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Simultaneous high-throughput determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with potentiometric detection

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Abstract

Potentiometric detection of clenbuterol, ambroxol and bromhexine in marketed pharmaceuticals was described in six isocratic HPLC systems. The podant- and macrocyclic-type neutral ionophores, *N*,*N*,*N*′,*N*′-tetracyclohexyl-oxybis(*o*-phenyleneoxy)diacetamide (TOPA) and hexakis(2,3,6-tri-*O*-octyl)-α-cyclodextrin (OCD), were applied in poly(vinyl)chloride (PVC)-based liquid membrane electrodes. Both types of neutral ionophores improve the sensitivity for all mentioned drugs when compared with a tetrakis(*p*-chlorophenyl)borate (BOR)-based electrode as well as with single wavelength UV detection. Detection limits ($S/N = 3$) of 2.6×10^{-10} mol l^{-1} (injected concentration) for the highly hydrophobic bromhexine were achieved with the TOPA-based electrode and a cyano reversed-phase (RP)-HPLC with Uptisphere® UP5CN-25QS silica column (250 × 4.6 mm i.d.) eluted with acetonitrile (AcN)–ethanol–perchloric acid (1.66 mM) (60:2:38, v/v/v) (pH* 2.45). Comparable result was obtained with OCD-based electrodes and an XTerra™ RP18 hybrid silica–polymer column eluted with AcN–phosphoric acid (20 mM) (25:75, v/v) (pH* 2.60). In the mobile phases containing 60–75% v/v AcN or methanol, stable and reproducible response of both types of neutral ionophore-based electrodes was observed for at least 3 days. The results of the validated procedure for reliable simultaneous determination of the drugs in fortified representative samples of pharmaceuticals were also presented.

Keywords: Potentiometric detection; HPLC; Ambroxol; Clenbuterol; Mucolytic drugs

1. Introduction

Mucolytic drugs as ambroxol—a derivative of the alkaloid visicyn—and its metabolite bromhexine, alone or incorporated in combined pharmaceutical preparations, reduce exacerbations, illness days, and antibiotic use in chronic bronchitis and

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chronic obstructive pulmonary disease [1]. Both mentioned drugs are considered to be beneficial in various mucoregulatory therapies of human and animal airways and are mostly administered using oral formulations, by inhalation, injection or rectally. However, ambroxol indicates also some other clinically most significant properties as the radical scavenger activity [2,3], in vivo inhibition of doxorubicin-induced lipid peroxidation [3], blocking generation of reactive oxygen species in placenta [4] and bronchoalveolar cells [5]. It also prevents *Helicobacter pylori* associated gastric carcinogenesis [2] and has valuable anti-inflammatory activity by inhibiting the release of inflammatory mediators from human leukocytes and mast cells [6]. Recently, Severina et al. [7] reported experimental evidences that the therapeutic action of ambroxol involves inhibition of endogenous nitric oxide-dependent activation of soluble guanylate cyclase. However, the exact, specific pharmacologic impact of ambroxol on the stimulation of surfactant synthesis and secretion in alveolar type II cells is still unclear until now [8].

Combinations of clenbuterol as an efficient beta2-adrenergic bronchodilator with a potent bronchosecretolytic drug such as ambroxol are commercially available in tablets and syrups with trade names Spasmo-Mucosolvan®, Mucosolvan compositum®, Vaksan compositum®, Mucovibrol®, Oxibron NF®, Broncoxan®, Calox-McK®, Allbron®, Ciliotussin compositum®, Pectorol® in Germany, Japan, South Korea and most countries of Latin America [9]. Ambroxol improves the broncho-spasmolytic activity of clenbuterol and combination of these drugs is frequently prescribed in acute and chronic respiratory tract diseases, spasmolitic stenosis and asthma [9,10]. However, in some European countries the distribution, prescription and treatment of clenbuterol, indicating the severe adverse anabolic effects during abuse [11], for any human therapeutics and veterinary administration is presently strictly forbidden by law regulations [12]. This implies that development of highly specific, reliable and reduced-cost methodologies for rapid determination of clenbuterol in diverse matrices is still urgently needed.

