

Voltammetric behaviour of bromhexine and its determination in pharmaceuticals

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Received 16 March 2007; received in revised form 3 May 2007; accepted 11 May 2007
Available online 21 May 2007

Abstract

A complete electrochemical study and a novel electroanalytical procedure for bromhexine quantitation are described. Bromhexine in methanol/0.1 mol L⁻¹ Britton–Robinson buffer solution (2.5/97.5) shows an anodic response on glassy carbon electrode between pH 2 and 7.5. By DPV and CV, both peak potential and current peak values were pH-dependent in all the pH range studied. A break at pH 5.5 in E_p versus pH plot revealing a protonation–deprotonation (pK_a) equilibrium of bromhexine was observed. Spectrophotometrically, an apparent pK_a value of 4.3 was also determined.

An electrodic mechanism involving the oxidation of bromhexine via two-electrons and two-protons was proposed. Controlled potential electrolysis followed by HPLC–UV and GC–MS permitted the identification of three oxidation products: *N*-methylcyclohexanamine, 2-amino-3,5-dibromobenzaldehyde and 2,4,8,10-tetrabromo dibenzo[*b,f*][1,5] diazocine.

DPV at pH 2 was selected as optimal pH for analytical purposes. Repeatability, reproducibility and selectivity parameters were adequate to quantify bromhexine in pharmaceutical forms. The recovery was $94.50 \pm 2.03\%$ and the detection and quantitation limits were 1.4×10^{-5} and 1.6×10^{-5} mol L⁻¹, respectively. Furthermore, the DPV method was applied successfully to individual tablet assay in order to verify the uniformity content of bromhexine. No special treatment of sample were required due to excipients do not interfered with the analytical signal. Finally the method was not time-consuming and less expensive than the HPLC one.

Keywords: Bromhexine; Differential pulse voltammetry; Tablets; Oxidation mechanism

1. Introduction

Bromhexine, 2-amino-3,5-dibromo-*N*-cyclohexyl-*N*-methylbenzenemethanamine (Fig. 1), is a mucolytic agent used in the treatment of respiratory disorders associated with viscid or excessive mucus [1,2]. The drug is well absorbed by oral route and spreads to the tissues, included the bronchial epithelium, where reaches sufficient concentrations to act locally. It undergoes oxidation and conjugation hepatic bio-transformation, and the drug and its metabolites are excreted in urine and feces [3]. Its pharmacological effect begins at 24–48 h and reaches its maximum to 5–7 days.

The drug has been quantified using different methods, in pharmaceutical forms [4–18] and in biological fluids [19–22]. For these purposes, UV–vis spectrophotometry [4–9], flow injection analysis with ion-selective electrodes [5], liquid chromatography [10–15,19,20], inductively coupled plasma mass spectrometry [20], capillary isotachopheresis [16], electrokinetic chromatography [17], liquid–gas chromatography [10,18] and gas chromatography with mass detection [22] have been employed.

From the electrochemical point of view, bromhexine has been also assayed by using chromatography with potentiometric detection [11] without an electrochemical characterization. On the other hand, its active metabolite, ambroxol (4-[(2-amino-3,5-dibromophenyl)methyl]amino)cyclohexanol), has been assayed in human serum using chromatography with amperometric detection [23]. Also, the voltammetric oxidation

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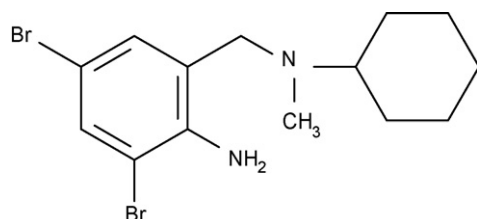


Fig. 1. Chemical structure of bromhexine.

of ambroxol and a differential pulse voltammetric method for its quantification in pharmaceuticals has been carried out [24]. Finally, a bromhexine-selective PVC membrane electrode based on bromhexinium tetraphenylborate has been also described [25].

