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Article

Electrochemical Reduction and Stripping Voltammetric Determination of the Anti-Glaucoma Drug Levobunolol HCl in Formulation and Human Serum at the Mercury Electrode

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Levobunolol HCl é um agente bloqueador ß-adrenoceptor potente e não seletivo usado no tratamento tópico de pressão intraocular aumentada em pacientes com glaucoma de ângulo aberto crônico ou hipertensão ocular. Um método voltamétrico de onda quadrada e redissolução catódico adsortivo (SW-AdCSV) preciso, rápido e sem extração foi descrito para quantificação de traços de levobunolol HCl puro, formulações comerciais (gotas oftalmológicas) e sérum humano. Os limites de quantificação (LOQ) de $1{,}0 \times 10^{-10}$ mol L^{-1} (na forma pura) e $2{,}5 \times 10^{-10}$ mol L^{-1} levobunolol HCl (em sérum humano adulterado) foram obtidos pelo método descrito. Foram obtidas interferências não significativas dos excipientes associados à formulação de levobunolol HCl e de alguns íons metálicos comuns, medicamentos co-administrados, alguns outros agentes ß-bloqueadores e seu metabolito diidrolevobunolol, que possivelmente estão presentes em fluidos biológicos. O método SW-AdCSV descrito é sensível o suficiente para determinar o medicamento em sérum humano, comparado à maioria dos métodos relatados.

Levobunolol HCl is a potent non-selective ß-adrenoceptor blocking agent used for the topical treatment of increased intraocular pressure in patients with chronic open angle glaucoma or ocular hypertension**.** Precise, rapid and extraction-free square-wave adsorptive cathodic stripping voltammetry (SW-AdCSV) method has been described for trace quantitation of levobunolol HCl in bulk form, commercial formulation (ophthalmologic drops) and human serum. Limits of quantification (LOQ) of 1.0×10^{-10} mol L^{-1} (in bulk form) and 2.5×10^{-10} mol L^{-1} levobunolol HCl (in spiked human serum) were achieved by the described method. Insignificant interferences from excipients associated with formulation of levobunolol HCl and from some common metal ions, co-administrated drugs, some other ß-blocker agents and its metabolite dihydrolevobunolol that are likely to be present in the biological fluids were obtained. The described SW-AdCSV method is sensitive enough to assay the drug in human serum compared to most of the reported methods.

Keywords: levobunolol HCl, electrode reaction, determination, stripping voltammetry

Introduction

Glaucomas are a family of "silent diseases" at least until the later stages and if not treated, invariably result in irreversible blindness. The early detection and adequate treatment minimizes the visual morbidity from these conditions. The current management of glaucoma is directed to lower intraocular pressure (IOP) and the medical therapy is always the first line treatment for the management of primary open angle glaucoma. The most frequently used medical treatment in lowering IOP is a topical beta-blocker (ß-blocker). Levobunolol HCl (LV.HCl, 5-[(2*S*)-3-(*tert*-butylamino)-2-hydroxypropoxy]-1,2,3,4-tetrahydronaphthalen-1-one hydrochloride) is an ophthalmic potent non-selective beta-adrenoceptor blocking agent with long duration of action[1,2] (Scheme 1A).

It has been shown that the metabolic reduction of the cyclohexanone functional group to form dihydrolevobunolol (5-[(2*S*)-3-(*tert*-butylamino)-2-hydroxypropoxy]-1,2,3,4-tetrahydronaphthalen-1-ol, Scheme 1B) is mediated by

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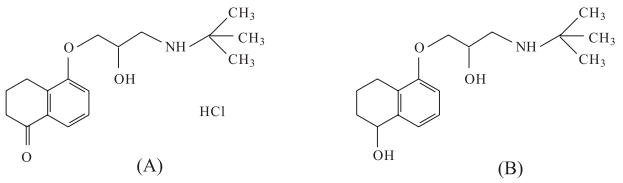


Scheme 1: Chemical structure of levobunolol HCl (LV.HCl) molecule (A) and its metabolite dihydrolevobunolol (B).

NADPH-dependent ketone reductase in ocular tissues including cornea.[3] As dihydrolevobunolol is equipotent to levobunolol, levobunolol produced greater reduction in IOP[4,5] and could therefore be a better alternative to the other beta-blockers (betaxolol and timolol).[6]

The mean plasma levels of levobunolol in normal volunteers after a single topical instillation of 0.5 or 1% m/v levobunolol in both eyes, ranged from 0.1 to 0.3 ng L^{-1} (3.05×10^{-10} to 9.15×10^{-10} mol L^{-1}) for 0.5% concentration and 0.3 to 0.6 ng L^{-1} (9.15×10^{-10} to 1.83×10^{-9} mol L^{-1}) for 1% concentration.[7]

The official method of determining LV.HCl in formulation was liquid chromatography.[8] There have also been reports for determination of the drug in formulation[9-11] including membrane electrodes[9] (limit of detection (LOD) in bulk = 5.50×10^{-7} mol L^{-1}), spectrophotometry[10,11] (LOD in bulk = 4.57×10^{-5} mol L^{-1} and 9.15×10^{-6} mol L^{-1}). Besides, few studies have been reported for determination of the drug in biological fluids[12-14] including high-performance liquid chromatography (HPLC)[12,13] (LOD in plasma = 1.5×10^{-8} mol L^{-1}) and liquid chromatography/tandem mass spectrometry (LOD in urine = 3.0×10^{-8} mol L^{-1}).[14] The reported spectrophotometric methods are either non-specific, time-consuming or indirect (based on formation of colored chromogens prior to the analysis). The chromatographic methods need time-consuming extraction steps. They have also expensive instrumentation and running costs. Besides, liquid chromatography/tandem mass spectrometry is not economically feasible for routine analysis. Moreover, the limits of quantification (LOQ) of these reported analytical methods were inadequate for clinical blood samples after ophthalmic doses.[7]

However, adsorptive stripping voltammetry has been shown to be an efficient electroanalytical technique for the determination of sub-nanomolar levels of a wide range of drugs that have an interfacial adsorptive character onto the working electrode surface.[15] To our knowledge, no information is reported in the literature to date concerning the stripping voltammetric quantification of LV.HCl.