Spectrophotometric [13,14], gas chromatographic [15,16], flow-injection [14], TLC [17], HPLC [18–28], capillary electrophoretic [29] and capillary isotachophoretic [30] procedures have been developed for the individual determination of ambroxol hydrochloride or bromhexine hydrochloride in pharmaceutical preparations and in biological samples. Simultaneous determination of bromhexine in pharmaceutical samples containing antibiotics [13], salbutamol [17], antimicrobial preservatives [18] or sulfonamides [29] was presented by using, respectively, an indirect photometric method [13], TLC [17], HPLC [18] and capillary zone electrophoresis [29]. In HPLC procedures, single wavelength UV detection at 246 nm was mostly involved for individual detection of ambroxol or bromhexine with a sensitivity of 2×10^{-7} mol l^{-1} [18–28]. Flores-Murrieta et al. [22] and Lau and Mok [26] described the efficient use of amperometric and indirect conductometric detection of ambroxol and bromhexine, respectively, with improved limits of detection down to 2.5×10^{-9} mol l^{-1} in a reversed-phase (RP)-HPLC system. This enabled reliable pharmacokinetic studies of both mucolytic drugs [22,26].

Electrochemical methods such as amperometry [31], coulometry [32], batch potentiometry [33] and differential-pulse voltammetry [34–36] using porous carbon [31,37], polyaniline/poly(vinyl)chloride (PVC) membrane surface modified glassy carbon [33], Nafion®-modified carbon paste [34,35] and molecularly imprinted composite electrodes [36] were used for detection of clenbuterol in pharmaceutical formulations and biological matrices.

Potentiometric detectors based on liquid membrane electrodes offer an attractive alternative in the HPLC and CE determination of organic cations [38–42]. The effect of matrix interference for UV-absorbing compounds which are early eluted from HPLC columns [43–45] can be strongly reduced by application of potentiometric detection [38,39]. Compared with the amperometric detectors, potentiometric detection is not restricted to electroactive compounds. It also does not need ion suppressor systems, as is the case for conductometric detection [46]. Moreover, in contrast to amperometric detectors [47], the response