At the best of our knowledge the electrochemical oxidation of bromhexine has not been previously reported. In this paper, we reveal the electrooxidation of bromhexine at the glassy carbon electrode focused to both clarifying the electrode mechanism and proposing a new method for its determination in pharmaceutical forms.

2. Experimental

2.1. Reagents

Bromhexine (99.89% chromatographically pure) was supplied by Labomed Laboratories (Santiago, Chile). Commercial tablets of Bisolvon[®] (declared amount per tablet 8.0 mg bromhexine, Boheringer Ingelheim S.A. Laboratories, Santiago, Chile) were obtained commercially.

All others reagents were of analytical grade unless indicated otherwise. Sodium hydrogen phosphate, phosphoric acid and acetonitrile HPLC grade were obtained from Merck. All solutions were prepared with ultrapure water ($\rho = 18 \text{ M}\Omega$) from Millipore-MilliQ system.

2.2. Solutions preparation

2.2.1. Buffer solutions

0.1 mol L⁻¹ Britton–Robinson buffer (acetic/boric/phosphoric acids mixture) for voltammetric experiments was used, and desired pH was adjusted with concentrate solutions of NaOH or HCl. For HPLC 0.01 mol L⁻¹ buffer phosphate solution (disodium hydrogen phosphate anhydrous salt) adjusted at pH 3.0 with phosphoric acid was used.

2.2.2. Stock drug solution

Approximately 154 mg bromhexine was dissolved and diluted up to 10 mL with methanol, to obtain a final concentration around of $4 \times 10^{-2} \text{ mol L}^{-1}$ bromhexine. The solution was protected from light by using amber glass material.

2.2.3. Work solution

An aliquot (12.5 μL) of the stock solution was taken and diluted to 10 mL with methanol–Britton–Robinson buffer solution (0.1 mol L⁻¹) (2.5:97.5, v/v), for both UV–vis spectroscopy

and electrochemical experiments or with acetonitrile-phosphate buffer solution (pH 3.0; 0.01 mol L⁻¹) (30:70, v/v) for HPLC experiments.

2.3. Apparatus

2.3.1. Voltammetric analyser

Differential pulse voltammetric (DPV) and cyclic voltammetric (CV) experiments were performed with a totally automatized workstation (BAS 100 B/W). A 25-mL thermostated BAS measuring cell with a glassy carbon electrode (GCE) ($\varnothing = 3 \text{ mm}$, BAS) as working electrode were used. A platinum wire and an Ag/AgCl were used as counter and reference electrodes, respectively. The operating conditions were: sensitivity 10–100 $\mu\text{A V}^{-1}$; potential range 0–1500 mV; sweep rate 100–1000 mV s⁻¹ for CV experiments. The working electrode surface was polished with 0.3 and 0.05 μm alumina slurries before each measurement [26].

2.3.2. Controlled potential electrolysis

Assays were carried out using a totally automatized assembly (BAS CV-100W), composed by a 10-mL electrolysis cell, a reticulated vitreous carbon (RVC) as a working electrode, an Ag/AgCl 3 M KCl and Pt as reference and auxiliary electrodes, respectively. The electrolysis potential was set at 1200 mV at pH 2, and the experiments were carried out in triplicate.

2.3.3. HPLC

Measurements were carried out by using a Waters assembly equipped with a model 600 Controller pump and a model 996 Photodiode Array Detector. The acquisition and treatment of data were made with the Millennium version 2.1 software. As chromatographic column a Bondapak/Porasil C18 column of 3.9 mm \times 150 mm was used. As column guard a C18 Bondapak (30 mm \times 4.6 mm) was employed. The injector was a 20 μL Rheodyne valve. UV detection at 245 nm was employed and the column was kept at constant temperature using a Waters column heater cartridge model 600.

An isocratic elution composed by a solution consisting of acetonitrile-phosphate buffer (pH 3.0; 0.01 mol L⁻¹) (30:70, v/v) mobile phase was used. The flow was 1.0 mL min⁻¹ and the working temperature was kept constant at $40 \pm 1 \text{ }^\circ\text{C}$. Below these conditions, bromhexine exhibited a retention time of $5.996 \pm 0.030 \text{ min}$.