In this work, LV.HCl is not responded at the carbon paste and glassy carbon electrodes, which may be due to its reduction potential being beyond the potential range of these electrodes. Mercury is a very attractive choice of material because it has a high hydrogen overvoltage (which facilitates the study of electroreduction of substances of very high negative reduction potentials in aqueous electrolyte (e.g. >C=O group in the examined LV.HCl molecule), possesses a highly reproducible, readily renewable, and smooth surface.

Therefore, the present work aimed to study the electroreduction of LV.HCl at the hanging mercury dropping electrode (HMDE) and to develop a simple and reliable square-wave adsorptive cathodic stripping voltammetric (SW-AdCSV) method for its trace quantification in bulk form, commercial formulation (eye drops) and in body fluids without prior extraction.

Experimental

Equipments

Computer-controlled electrochemical analyzers models 263A and 273-PAR (Princeton Applied Research, Oak Ridge, TN, USA) with the software package 270/250-PAR were used for the voltammetric measurements. An electrode assembly (303A-PAR) incorporated with a micro-electrochemical cell and a three-electrode system comprising of a hanging mercury drop electrode (HMDE) as a working electrode (surface area = 0.026 cm^2), an $Ag/AgCl/KCl_s$ reference electrode and a platinum wire auxiliary electrode were used. A magnetic stirrer (305-PAR) was used to provide the convective transport during the accumulation step.

Materials and solutions

LV.HCl was supplied from Sigma-Aldrich. A stock standard solution of 1.0×10^{-3} mol L^{-1} bulk LV.HCl was prepared in distilled water, and then stored at 4 °C. Working solutions of LV.HCl were prepared daily by appropriate dilution with distilled water just before use.

The commercial sample analyzed was Betagan®; 0.5% m/v LV.HCl eye drops solution (Westport, Allergan Pharmaceutical, Co. Mayo, Ireland) which was purchased from a local pharmacy. One mL of the drug formulation, equivalent to 5 mg LV.HCl was accurately transferred into a 10 mL volumetric flask and diluted to the mark with distilled water. Appropriate dilution with distilled water was carried out just before use.

Six serum samples of three healthy subjects (two samples from each subject) were stored frozen until assay. Into each of 10 centrifugation tubes (3.0 mL-volume polypropylene micro-centrifuge tubes) containing 1.0 mL-volume of the

human serum and a certain concentration of LV.HCl, 1.0 mL of methanol was transferred and mixed well to denature and precipitate proteins. The solutions were centrifuged (using an Eppendorf centrifuge 5417C, Hamburg, Germany) for 3 min at 14000 rpm to separate out the precipitated proteins. The clear supernatant layers of the solutions were filtered through 0.45 μm Millipore filters to produce protein-free human serum samples. Appropriate dilution with Britton-Robinson (BR) universal buffer at pH 7.0 was carried out just before use to obtain serum samples spiked with various concentrations of LV.HCl (1.0×10^{-10} to 1.0×10^{-6} mol L^{-1}).

A series of Britton-Robinson (BR) universal buffer at pH 2.0 to 11.5 (a mixture of 0.04 mol L^{-1} solution each of acetic, orthophosphoric and boric acids adjusted to the required pH with 0.20 mol L^{-1} sodium hydroxide solution) as a supporting electrolyte was prepared. A pH-meter (Crison, Barcelona, Spain) was used for the pH measurements. Deionized water was supplied from a Purite-Still Plus de-ionizer connected to an AquaMatic double-distillation water system (Hamilton Laboratory Glass LTD, Kent, UK).

Results and Discussion

Electrochemical and adsorptive behavior of levobunolol HCl

Cyclic voltammograms of 1.0×10^{-4} mol L^{-1} LV.HCl were recorded at the HMDE in the BR universal buffer of various pH values at various scan rates (100-500 mV s^{-1}). The voltamograms exhibited a main single reduction peak in the pH range 4.0 to 9.0 (e.g., Figure 1). No anodic peaks were obtained on the reverse scan indicating irreversible nature of reduction process. Since LV.HCl is a ketonic drug that is efficiently reduced to corresponding alcohols *in vivo*,[7] its electroreduction at the mercury electrode was attributed to reduction of its >C=O double bond (via the consumption of two electrons as confirmed also from controlled-potential electrolysis). However, at pH ≥ 10.0, an ill-defined second small reversible reduction peak appeared at more negative potentials beside the main one (Figure 1).

The peak potential E_p of the main peak shifted to more negative values with the increase of pH, which denotes that the protons are involved in the electrode reaction process and the proton-transfer reaction precedes the electron transfer process.[16] Rectilinear plots of the peak potentials E_p *vs.* pH were obtained; their corresponding regression equations were:

$$E_p \text{ (V)} = 0.045 \text{ pH} + 1.34,$$
$$r = 0.992 \text{ and } n = 6 \text{ (pH 4.0 to 9.0)} \quad (1)$$

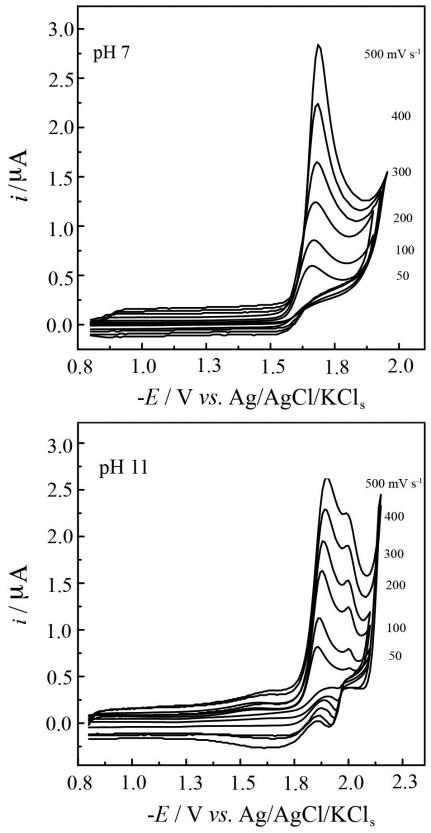


Figure 1. Voltammograms of 1.0×10^{-4} mol L^{-1} LV.HCl in the BR universal buffer of various pH values at various scan rates.

$$E_p \text{ (V)} = 0.075 \text{ pH} + 1.04,$$
$$r = 0.981 \text{ and } n = 3 \text{ (pH 9.0 to 11.0)} \quad (2)$$