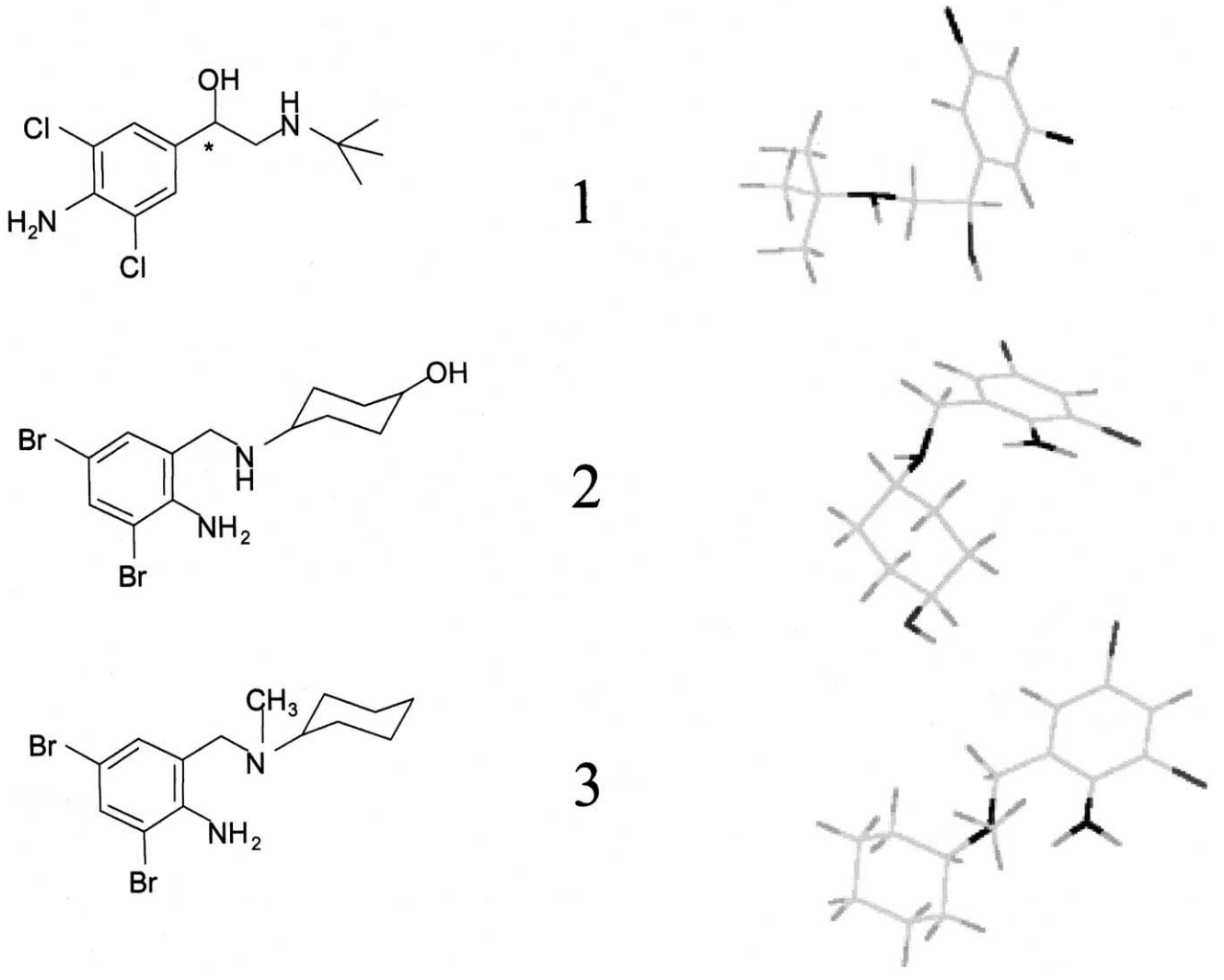

Fig. 1. Molecular structure of (1) clenbuterol, (2) ambroxol and (3) bromhexine, and their optimized conformers calculated by the MM^{+} method.