2.3.4. Spectrophotometer

Spectrophotometric measurements were carried out with an UV–vis spectrophotometer ATI Unicam model UV3, using 1 cm quartz cell and equipped with a 486 computer with Vision acquisition and treatment program.

2.3.5. Gas chromatography (GC)

GC measurements were carried out in a Hewlett Packard GC 5890 with MSD 5972 mass detector (mode EI) and autosampler Hewlett Packard 7673A. The operating conditions were: injector temperature 250 $^\circ\text{C}$; detector temperature 310 $^\circ\text{C}$; program temperature: initial temperature 90 $^\circ\text{C}$ (1 min), 15 $^\circ\text{C min}^{-1}$, final temperature 310 $^\circ\text{C}$ (3 min). Run time: 18.67 min.

2.4. Analytical procedure

2.4.1. Calibration curve preparation

2.4.1.1. DPV and UV spectroscopy. By diluting the bromhexine stock solution with methanol–Britton–Robinson buffer (0.1 mol L⁻¹) (2.5:97.5, v/v) (pH 2), working solutions ranging between 2×10^{-5} and 1×10^{-4} mol L⁻¹ were prepared.

2.4.1.2. HPLC. By diluting the bromhexine stock solution with mobile phase, working solutions ranging between 3×10^{-6} and 3×10^{-5} mol L⁻¹ were prepared. The solutions were injected and chromatographed according to the working conditions previously given. UV detector was operated at $\lambda = 245$ nm.

2.4.2. Synthetic samples

Excipients (cornstarch, magnesium stearate, lactose, sodium lauryl sulfate and microcrystalline cellulose) were added to the drug for recovery studies, according to manufacturer's batch formulas for 8.0 mg bromhexine per tablet.

2.4.3. Individual tablet assay procedure

2.4.3.1. DPV. No less than 10 commercial tablets of bromhexine (Bisolvon[®], amount declared 8.0 mg bromhexine per tablet) were used. Every tablet was independently suspended in 10 mL methanol with ultrasonic agitation to assure the complete dissolution of the drug and diluted to a final volume of 100 mL with the same solvent. Then, 250 μ L were taken and diluted to 10 mL with methanol–Britton–Robinson buffer solution (0.1 mol L⁻¹) (2.5:97.5, v/v) pH 2. Finally, the sample solution was transferred to a voltammetric cell and recorded at least twice from 0 to 1500 mV. The amount of bromhexine (mg) in the sample solution was calculated using the standard calibration curve.

2.4.3.2. HPLC. The same procedure above described was applied for HPLC analysis, but the solution was filtered previous to inject on chromatograph. The amount of bromhexine (mg) in the sample solution was calculated from the corresponding prepared standard calibration curve.

2.4.4. Selectivity studies [27]

2.4.4.1. Degradation trials. Hydrolysis. Individually ca. 15 mg bromhexine were dissolved in 10 mL 1 mol L⁻¹ HCl in a 25 mL-distillation flask or 10 mL water for acid or neutral hydrolysis, respectively. Each solution was boiled for one hour at reflux.

Photolysis. (a) Bromhexine raw material: ca. 15 mg bromhexine were put on a black box and irradiated with UV light (UV Black-Ray long wave ultraviolet lamp, UVP model B 100 AP (50 Hz, 2.0 A) with a 100 W Par 38 Mercury lamp equipped with a 366 nm filter). Fifteen centimeter distance for 8 h (1.2×10^{19} quanta/s, determined by using the potassium ferrioxalate chemical actinometer [28]) were selected as experimental conditions. (b) Bromhexine solution: 10 mL of 1×10^{-3} mol L⁻¹ bromhexine solution (methanol–water

(30:70, v/v)) was bubbled by 2 min with nitrogen and transferred to a black box and then irradiated with UV light as the raw material.

Thermolysis: ca. 15 mg bromhexine were heated at 105 °C for 5 h.