The break of E_p *vs.* pH plot around pH 9.0 (Figure 2a) would indicate a change in the protonation of the electroactive species and can be related to the equilibrium constant of LV.HCl (pK_a = 9.40 to 9.66).[9,17] Most beta blockers are basic in nature and contain a secondary amine group able to gain a proton from media and have acidic pK_a (proton lost) values in the range 8.60 to 9.70.[9] So, the LV.HCl acidic pK_a value of 9.40 to 9.66[9,17] is due to the formation of nonprotonated amino group. This value is of importance during formulation development, as the pH at which the drug is ionized may affect its distribution in biological fluids and within or across membranes.[18]

It was also observed that as pH increased, there was an increase of the peak current magnitude (i_p) of the main reduction peak up to pH 10.0 then remained constant (Figure 2b). As both the acidic (I) and basic (II) forms of LV.HCl are electroactive, this behavior may be due to strong contribution of the less solvated[19] and/or of strong adsorptive deprotonated (basic) form of LV.HCl[20] as pH increases. On one side, the small values of peak current magnitude in acid media (where protonated form is

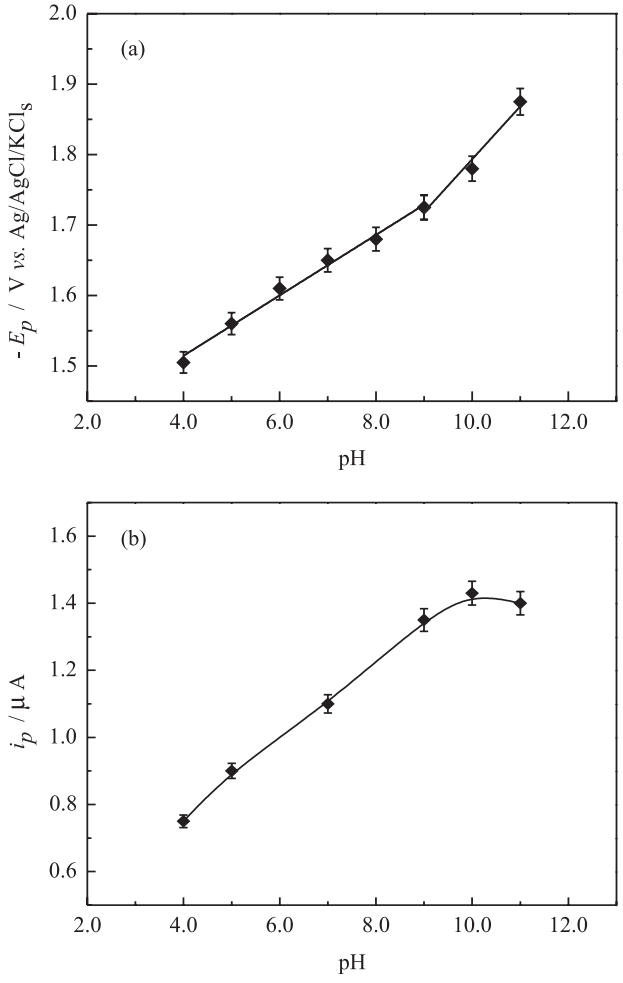
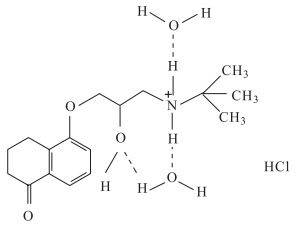


Figure 2. (a) E_p *vs.* pH and (b) i_p *vs.* pH plots for LV.HCl at various pH values (scan rate = 200 mV s^{-1}).

predominant) may be due to the decrease of diffusion rate of the protonated reactant species because of stereoelectronic and solvation effects through hydrogen bonding of the conjugate acid of LV.HCl (positively charged ammonium ion (acidic form) is more effectively solvated than an uncharged amine (basic form)), Scheme 2.[19]



Scheme 2. Solvation of the conjugate acid (I) of LV.HCl.

On the other side, the adsorption of the drug on the electrode might also be pH-dependent.[20,21] It may be that adsorption of reducible species is more efficient in neutral and alkaline media than in acidic one.[21] This assumption can be confirmed from the dependence of peak current (i_p) on scan rate (v) at different pH values.

According to the Randles-Sevcik equation for irreversible process:[22,23]

$$i_p = (2.99 \times 10^5)\ n\ (\alpha n_a)^{1/2} A C^0 D^{1/2} v^{1/2} \quad (3)$$

the peak current i_p is proportional to the square root of scan rate $v^{1/2}$ (semi-infinite diffusion),[23] while for an adsorption-controlled reaction,[22,24] the peak current i_p is proportional to the applied scan rate v (thin layer behavior), according to the following equation:[22]

$$i_p = (n^2F^2A\Gamma^0 / 4RT)\ v \quad (4)$$

where n_a is the number of electron transfer in the rate-determining step, A (cm^2) is the surface area of the working electrode, D ($cm^2\ s^{-1}$) is the diffusion coefficient, C^0 (mol cm^{-3}) is the bulk concentration of analyte, n is the total number of electrons consumed in the reduction process and Γ^0 (mol cm^{-2}) is the mole of analyte adsorbed onto surface of the electrode. Linear plots of i_p (μA) *vs.* scan rate v (V s^{-1}) were obtained at different pH values with slope values of 1.94 (pH 5.0) to 5.80 (pH 10.0) μA V^{-1} s (r = 0.993 ± 0.003 and n = 6) indicating that the reduction process of LV.HCl at the HMDE is controlled by adsorption (surface-controlled process).[22,24] Moreover, the slope value of i_p *vs.* v plots increased as the pH increased indicating again that the adsorption effects of the process become evident in neutral and alkaline media than in acidic one. Besides, the slope values of log i_p *vs.* log ν plots were in the range of 0.41-0.45 μA mV^{-1} s in acidic media (pH < 7.0) which are close to the theoretically expected value of 0.50 for a diffusion-controlled process.[23,24] However, as the pH was increased, the slope values increased from 0.70 μA mV^{-1} s (pH 7.0) to 0.79 μA mV^{-1} s (pH 10.0), which are relatively close to the theoretically expected value of 1.0 for an adsorption controlled process.[22,24]