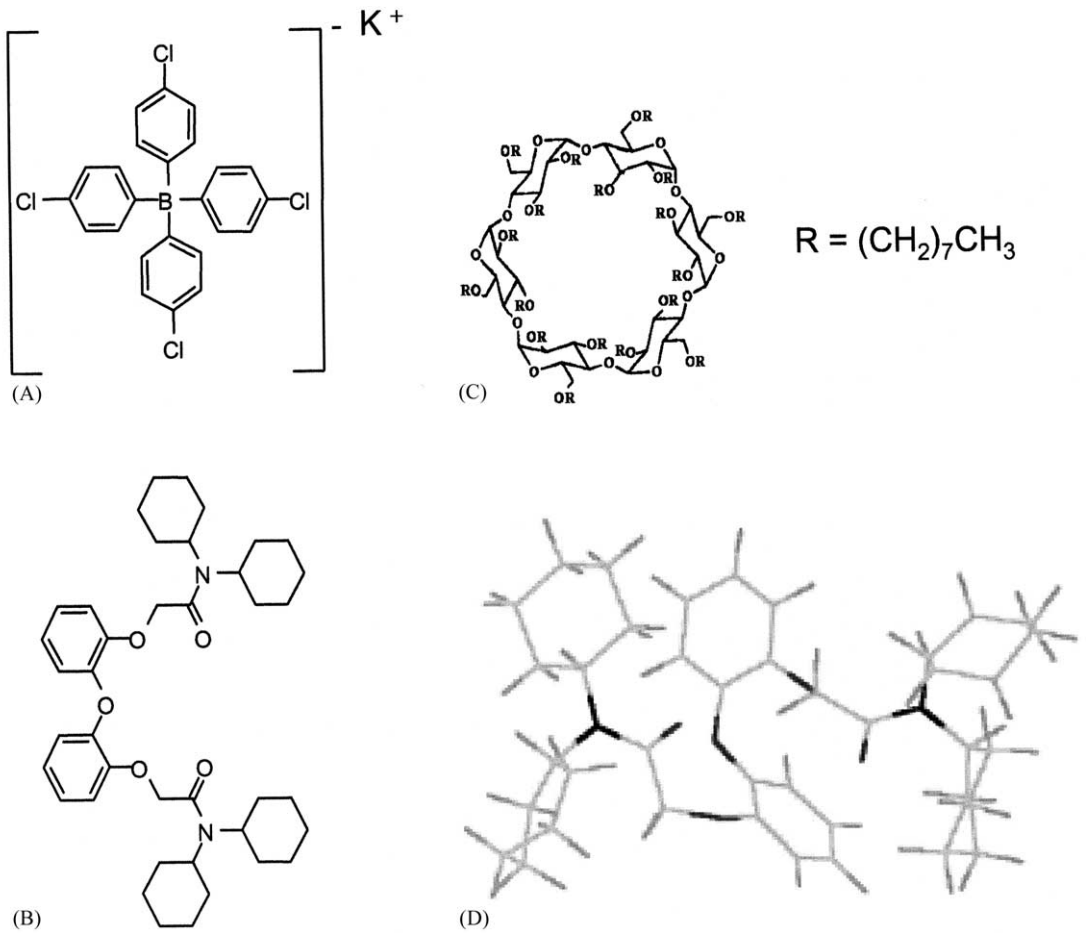

Fig. 2. Molecular structure of cation sensing ionophores: (A) BOR; (B) TOPA; (C) OCD. For the TOPA ionophore the model of the optimized conformer (D) calculated by MM^+ method is also shown.

of potentiometric detector is quasi-independent on a flow rate which creates possibilities for use in miniaturized systems [38] as well as in turbulent flow or ultra-high flow rate HPLC systems which are currently recommended as effectively decreasing sample analysis cycle time [48].

The aim of the present work was to develop the potentiometric detection procedure for reliable HPLC determination of clenbuterol, ambroxol and bromhexine alone, or in different combinations, used in tablet formulation. The evaluation of potentiometric detection performance for these compounds in different HPLC modes employing mobile phases with high content ($>50\%$ v/v) of polar organic modifiers was especially addressed in the present studies. Special interest was taken to search of neutral ionophores applicable in construction of liquid membrane electrodes with improved response for highly lipophilic compounds such as bromhexine with a log P value of 6.40 [16].

2. Experimental

2.1. Reagents

Ambroxol (*trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol) and bromhexine (*N*-cyclohexyl-*N*-methyl-(2-amino-3,5-dibromobenzy-

l)amine), both as the hydrochloride salts, were supplied from Sigma (St. Louis, MO). Racemic clenbuterol (*R*,*S*-1-(4-amino-3,5-dichlorophenyl)-2-*tert*-butylaminoethanol) was purchased from ICN Biomedicals (Aurora, OH). The stock solutions of all mentioned drugs (chemical structure shown in Fig. 1) were prepared at concentrations of 3.1×10^{-3} mol l^{-1} by dissolving in the appropriate mobile phase. Working solutions were prepared by dilution of stock solutions in the respective fresh mobile phases just before use by applying 250 μl Hamilton 725-type microsyringe (Hamilton, Bonaduz, Switzerland) and 2 ml RAM glass vials with TFE/butyl screw caps (Alltech Associates, Lokeren, Belgium).

HPLC-grade acetonitrile (AcN), methanol, 1-heptanesulfonic acid sodium salt (98%), dodecyl sulfate sodium salt (99%) (all from Acros Organics, Geel, Belgium), absolute ethyl alcohol (Carlo Erba Reagents, Rodano, Italy), concentrated reagent grade *ortho*-phosphoric acid (min. 89% w/w, $d = 1.75$ g ml^{-1} from UCB, Leuven, Belgium), trifluoroacetic acid (Fluka, Buchs, Switzerland), glacial acetic acid ($d = 1.0490$ g ml^{-1}) and perchloric acid (70% w/w in water, $d = 1.67$ g ml^{-1} from Merck, Darmstadt, Germany) were used for preparation of the respective HPLC mobile phases. Double-distilled and deionized water with conductivity 1.8 μs was used throughout all experiments.