Appropriate volumes of each obtained solution from degradation trials or the corresponding mg from thermolysis and photolysis in raw material assays were taken and completed to a final volume with methanol–Britton–Robinson buffer solution (0.1 mol L⁻¹) (2.5:97.5, v/v) (pH 2) to obtain a theoretical concentration of 5×10^{-5} mol L⁻¹ bromhexine. Samples from these studies were stored at -20 °C and protected from light prior to voltammetric and HPLC analysis. Each sample was analyzed by duplicate.

2.4.5. Statistic analysis

Comparison between different techniques, as well as the comparison with standard deviations, was carried out by means of the Student's *t*-test, and using significance limits between 95 and 99% of confidence [29,30].

3. Results and discussion

3.1. Electrochemical characterization

Bromhexine in methanol/0.1 mol L⁻¹ Britton–Robinson buffer solution (2.5/97.5) shows an anodic response on glassy carbon electrode in the pH range 2–7.5 (Fig. 2). Above this pH

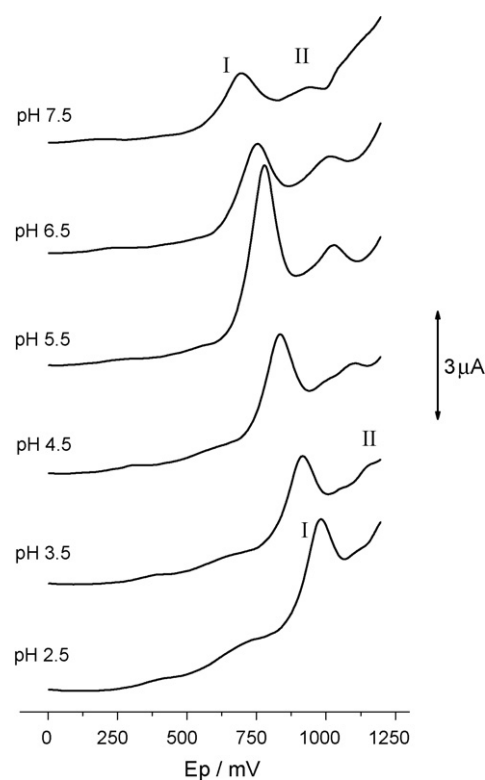


Fig. 2. Differential pulse voltammograms of 5×10^{-5} mol L⁻¹ bromhexine solution at different pHs (methanol/0.1 mol L⁻¹ Britton–Robinson buffer: 2.5/97.5).

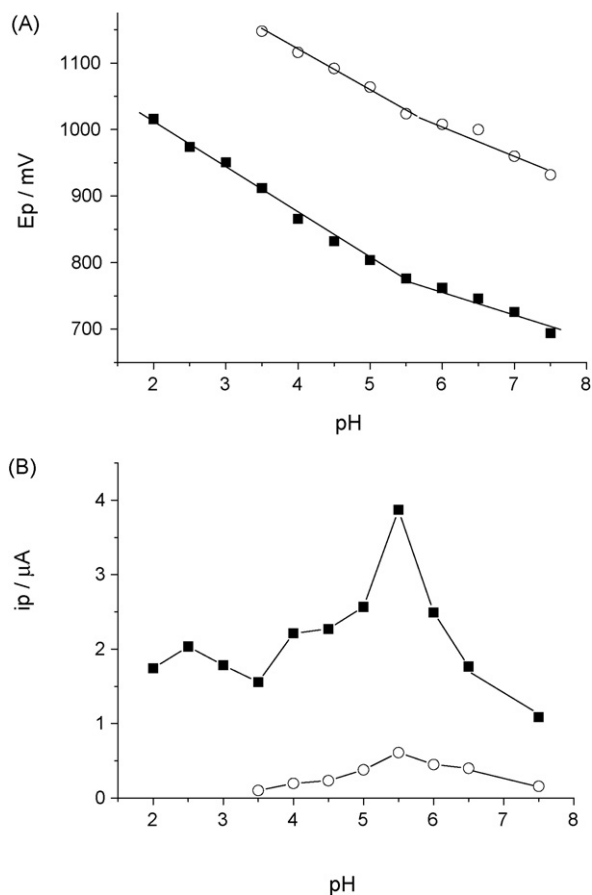


Fig. 3. (A) Peak potential evolution of $5 \times 10^{-5} \text{ mol L}^{-1}$ bromhexine solution at different pHs (methanol/0.1 mol L⁻¹ Britton–Robinson buffer: 2.5/97.5) and (B) i.p.–pH graph: (■) peak I, (○) peak II.

