefore, it can be concluded (in agreement with off-line experiments, see above) that chemical nature of P1 and process of its formation differ from those of the other oxidation products. The fact is obvious since P1 is formed by demethylation of ZOP (no extraneous substituent incomes to its molecule) while introduction of one or two oxygen atom(s) leads to formation of all remaining products (P2-P4, as well as a specific product P5 formed solely in alkaline solution in the presence of ammonia). Some oxidation products identified in electrochemical oxidation are similar to oxidation products from in vitro metabolic experiments (i.e., *N*-desmethyl zopiclone and zopiclone *N*-oxide) [1].

Conclusion

Voltammetric measurements reveal that electrochemical oxidation of zopiclone is quite complicated process influenced by kinetics and adsorption phenomena. Controlled potential electrolysis combined with mass spectrometry identification of the reaction products is a powerful tool for elucidation of electrochemical oxidation of zopiclone. Four products were found in electrolysed samples by means of off- and on-line tandem mass spectrometry. *N*-Desmethyl zopiclone was identified and three other oxidation products formed by an introduction of oxygen atom(s) to the molecule of zopiclone (including zopiclone *N*-oxide) were characterized. *N*-Desmethyl zopiclone and zopiclone *N*-oxide were formerly confirmed as in vitro metabolic products of zopiclone. The fact gives great credibility to the combination of electrochemistry with mass spectrometry for metabolic studies.

Experimental

Reagents

Zopiclone (ZOP) was obtained from Farmak (98 %, Olomouc, Czech Republic), zopiclone *N*-oxide (European Pharmacopoeia Reference Standard) was purchased from Fluka. A stock solution of 1×10^{-3} mol dm^{-3} ZOP was prepared in acetonitrile (HPLC grade, Sigma-Aldrich Czech Republic) and kept in a refrigerator. Britton-Robinson (BR) buffer solutions were prepared from phosphoric acid, acetic acid, and boric acid (0.04 mol dm^{-3} each, analytical grade, Lachema, Czech Republic). Desired pH values were adjusted with sodium hydroxide (0.2 mol dm^{-3}, analytical grade, Lach-Ner, Czech Republic). Ammonium acetate (p.a., >98.0 %, Lach-Ner, Czech Republic) was used as supporting electrolyte for MS experiments. Desired pH values were adjusted with acetic acid or ammonia (p.a., 25 % in water, Lach-Ner, Czech Republic).

Voltammetric experiments

An AutoLab PGSTAT128N electrochemical analyser (Metrohm Autolab, Utrecht, The Netherlands) with

software NOVA 1.10 was used for voltammetric experiments with three-electrode system consisting of a glassy carbon working electrode (GCE, disc diameter 3.0 mm, Bioanalytical Systems, USA) or rotating GCE (disk diameter 2.0 mm, Metrohm, The Netherlands), platinum wire auxiliary electrode, and saturated calomel reference electrode. GCE was polished using aqueous suspension of alumina powder (0.05 μm particles, Sigma-Aldrich) on a wet microcloth (Buehler, USA) and sonicated in distilled water for 30 s prior to each measurement. Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were performed at different scan rates over the range 0.02–0.8 V s^{-1} and at different pH values of the supporting buffer solution. Differential pulse voltammograms were recorded at pulse amplitude of 25 mV, pulse width 500 ms and scan rate 0.02 V s^{-1}. Hydrodynamic measurements were carried out with angular velocity ranged from 52 to 314 rad s^{-1} and scan rates 0.02 and 0.4 V s^{-1}. All experiments were performed in supporting electrolytes containing BR buffer solutions of desired pH and acetonitrile (1:1, v/v). Hydrodynamic voltammograms were processed using el-Chem Viewer software [26].

Controlled potential electrolysis

Potentiostat 100 mA (L-Chem, Horka nad Moravou, Czech Republic) with three-electrode system consisted of platinum gauze working electrode, platinum auxiliary electrode placed in a separate cathode compartment and reference SCE electrode separated from the bulk solution with a porous ceramic frit. The electrolysis was performed in mixture of acetonitrile/ammonium acetate buffer solution (1:1, v/v) of different pH values and at different potentials: pH 3.5 (0.2 V, 1.4 V), pH 6.8 (0.2 V, 1.2 V), pH 9.5 (0.2 V, 1.2 V). All samples were electrolysed in stirred solutions containing 5×10^{-4} mol dm^{-3} ZOP for 30 min.

MS and on-line EC/MS analysis

Mass spectrometer Agilent 1100 Series LC/MSD Trap (Agilent Technologies, Palo Alto, CA, USA) with electrospray ionization (ESI) was used for analysis of electrolysed solutions of ZOP. Parameters of ESI source working in the positive mode were as follows: capillary voltage 2.4 kV, source temperature 150 °C, pressure of desolvation gas 10 psi, desolvation gas flow rate 180 dm^3 h^{-1}. Nitrogen was used as desolvation gas and helium as collision gas.

On-line electrochemistry/mass spectrometry measurements were performed using ESA Conditioning cell 5021A (ESA, Chelmsford, MA, USA) connected to the potentiostat Detector ADLC 1 (Laboratorní přístroje Praha, Czech Republic). Working ZOP solution (5×10^{-4} mol dm^{-3}) in 1:1 (v/v) acetonitrile/ammonium acetate buffer solution (pH 3.5, pH 6.8, and pH 9.5) was used for on-line oxidation in the potential range from 0 to 0.8 V (vs. Pd/H_2).

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