Furthermore, the irreversible nature of the electrode reaction was also confirmed from the shift of peak potential E_p to more negative values upon the increase of scan rate v (100-500 mV s^{-1}) at different pH values.[22,25] Plots of E_p *vs.* ln v at different pH were linear; their corresponding regression equation was:

$$E_p\ (V) = (0.015 \text{ to } 0.018)\ \ln(v\ (mV\ s^{-1})) + (1.56 \text{ to } 1.91),$$
$$r = 0.988 \pm 0.144 \text{ and } n = 6 \quad (5)$$

Values of αn_a (product of symmetry transfer coefficient α and number of electrons n_a transferred in the rate-determining step) of 0.71 to 0.86 were estimated from slope values of the obtained E_p *vs.* ln v plots according to following equations

of the totally irreversible electrode reaction:[22,25]

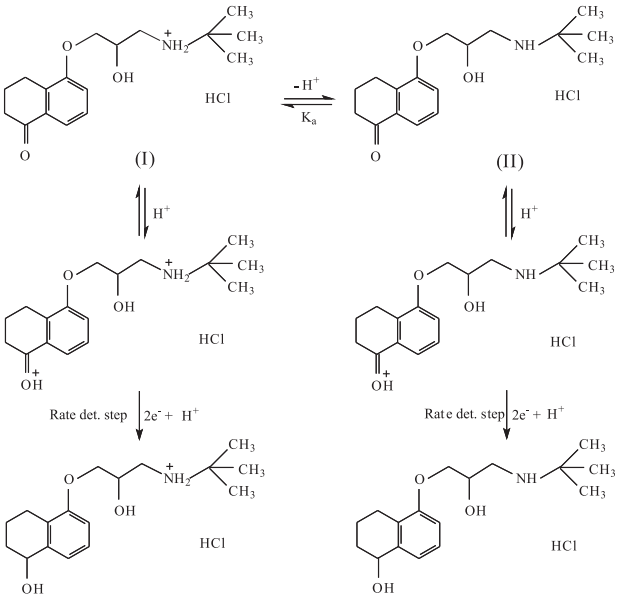
$$E_p = E^o + (RT / 2\alpha n_a F) \ln(RTkf^o / \alpha n_a F) - (RT / 2\alpha n_a F) \ln v \quad (6)$$

$$\Delta E_p \text{ (V)} / \Delta \ln v \text{ (mV s}^{-1}) = 0.02569 / 2\alpha n_a \quad (7)$$

The most probable values of transfer coefficient α (0.36 to 0.43) were estimated at various pH values, for the number of electrons ($n_a = 2$) transferred in the rate-determining step for the electroreduction of the >C=O double bond of the analyte.[16] This is confirming again the irreversible nature of the electrode reaction of LV.HCl at the HMDE.

The number of protons (Z_{H^+}) involved in the rate-determining step was estimated from slope value of the E_p *vs.* pH plot using the relation[26] ΔE_p (V) / ΔpH = (0.0591 / αn_a) Z_{H^+} and was found to be equal one ($Z_{H^+} = 1$).

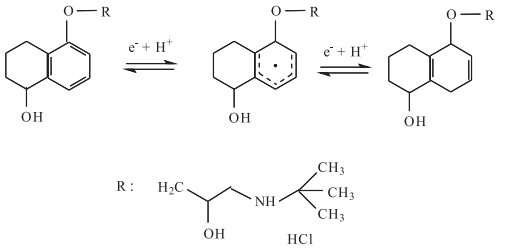
Since both the acidic and basic forms of LV.HCl are electroactive, the suggested electrode reaction mechanism for the main reduction peak (which corresponds to the electroreduction of >C=O double bond of the acidic (I) and the basic (II) forms in strong acidic and alkaline media, respectively) is shown in Scheme 3.



Scheme 3. Electrode reaction mechanism of the first reduction process of LV.HCl at the mercury electrode.

However, in solutions of intermediate pH values where LV.HCl molecule is expected to be present in an acid-base equilibrium, the reduction of both the acidic (I) and the basic (II) forms (depending on the extent of equilibrium as the pH increases) takes place via one cathodic step.

On the other hand, the second small reversible reduction peak at more negative potential at pH ≥ 10.0 may be due to the reduction of the aromatic ring,[27-30] which is simply the electrochemical equivalent of the Birch reaction.[30,31] The reduction of the phenyl ring of LV.HCl was so difficult that its corresponding cathodic peak appeared only in solution of high pH values (pH ≥ 10.0, Scheme 4).



Scheme 4. Electrode reaction mechanism of the second reduction process of LV.HCl at the mercury electrode.

On the other side, the interfacial adsorptive affinity of LV.HCl onto the HMDE surface was also designated by recording the cyclic voltammograms of 1.0×10^{-6} mol L^{-1} LV.HCl at 100 mV s^{-1} in the BR universal buffer at pH 7.0 following its preconcentration by adsorptive accumulation onto the HMDE under open circuit conditions (Figure 3, curve a), and then at preconcentration potential (E_{acc}) of –0.8 V (*vs.* Ag/AgCl/KCl_s) for 20 s (Figure 3, 1st cycle, curve b and 2nd cycle, curve c). As shown in Figure 3, enhanced peak current magnitude was observed following preconcentration of the analyte by adsorptive accumulation onto the HMDE (1st cycle, curve b) compared to that recorded following accumulation of the drug at open circuit (curve a) confirmed the interfacial adsorptive character of LV.HCl onto the mercury electrode. Whereas in the 2nd cycle (curve c) the voltammogram exhibited very small peak current which may be attributed to desorption of LV.HCl from the mercury electrode surface.

Furthermore, the electrode surface coverage (Γ^o

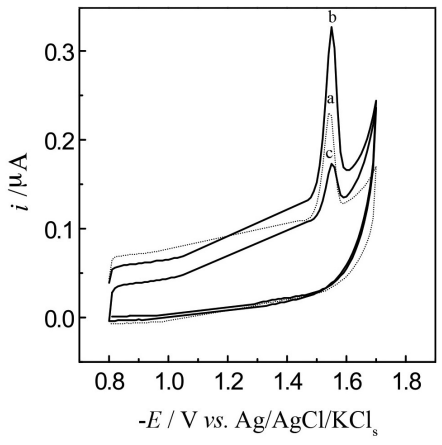


Figure 3. Cyclic voltammograms of 1.0×10^{-6} mol L^{-1} LV.HCl in the BR universal buffer at pH 7.0 recorded following its preconcentration onto HMDE by adsorptive preconcentration under open circuit conditions (a) and then at E_{acc} = –0.8 V for 20 s (1st cycle (b) and 2nd cycle (c)); scan rate = 100 mV s^{-1}.