bromhexine precipitated. At $\text{pH} < 3.5$ bromhexine exhibits only one signal (peak I) and above $\text{pH} 4$ a second one appears at higher potentials (peak II). Peak I is pH-dependent in all the pH range studied (Fig. 3A), however a break in the E_p versus pH plot at $\text{pH} 5.5$ is observed, presumably due to a $\text{p}K_a$ of bromhexine. To confirm this assumption, the spectrophotometric behaviour of bromhexine was evaluated determining an apparent $\text{p}K_a$ value of 4.3 from the absorbance–pH plot (data not shown). On the other hand peak II shows to be pH-dependent between $\text{pH} 3.5$ and 7.5 but its intensity is very low compared with peak I. Fig. 3B shows the peak current dependence with pH for both signals. The peak current intensity of signal I increases up to $\text{pH} 5.5$ and then decreases probably due to the precipitation of bromhexine that it occurs at alkaline pHs. On the other hand, the peak current of signal II follows a similar pattern that signal I but with a lower intensity of current.

The electrooxidation of bromhexine was also studied by cyclic voltammetry (Fig. 4). Experiments were conducted at two pH values (2 and 5) at different sweep rate values ($100\text{--}1000 \text{ mV s}^{-1}$) and demonstrated that the bromhexine oxidation is irreversible. Furthermore, $\log i.p.$ versus \log sweep rate plot exhibits a slope value ~ 0.9 evidencing that bromhexine undergoes an adsorption processes on the electrode surface [31].

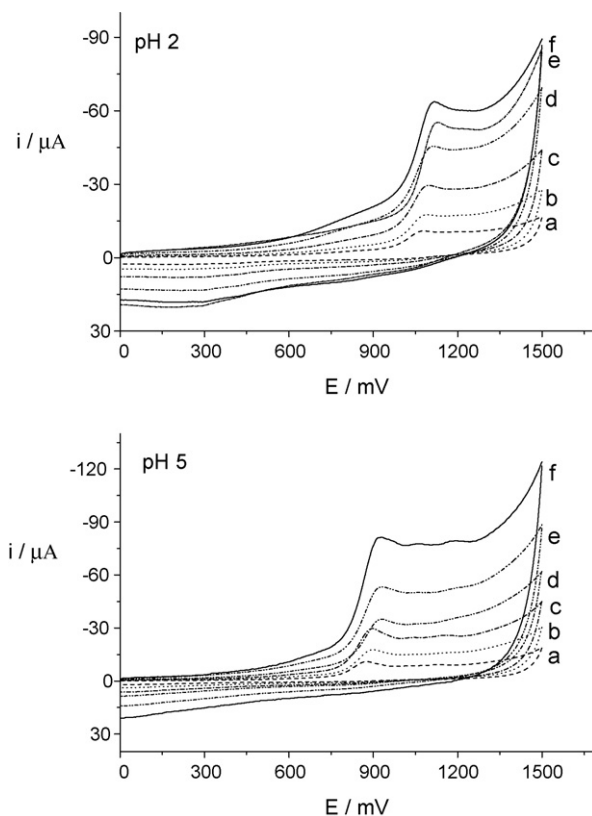


Fig. 4. Cyclic voltammograms of $1 \times 10^{-3} \text{ mol L}^{-1}$ bromhexine solution in methanol/0.1 mol L⁻¹ Britton–Robinson buffer: 2.5/97.5 at different sweep rate ($a = 100 \text{ mV s}^{-1}$, $b = 200 \text{ mV s}^{-1}$, $c = 400 \text{ mV s}^{-1}$, $d = 600 \text{ mV s}^{-1}$, $e = 800 \text{ mV s}^{-1}$, $f = 1000 \text{ mV s}^{-1}$) at $\text{pH} 2$ and $\text{pH} 5$.

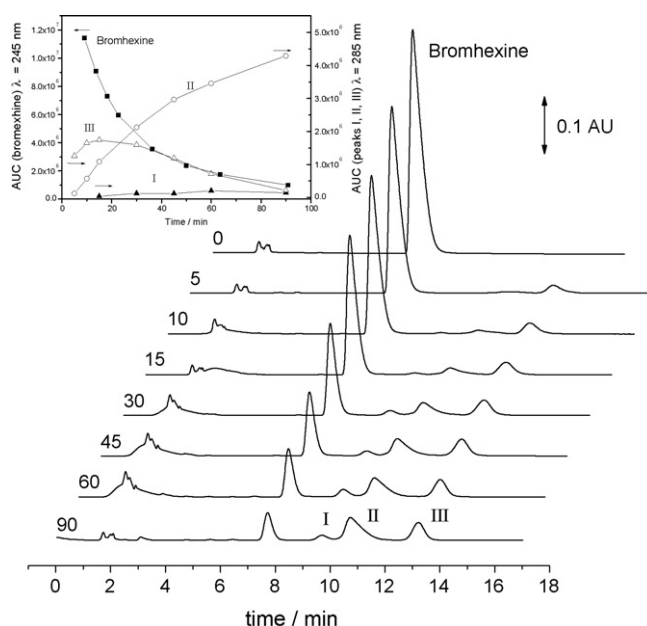
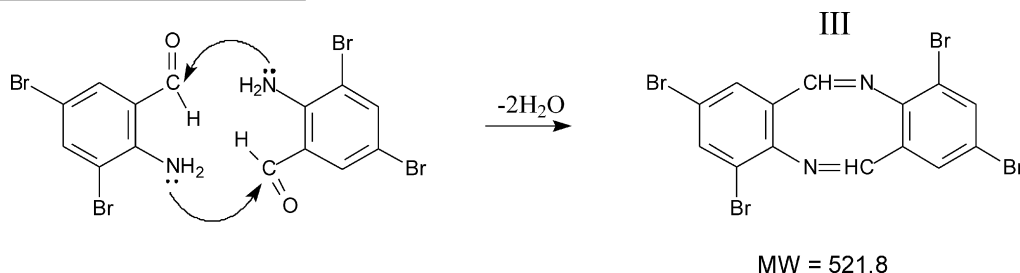


Fig. 5. Time course (min) of bromhexine electrolysis followed by HPLC–UV at $\lambda = 245 \text{ nm}$. Electrolysis potential = 1200 mV . Inset: area under curve (AUC) of each chromatographic signal vs. electrolysis time: (■) Bromhexine_{245 nm}, (▲) peak I_{285 nm}, (○) peak II_{285 nm}, (△) peak III_{285 nm}.

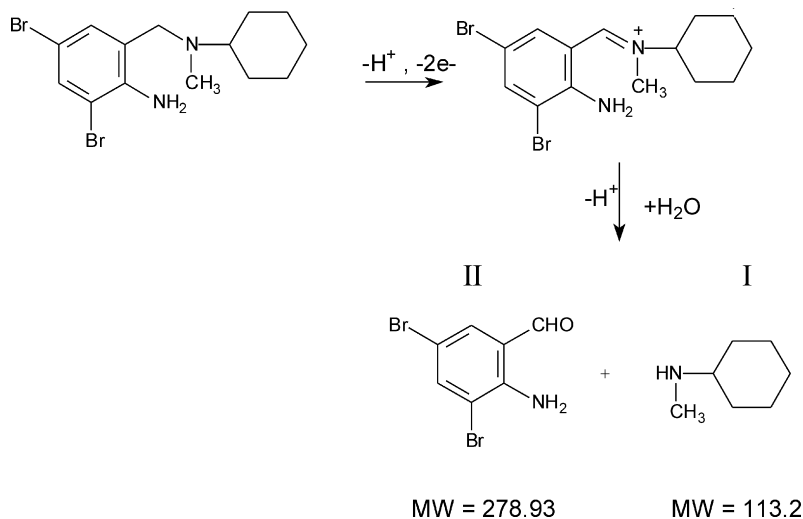
Controlled potential electrolysis experiments coupled with HPLC–UV were conducted to elucidate the mechanism of bromhexine electrooxidation. Concomitant with this experiment and using a rotating disk electrode a diffusion coefficient value of $1.22 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ was calculated for bromhexine solution at pH 2. From the total charge transferred after applied a constant potential of 1200 mV and using the Faraday equation [31], was possible to conclude that two electrons are involved in the electrodic process. Furthermore, at large electrolysis