(mol cm^{-2})) of LV.HCl in the BR universal buffer at pH 7.0 was estimated using the equation:[22]

$$\Gamma^{o} = Q / nFA \quad (8)$$

where Q (C) is the charge consumed by the surface process, which was estimated by the integration of the area under the peak corrected to the residual current, n is the number of electrons consumed in the reduction of >C=O group to the >CHOH via the consumption of 2 electrons (n = 2), F is the Faraday constant (96487 C) and A is the surface area of the working electrode (0.026 cm^2). On dividing the amount of charge (Q) consumed by the surface process, 1.06×10^{-6} C, by the conversion factor nFA (5017.324 mol C cm^{-2}), a monolayer surface coverage of 2.11×10^{-10} mol cm^{-2} was estimated. Each adsorbed LV.HCl molecule thus occupied an area of 0.79 nm^2.

Electroanalytical studies

Based on the adsorption behavior of LV.HCl onto the mercury electrode surface, square-wave adsorptive stripping voltammetry (SW-AdCSV) method was optimized for its trace determination. The optimum operational conditions for its analytical determination were studied as:

Effect of pH of the medium

This was carried out by recording voltammograms of 7.0×10^{-9} mol L^{-1} bulk LV.HCl at the HMDE in the BR universal buffers at various pH values using square-wave potential-waveform. A better developed peak current was achieved in the BR universal buffer at pH 7.0 following preconcentration of LV.HCl by adsorptive accumulation onto the HMDE at −0.7 V for 20 s. Therefore, BR buffer at pH 7.0 was chosen as a supporting electrolyte for the rest of the present analytical study.

Effect of preconcentration conditions

Voltammograms of 7.0×10^{-9} mol L^{-1} LV.HCl were recorded using square-wave potential-waveform following preconcentration by adsorptive accumulation onto the HMDE for 20 s at various accumulation potentials E_{acc} (−0.4 to −1.3 V). A better developed peak current was achieved over the potential range of (−0.8 to −1.1 V), Figure 4A. This is because of an increase of the accumulation rate, due to the more favorable alignment of the molecules by the electric field at the electrode solution interface.[32,33] However, at more negative potentials the peak current decreased indicating that the reactant species were no longer strongly adsorbed at potentials where the mercury is negatively charged with respect to the point of zero charge potential.[33] Therefore, a preconcentration potential of −0.8 V (*vs.* Ag/AgCl/KCl$_s$) was chosen for the rest of the present analytical study.

On the other hand, the dependence of SW-AdCSV peak current magnitudes of 7.0×10^{-9}, 1.0×10^{-9}, 8.0×10^{-10} and 1.0×10^{-10} mol L^{-1} LV.HCl on the preconcentration time (t_{acc}) of the analyte at E_{acc} = −0.8 V was studied. Figure 4B shows that at fixed preconcentration time the growth of the adsorbed LV.HCl layer is faster as its bulk solution concentration increases and consequently peak current increases.[21] The linear response of SW-AdCSV peak current magnitudes was extend up to 30 s then leveled off. This indicates that the adsorptive equilibrium onto the mercury electrode surface was achieved.[34] The subsequence observed decrease in the peak current magnitude with preconcentration times (curves a and b) probably owing to an inhibition of the voltammetric process occurring after saturation of mercury drop[34] or interactions among the molecules in the adsorbed state become noticeable.[35] This could be explained by the release of an amount of the adsorbed molecules due to possible repulsive interaction between the adsorbed species when coverage of the electrode has been reached.[35] So, in the present analytical investigations, preconcentration time of 20 s was applied to avoid the achievement of saturation of the electrode surface.

Effect of pulse parameters

The influence of pulse parameters (frequency f = 10 to 120 Hz, scan increment ΔE_s = 2 to 12 mV and pulse height a = 5 to 30 mV) on the peak current response were studied in BR universal buffer at pH 7.0 and E_{acc} = −0.8 V for 20 s. Well-developed peak current was achieved at pulse parameters of: f = 80 Hz, ΔE_s = 10 mV and a = 25 mV.

The influence of the rest time was also considered and a time period of 5 s was chosen. On the other hand, square-wave signal was found to increase as the area of the HMDE was increased (0.010 to 0.026 cm^2); therefore, the present study was carried out at an HMDE area of 0.026 cm^2.

Accordingly, the optimal conditions of the described SW-AdCSV method were: f = 80 Hz, ΔE_s = 10 mV and a = 25 mV. Besides, the optimal preconcentration conditions were: E_{acc}= −0.8 V (*vs.* Ag/AgCl/KCl$_s$) and t_{acc} = 20 s using the BR universal buffer at pH 7.0 as a supporting electrolyte.

Method validation

Validation schemes of the analytical methods for the determination of various analytes are defined in ICH guidelines and some pharmacopoeias such as United

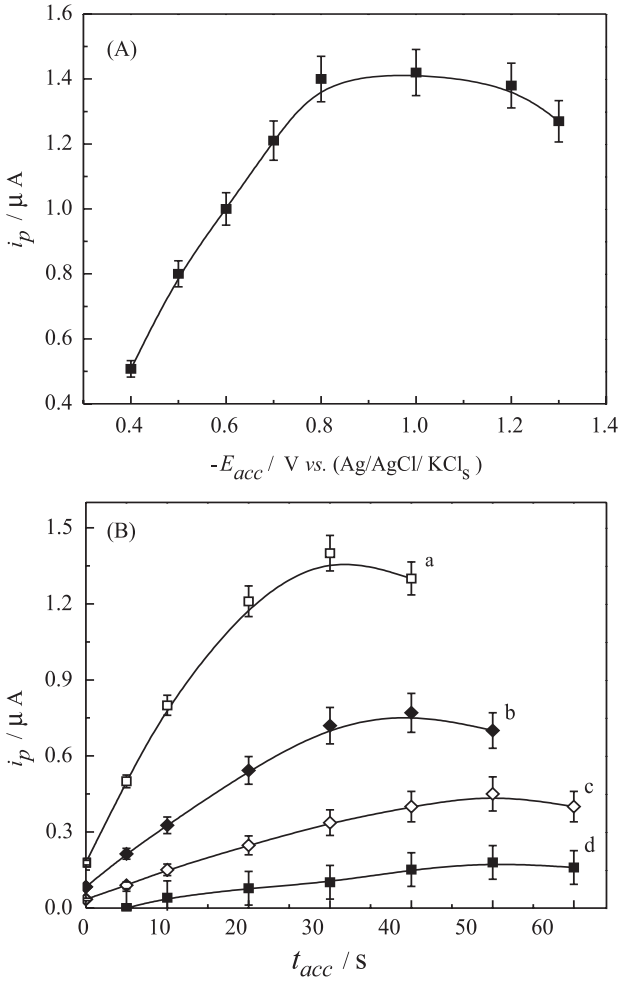


Figure 4. (A) Effect of preconcentration potential (E_{acc}) on SW-AdCSV peak current of 7×10^{-9} mol L^{-1} LV.HCl in the BR universal buffer at pH 7.0; t_{acc} = 20 s; (B) effect of preconcentration time (t_{acc}) on peak current of: (a) 7.0×10^{-9}; (b) 1.0×10^{-9}; (c) 8.0×10^{-10} and (d) 1.0×10^{-10} mol L^{-1} LV.HCl (f = 80 Hz, ΔE_s = 10 mV and a = 25 mV) in the universal buffer at pH 7.0 at E_{acc} = –0.8 V.

States (USP) or European (EP) Pharmacopoeias. Most of the validation schemes described in ICH guidelines[36] and USP[37] were applied in the present work.

Voltammograms of various concentrations of LV.HCl were recorded under the optimized operational conditions of the developed stripping voltammetry method. Linear variation of the peak current i_p (µA) with concentrations C (µmol L^{-1}) of bulk LV.HCl over the concentration range 1.0×10^{-10} to 3.0×10^{-8} mol L^{-1} was obtained. Characteristics of the calibration curves and the achieved limits of detection (LOD) and quantification (LOQ) by means of the developed SW-AdCSV method are reported in Table 1. LOD and LOQ of bulk LV.HCl were estimated using the following expressions:[38]

$$\text{LOD} = 3\ \text{SD} / b \text{ and } \text{LOQ} = 10\ \text{SD} / b \qquad (9)$$

where SD is the standard deviation of the intercept of the calibration curve (or the blank) and b is the slope of the calibration curve.

Table 1. Characteristics of the calibration curves of SW-AdCS voltammetric determination of LV.HCl in bulk form in a BR universal buffer at pH 7.0; $t_{acc.}$ = 20 s, $E_{acc.}$ = –0.8 V, at 25 °C (n = 6)

Linearity range/ (mol L^{-1})	1.0×10^{-10}-3.0×10^{-8}
Slope / (µA µmol^{-1} L)	179.96
SD	1.15×10^{-3}
SE	4.69×10^{-4}
Intercept / µA	0.078
SD	1.80×10^{-3}
SE	7.35×10^{-4}
Correlation coefficient (r)	0.999 ± 0.002
Mean LOQ / (mol L^{-1})	1.0×10^{-10}
Mean LOD / (mol L^{-1})	3.0×10^{-11}

SE: standard error.

LOD and LOQ of 3.0×10^{-11} and 1.0×10^{-10} mol L^{-1} of bulk LV.HCl, respectively, were achieved by means of the described SW-AdCSV method. The results indicated the reliability of the developed stripping voltammetric method for quantification of bulk LV.HCl. LOD value obtained for LV.HCl using the herein proposed SW-AdCSV method is much lower than those obtained by other reported methods.[9-14]

Repeatability (intra-day assay), reproducibility (inter-day assay), precision and accuracy[36,37] of the described SW-AdCSV method (Table 2) were evaluated by performing five replicate measurements for various concentrations of bulk LV.HCl over 1 day (intra-day assay) and for 3 days (inter-day assay). Insignificant differences were observed between the amounts of LV.HCl taken and found. The mean percentage recoveries (%R = [(found) / (taken)] × 100) were estimated as percent of the nominal concentrations in the standard solutions, and precision was assessed from the relative standard deviations (RSD) in percent of the mean recoveries. Whereas bias was estimated as the difference between the mean value determined for the analyte of interest and the accepted true value or known level actually present (Bias% = {[(found) – (taken)] / (taken)} × 100). Satisfactory mean recoveries, relative standard deviations and bias percent were achieved indicating the repeatability, reproducibility, precision and accuracy of the described method.

The robustness[36,37] of the developed stripping voltammetric methods was examined by studying the effect of variation of some of the neck operational conditions such as pH (7.0 to 7.5), preconcentration potential (–0.8 to –1.0 V) and preconcentration time (15 to 25 s) on mean

Table 2. Results of intra-day and inter-day assays of various concentrations of bulk LV.HCl by the described SW-AdCSV method at HMDE (n = 5)

Day	[Taken] / (mol L^{-1})	Mean [found] / (mol L^{-1})	Recovery / %	Bias / %	Precision RSD / %
Intra-day					
1	4.0×10^{-10}	40.02×10^{-11}	100.05	0.05	0.87
	1.0×10^{-9}	10.01×10^{-10}	100.10	0.10	0.62
	1.0×10^{-8}	9.91×10^{-9}	99.10	–0.90	1.03
Inter-day					
1	4.0×10^{-10}	39.53×10^{-11}	98.83	–1.18	1.97
	1.0×10^{-9}	10.08×10^{-10}	100.80	0.80	1.57
	1.0×10^{-8}	10.06×10^{-9}	100.60	0.60	0.64
2	4.0×10^{-10}	39.81×10^{-11}	99.53	–0.48	1.11
	1.0×10^{-9}	10.02×10^{-10}	100.20	0.20	1.33
	1.0×10^{-8}	9.86×10^{-9}	98.60	–1.40	0.56
3	4.0×10^{-10}	39.77×10^{-11}	99.43	–0.57	1.40
	1.0×10^{-9}	10.29×10^{-10}	102.90	0.90	0.62
	1.0×10^{-8}	10.09×10^{-9}	100.90	0.90	1.65

percentage recovery (%R) and relative standard deviation (RSD) of different concentrations of bulk LV.HCl. The obtained mean %R and RSD based on five replicate measurements of 4.0×10^{-10} to 7.0×10^{-9} mol L^{-1} of bulk LV.HCl under the varied conditions were 98.96 ± 1.14 to 99.55 ± 1.14. Since the mean percentage recoveries and relative standard deviations obtained within the studied range of variation of the operational conditions were insignificantly affected, the developed adsorptive stripping voltammetric method is reliable for quantitation of LV.HCl and could be considered robust.