This proposed mechanism explains the appearance of products I and II (*N*-methylcyclohexanamine and 2-amino-3,5-dibromobenzaldehyde, respectively). To elucidate the nature of the product III, we have considered its higher retention time and also its visible absorption band, concluding that the product must be more lipophilic and conjugated than bromhexine. According to that, we propose that product II reacts itself to form a dimeric structure via a dehydration reaction to generate product III (2,4,8,10-tetrabromodibenzo[*b,f*][1,5] diazocine) according to:



time ($t > 30$ min) the colourless solution turned red. Fig. 5 shows the time course of the electrolysis procedure followed by HPLC–UV at $\lambda = 245$ nm. In this figure, it can be appreciated that the main peak of bromhexine ($R_t = 7.4$ min) decreases as the electrolysis time increases; and three new peaks at R_t of 9.6 min (peak I), 10.8 min (peak II) and 13.3 min (peak III), appeared. Insert in Fig. 5 shows the electrolysis time evolution, expressed as area under curve at $\lambda = 285$ nm, of each new chromatographic signal (peaks I, II and III). As it can be seen, peak III increases up to 15 min of electrolysis and then decrease until almost disappeared after 90 min of electrolysis. The UV–vis spectra of each product reveals that both products I and II exhibit only one absorption band with a maximum near to $\lambda = 300$ nm. On the other hand, peak III exhibits the higher retention time, presenting an absorption band in the visible region (~ 400 nm) being coherent with the above mentioned color change in the solution. Obviously this color change suggests that this oxidation product has higher conjugation than the parent bromhexine (data not shown).

On the basis of these experimental evidences and the literature report [32], the following overall electrode mechanism was proposed for the bromhexine oxidation:



To confirm this mechanism, an identification of the final products by GC–MS was performed. From GC chromatogram, three products were detected at 2.191 min (product I, $M = 113$) and 7.62 min (product II, $M = 279$; $M + H^+ - CHO = 251$; $M - CHO - Br = 170$; $M - CHO - 2Br = 90$). Product II exhibited the typical isotopical relation of a 2Br-containing molecule [33]. Also it was possible to distinguish a signal corresponding to product III at 14.382 min ($M = 522$; $M + H^+ - Br = 443$; $M + H^+ - 2Br = 363$), which exhibited the typical isotopical relation of a 4Br-containing molecule [33]. In conclusion, the three products proposed as final products of the bromhexine electrooxidation were clearly identified by GC–MS, supporting the proposed electrochemical oxidation mechanism.

3.2. Selectivity studies

In order to prove the selectivity of the method, we tested typical excipients used in oral formulations (corn starch, magnesium stearate, lactose, sodium lauryl sulfate and microcrystalline cellulose) and follow classical degradation trials: hydrolysis (acidic and neutral), photolysis and thermolysis

Table 1
Analytical parameters for the developed methods

Parameter	DPV ($E_p = 1016$ mV)	HPLC–UV ($\lambda = 245$ nm)
Repeatability, CV (%) ^a	0.46	0.24
Reproducibility, CV (%) ^a	0.57	0.39
Recovery (%) ^b \pm S.D.	94.50 \pm 2.03	91.0 \pm 0.16
Concentration range (M)	$2.0 \times 10^{-5} - 1.0 \times 10^{-4}$	$2.0 \times 10^{-5} - 1.0 \times 10^{-4}$
Calibration curve	I_p (μ A) = $0.03684 C$ (M) – 4.0923×10^{-7}	AUC = $1.25 \times 10^{10} C$ (M) – 107801
Detection limit (M)	1.4×10^{-5}	1.1×10^{-6}
Quantitation limit (M)	1.6×10^{-5}	1.5×10^{-6}

^a Concentration level of 5.0×10^{-5} M.