The inter-laboratory precision[36,37] was also examined for analysis of different concentrations of 4.0×10^{-10} to 7.0×10^{-9} mol L^{-1} LV.HCl by means of the described SW-AdCSV method, using two potentiostats (PAR) models 273A (lab 1) and 263A (lab 2) at different elapsed time by two different analysts. The obtained mean recoveries (97.88 ± 1.57 to 98.67 ± 1.66) were found reproducible.

The selectivity[36,37] of the described stripping voltammetric method was identified through possible interferences from excipients usually present in the formulations. Betagan® eye drops contain the active substance (LV.HCl 0.5 or 1.0% m/v) and other inactive ingredients. So, the effect of the preservative (benzalkonium chloride) and inactive additives (polyvinyl alcohol, disodium edentate, sodium metabisulfite, sodium phosphate dibasic heptahydrate, potassium phosphate, monobasic; sodium chloride, hydrochloric acid or sodium hydroxide to adjust pH) associated with LV.HCl in its formulation were tested using the developed method. This was carried out by recording voltammograms of various concentrations of standard solutions of bulk LV.HCl (excipients are absent) and of the tested commercial formulation (Betagan® eye drops; 0.5% m/v LV.HCl) containing such excipients. The voltammograms of all the tested solutions were similar and showed no any voltammetric peaks due to any of the frequently encountered excipients over the applied potential range (–0.8 to –1.9 V *vs.* Ag/AgCl/3 mol L^{-1} KCl). Furthermore, insignificant differences in the percentage recoveries and relative standard deviations (%R ± RSD) were achieved in the absence (98.45 ± 1.15 to 99.97 ± 1.08) and in the presence of such excipients (97.87 ± 1.12 to 98.58 ± 1.32). This indicates that the proposed method does not suffer any interference from such commonly associated inactive additives and preservative agents in the level found in the preparation of eye drops under consideration and consequently, it is highly selective towards LV.HCl under the optimized experimental conditions.

Analytical application

Assay of LV.HCl in commercial formulations

The described SW-AdCSV method was used in the determination of various concentrations of LV.HCl in Betagan® 0.5% m/v eye drops without the necessity for samples pretreatment and/or time-consuming extraction steps prior to the analysis by applying the calibration curve method. Non-significant difference between the slopes of the calibration curves for the bulk and Betagan® 0.5% m/v eye drop solutions was observed. The validity of the described method was further assessed by applying standard addition method[39] for three different standard LV.HCl solutions added to a pre-analyzed one of the investigated commercial formulation in the concentration

Table 3. Assay of standard solutions of 1.0×10^{-8} mol L^{-1} LV.HCl in its formulation (0.5% m/v betagan® eye drops) by means of the described SW-AdCSV method in comparison with official method[8]

Method	Described SW-AdCSV method		Official method[8]	
	(A)	(B)	(A)	(B)
[Found] / ($\times 10^{10}$ mol L^{-1})	99.92	99.89		
	99.78	99.98		
	100.88	102.00		
	99.94	99.28		
Mean [found] / ($\times 10^{10}$ mol L^{-1}) ± SD	100.13 ± 0.006	100.30 ± 0.012		
Mean %R ± RSD	100.13 ± 0.60	100.30 ± 1.20	99.20 ± 0.80	100.60 ± 0.70
F-value	1.36	2.94		
t-test	1.14	0.48		

A: Calibration curve method; B: standard addition method. The theoretical values of *F* and *t*-tests at 95% confidence limit (for $n_1 = 5$ and $n_2 = 5$) are 5.05 and 2.57, respectively.

range that fell within the linear concentration of LV.HCl. Insignificant differences were obtained between the concentrations taken and found. Besides, good recoveries were achieved ranging from 99.43 to 100.13% and from 99.27 to 100.30% using the calibration curve and standard addition methods, respectively, (Table 3). This indicates again that the matrix effect for the investigated commercial drops does not present any significant interference in determination of LV.HCl.

Moreover, the obtained results were statistically compared with those obtained by the official method.[8] Since the calculated *F*-value (variance ratio *F*-test) did not exceed the theoretical one at the 95% confidence level for 5 degrees of freedom (Table 3), there was insignificant difference between the optimized proposed SW-AdCSV method and the reference one[8] with respect to reproducibility.[40] Also, insignificant difference was noticed between the two methods regarding accuracy and precision as revealed by *t*-test value (Student's *t*-test)[40] (Table 3). The results demonstrated that the optimized SW-AdCSV method was quite reliable and sensitive enough for determination of LV.HCl in commercial formulation of various drug concentrations.

Assay of LV.HCl in spiked human serum

In contrast to the previous work in biological fluids,[12-14] herein a direct and more sensitive quantitative assay of LV.HCl spiked in human serum was carried out successfully by the described SW-AdCSV method without any necessity for sample pretreatment or time-consuming extraction steps prior to the analysis other than the centrifugal separation of precipitated proteins from the serum samples by methanol, prior to analysis of the drug. SW-AdCS voltammograms of various concentrations of LV.HCl spiked in 6 human serum samples of three healthy volunteers were recorded (e.g., Figure 5A) under the optimized operational conditions (E_{acc}= −0.8 V and t_{acc} = 20 s). No interfering peaks from endogenous human serum constituents were observed in the blank human serum within the studied potential range (e.g., Figure 5A, curve a). Linear variations of the peak currents (i_p) with concentrations (C) of LV.HCl in each of the spiked human serum samples were obtained (e.g., Figure 5B). Characteristics of some of the obtained calibration plots for the investigated samples are reported in Table 4. Satisfactory mean recoveries (%R = 97.89 to 100.53) and relative standard deviations (RSD = 0.88 to 2.02%) of various concentrations of LV.HCl in spiked human serum samples were obtained (Table 4) indicating insignificant differences between the spiked and the detected amounts of LV.HCl in human serum samples and consequently no interference from the competitively adsorbed surface active substances that may present in serum. The attained detection limit using SW-AdCSV method is about two orders of magnitude lower than that obtained using the other reported methods[12-14] for determination of LV.HCl in human biological fluids.

Moreover, interference encountered from various foreign species (such as some common metal ions, anions, some topical use or co-administrated beta-blockers drugs (talinolol, atenolol and propranolol), or some other typical co-administered drugs (such as vitamins C and E, paracetamol, aspirin, ibuprofen) and dihydrolevobunolol as a metabolite of levobunolol) that are likely to be present in complex matrices, such as biological fluids and which may affect the specificity of the proposed method, was also evaluated. The effect of various excess amounts of these foreign species on analysis of 5.0×10^{-9} mol L^{-1} LV.HCl spiked human serum was identified by means of the described SW-AdCSV method, (e.g., Table 5).

Table 4. Characteristics of calibration curves and assay of various concentrations of LV.HCl by the described SW-AdCSV method in spiked human serum samples of three volunteers (6 samples of each volunteer were analyzed)

Volunteer	1	2	3
Linearity range/ (mol L^{-1})	2.5×10^{-10}-1.0×10^{-8}	3.0×10^{-10}-1.0×10^{-8}	2.0×10^{-10}-1.5×10^{-8}
Slope / (μA μmol^{-1} L)	165.87	164.48	161.85
SD	0.02	0.08	0.01
SE	8.16×10^{-3}	3.27×10^{-2}	4.08×10^{-3}
Intercept / μA	0.12	0.13	0.10
SD	4.15×10^{-3}	4.98×10^{-3}	3.27×10^{-3}
SE	1.69×10^{-3}	2.03×10^{-3}	1.33×10^{-3}
Correlation coefficient (r)	0.998 ± 0.002	0.998 ± 0.001	0.999 ± 0.002
Mean LOQ / (mol L^{-1})	2.5×10^{-10}	3.0×10^{-10}	2.0×10^{-10}
Mean LOD / (mol L^{-1})	7.5×10^{-11}	9.0×10^{-11}	6.0×10^{-11}
%R[a]	100.42	99.23	100.23
RSD[a]	1.35	1.26	1.22
%R[b]	99.44	98.78	97.89
RSD[b]	1.02	1.64	0.88
%R[c]	100.13	99.67	98.74
RSD[c]	1.23	1.08	1.08

[a,b,c]For assay of 4.0×10^{-10}, 2.0×10^{-9} and 7.0×10^{-9} mol L^{-1} LV.HCl, respectively (n = 5). SE: standard error.

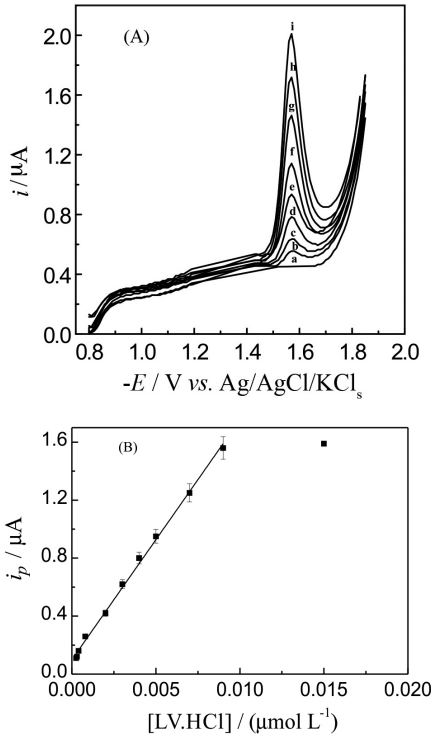


Figure 5. (A) Representative SW-AdCS voltammograms for various concentrations of LV.HCl spiked in human serum in the universal buffer at pH 7.0: (a) background; (b) 2.5×10^{-10}; (c) 4.0×10^{-10}; (d) 8.0×10^{-10}; (e) 2.0×10^{-9}; (f) 3.0×10^{-9}; (g) 5.0×10^{-9}; (h) 7.0×10^{-9} and (i) 9.0×10^{-9} mol L^{-1}. (B) Its calibration plot; $E_{acc} = -0.8$ V, $t_{acc} = 20$ s, $f = 80$ Hz, $\Delta E_s = 10$ mV and $a = 25$ mV.

The tolerance limit for foreign species was taken as the largest amount yielding a signal error of 5% for determination of LV.HCl. Results of the tolerance levels of each of the investigated species reported in Table 5 indicated that none of these substances was found to interfere with analysis of LV.HCl. This may be due to the fact that some of these foreign species are electro-inactive or they did not generate any voltammetric signal within the applied range of potential under the operational experimental conditions (talinolol, atenolol, propranolol, dihydrolevobunolol and the mentioned co-administered drugs are not electroreducible at the mercury electrode).[41,42] Therefore, the described voltammetric method can be successfully applied to assay of LV.HCl in biological fluids without interferences from foreign organic and inorganic species.

Table 5. Interferences from foreign species on analysis of 5.0×10^{-9} mol L^{-1} LV.HCl by the described SW-AdCS voltammetric method

Foreign species	Tolerance level[a] / (mol L^{-1})
Ca^{2+}, Mg^{2+}, Al^{3+}, Cd^{2+} and Fe^{3+}	1.0×10^{-3}
Zn^{2+} and Cu^{2+}	5.0×10^{-4}
Na^{+} and K^{+}	5.0×10^{-3}
Talinolol, atenolol, propranolol and dihydrolevobunolol	2.5×10^{-4}
Vitamins (C and E), paracetamol, aspirin and ibuprofen	5.0×10^{-4}

[a]For 5% signal error.

Conclusions

SW-AdCSV method has been described for trace quantitation of LV.HCl in pharmaceutical formulation

in the form of eye drops and human serum. The method was simple, rapid and does not require expensive and sophisticated apparatus or expensive solvents in comparison with other reported methods[8-14] for the study of levobunolol. According to the mean plasma levels of levobunolol in normal volunteers after a single topical instillation of 0.5% or 1.0% m/v levobunolol in both eyes,[7] the reported methods[9-14] are considered not efficient enough for the assay of LV.HCl in human plasma and at different therapeutic dose levels for pharmacokinetic studies as well as therapeutic drug monitoring. However, all the results achieved in this work indicated that the described SW-AdCSV method is reliable and sensitive enough (LOQ = 2.5×10^{-10} mol L^{-1}) for assay of LV.HCl in human plasma of real samples and for pharmacokinetic studies and can be recommended for quantification of LV.HCl in quality control and clinical laboratories.

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