^b Average on a concentration level of 5.0×10^{-5} M.

[27]. Results indicate that no new signal appeared in the voltammograms and also the signal of analyte is not disturbed due to neither the excipients nor the degradation trials for bromhexine. According with those results, it can be concluded that the proposed DPV method is sufficiently selective in order to be applied to bromhexine quantification and no previous separation or extractions were necessary.

3.3. Analytical studies

For developing an electroanalytical method to quantify bromhexine in pharmaceuticals we have selected peak I at pH 2 as the optimal analytical signal.

In order to provide a DPV quantitative procedure, the dependence between peak current (I_p) and bromhexine concentration was studied. A linear relation between 2×10^{-5} and 1×10^{-4} mol L⁻¹ was found. Above this concentration (1×10^{-4} mol L⁻¹) lost of linearity was observed, probably due to the absorption of bromhexine on electrode surface. The calibration curve was described by the following regression equation: I_p (μ A) = $3.684 \times 10^{-3} C$ (mol L⁻¹) – 4.0923×10^{-7} ($r = 0.9996$, $n = 8$). Within-day and inter-day reproducibilities were adequate with R.S.D. values lower than 3%.

For comparative purposes a HPLC method was developed. In Table 1, the analytical parameters achieved using both methodologies are summarized.

Our findings are rather different from those previously obtained using conventional and coated wire ion-selective electrodes [5]. There, the recoveries values were higher than ours. Methodological procedures could explain the differences since in our case bromhexine was tested in presence of typical excipients used in oral formulations and in Reference [5] it was tested in presence of cations, anions and others drugs.

3.4. Determination of bromhexine in tablets

Finally, the developed DPV method was applied successfully to the individual tablet assay in order to verify the uniformity content of bromhexine. For comparative purposes, also HPLC analysis was carried out. Table 2 shows the analytical results obtained by both methods which show a good agreement between them. The content for all assayed tablets fulfill the Pharmacopoeia requirement, i.e., for uniformity content of tablets (average) the content must be in the range of 85.0–115.0% of

Table 2
Individual tablet assay of bromhexine

	Differential pulse voltammetry ($E_p = 1016$ mV)	HPLC–UV ($\lambda = 245$ nm)
	mg/tablet	mg/tablet
	7.5	7.2
	7.4	7.3
	7.4	7.3
	7.5	7.3
	7.4	7.3
	7.4	7.3
	7.5	7.3
	7.4	7.3
	7.5	7.2
Average	7.4	7.3
S.D.	0.05	0.04
CV (%)	0.7	0.6

Declared amount per tablet: 8.0 mg bromhexine.

label claim and none individual value must be out of the range of 75.0–125.0% of label claim [34].

Using the Student's *t*-test; the average of both analytical methods were not significantly different, since the calculated *t*-test value (DPV: –1.47; HPLC: –1.34) were less than the tabulated value (2.262) at $p = 0.05$.

4. Conclusions

A two-electron two-proton mechanism for the electrochemical oxidation of bromhexine was revealed. *N*-methylcyclohexanamine, 2-amino-3,5-dibromobenzaldehyde and 2,4,8,10-tetrabromodibenzo[*b,f*][1,5] diazocine were identified as the oxidation products from bromhexine.

On the basis of the electrochemical response of bromhexine, a DPV method for its determination was proposed and compared with a HPLC one. The proposed DPV method was successfully applied to the determination of the drug in tablets without excipients' interference. Preparation of the sample was easy and the method is not time consuming and cheap.

Acknowledgements

Financial support from FONDECYT “Lineas Complementarias” (grant no. 8000016) and Chemical and Pharmaceutical

Sciences Faculty, University of Chile is gratefully acknowledged.

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