All membrane components were of the selectophore quality series available from Fluka (Buchs, Switzerland). These membrane components included potassium tetrakis(*p*-chlorophenyl)borate (BOR), the ionophores *N*,*N*,*N*′,*N*′-tetracyclohexyl-oxybis(*o*-phenyleneoxy)diacetamide (TOPA) and hexakis(2,3,6-tri-*O*-octyl)-α-cyclodextrin (OCD), the plasticizer dioctylsebacate (DOS), and tetrahydrofurane. The high relative molecular weight PVC was obtained from Janssen Chimica, Geel, Belgium. The structure of the lipophilic anion BOR and of the neutral ionophores are presented in Fig. 2.

2.2. Electrode maintenance

Three PVC matrix liquid membrane electrodes based on the plasticizer DOS and one of the cation sensing components (compare Fig. 2 for their respective structure) were prepared. The membrane composition (w/w) of the BOR-based electrode was as follows: PVC (26.09%), DOS (57.07%) and BOR (1.68%). The membrane composition of the TOPA-based electrode were PVC (35.10%), DOS (62.85%), TOPA (1.28%) and BOR (0.78%). The membrane composition of OCD-based electrode were PVC (32.45%), DOS (63.95%), OCD (3.08%) and BOR (0.52%). About 300 mg of each membrane cocktail was dissolved in 3 ml of tetrahydrofurane. BOR was used as the component of all electrodes in order to enhance membrane conductivity and reach proper permselectivity by anion exclusion. The molar ratio of BOR/neutral ionophore was 0.80 for TOPA- and 1.00 for OCD-based liquid membrane electrodes, respectively.

For preparation of solid-state electrodes the membrane cocktail was deposited directly on the surface of a platinum or glassy carbon substrate electrode with a diameter 3 mm. Three layers were deposited on the surface of the substrate electrode using a Pasteur pipette, at intervals of 20 min (40 μl of the membrane cocktail each). For each deposited layer, the tetrahydrofurane was allowed to evaporate under atmospheric conditions for 20 min and finally for at least 2 h. The prepared liquid membrane electrodes were stored overnight in distilled water. Before coating, the substrate electrodes (3 mm i.d.) containing platinum or glassy carbon mounted in plastic bodies, were carefully polished with a 5 μm grid polishing sheet (3 M, St. Paul, MN) and cleaned with distilled water and acetone (Carlo Erba Reagents).

Between experiments, the prepared liquid membrane electrodes were stored in 10 ml of freshly distilled water at laboratory temperature. Each electrode was equilibrated in the running mobile phase for 30 min before application in the HPLC flow cell. Contrary to the TOPA electrode, OCD electrodes needed an additional 3 days of conditioning in distilled water before starting the HPLC measurements [52]. The potential of BOR-based electrode was stabilized (as indicated potential drift of less than 6 mV h^{-1}) approximately 20 min after placing in the wall-jet detector and eluting with mobile phase at flow rate of 1 ml

min^{-1}. It was observed that after all-night storing of TOPA or conditioned OCD liquid membrane electrodes their potentials were stabilized (drift below 5 mV h^{-1}) in the wall-jet detector after 10 and 15 min, respectively.

2.3. HPLC measurements

HPLC analyses were made with a P200 isocratic pump and an AS100 autosampler (tsp—Thermo Separation Products, Riviera Beach, FL) fitted with a Rheodyne 7010 injection loop (20 μl). Before injection each sample was purged through a 0.45 μm syringe cellulose acetate filter (Alltech Associates, Deerfield, IL). The UV measurements were made with a multi-wavelength detector type SP8450 (Spectra Physics, Fremont, CA) operated at 246 nm. The DataJet type integrator (Spectra Physics) and data acquisition software PC1000 (tsp—Thermo Separation Products) were applied for monitoring potentiometric and/or UV detector signals. The following six HPLC systems were maintained:

1) Cation-exchange HPLC using the universal cation-exchange HPLC column (100 × 4.6 mm i.d.) (Alltech Associates) packed with a 7 μm silica-based support coated with polybutadiene–maleic acid copolymer. A guard pre-column (7.5 × 4.6 mm i.d.) module was placed before the analytical column. All HPLC separations were performed using daily prepared mobile phase consisting of AcN–phosphoric acid (40 mM) (85:15, v/v) (pH* 2.35 ± 0.02).
2) Ion-interaction HPLC using Lichrospher 100-5 RP 18ec (125 × 4.0 mm i.d.) (Macherey Nagel, Düren, Germany) packed with 5 μm octadecylsilica and suitable pre-column (14 × 4.6 mm^2 i.d.). The mobile phase was methanol–trifluoroacetic acid (2.74 mM) (70:30, v/v) (pH* 2.95 ± 0.02) with addition of 1-heptanesulfonic acid sodium salt (5.57 mM).
3) RP-HPLC with the Lichrospher 100-5 RP 18ec (125 × 4.0 mm i.d.) (Macherey Nagel) packed with 5 μm octadecylsilica and suitable pre-column (14 × 4.6 mm i.d.) The methanol–trifluoroacetic acid (2.8 mM) (70:30, v/v) (pH* 2.55 ± 0.02) was applied as mobile phase.
4) Cyano RP-HPLC applying the Uptisphere UP5CN-25QS (250 × 4.6 mm i.d.) (Interchim, Montluçon, France) with 5 μm cyanosilica with removable pre-column (17 × 4.6 mm^2 i.d.). The mobile phase consisted of AcN–ethanol–perchloric acid (1.66 mM) (60:2:38, v/v/v) (pH* 2.45 ± 0.02).
5) Micellar HPLC using the Lichrospher 100-5 RP 18ec (125 × 4.0 mm i.d.) (Macherey Nagel) packed with 5 μm octadecylsilica and suitable pre-column (14 × 4.6 mm i.d.). The mobile phase was, methanol–acetic acid (1.75 mM) (25:75, v/v) (pH* 4.05 ± 0.02) with addition of dodecylsulfate sodium salt (5 mM).
6) Hybrid RP-HPLC applying the XTerra™ RP18 column (50 × 3.0 mm i.d.) (Waters, Milford, MA) containing 3-(chlorodimethylsilyl)propyl-*N*-dodecylcarbamate bonded amorphous organosilica with spherical particles $d_p = 3.5$ μm without pre-column. The mobile phase consisted of AcN–phosphoric acid (20 mM) (75:25, v/v) (pH* 2.60 ± 0.02).

The apparent pH* of the mobile phases were controlled using a portable HI8314 pH-meter (Hanna Instruments, Germany) without any corrections. The mobile phases were filtered through a 0.2 μm a nylon membrane filter (Alltech Associates) and degassed by continuos bubbling of helium through the solution. In all HPLC systems (1)–(6) a flow rate of 1 ml min^{-1} was applied. The hold-up time of the columns was determined by injection of sodium nitrate solution (0.1 mol l^{-1}) as non-adsorbed solute. Retention times for the identification of analytes were established as the mean of four parallel measurements. All HPLC experiments were maintained at the ambient temperature of 20 ± 0.5 °C.

2.4. Potentiometric detection

The coated-wire solid-state platinum or glassy carbon electrodes with 3 or 1.5 mm i.d. were placed in a large-volume wall-jet type flow cell [41]. The distance from the LC tubing-outlet (PEEK, 100 μm i.d., Alltech Associates) to the

Table 1
Molecular parameters of analytes[a]

Compound	pK_a	log P	log D	HBA	HBD	μ (D)	P_o (Å^3)	H_a (kcal mol^{-1})
Clenbuterol	9.72	2.91	0.58	5	3	3.02	27.2	−2.89
Ambroxol	9.20	3.24	1.43	3	3	3.01	31.1	−5.54
Bromhexine	8.50	4.03 (6.40[b])	5.26	2	1	3.48	32.3	−3.25

[a] See Section 2 for calculation details.
[b] Experimental value from Ref. [15]. Symbols: pK_a—first acidic ionization constant; log P—n-octanol–water partition coefficient; log D—n-octanol-buffer distribution coefficient; HBA—number of hydrogen bond acceptors in analyte molecule; HBD—number of hydrogen bond donors in analyte molecule; μ—dipole moment; P_o—polarizability; H_e—hydration energy.

membrane was maintained at 100 μm. A Schotte (Hofheim, Germany) B3510 potassium chloride saturated calomel electrode was applied as a reference electrode. The potential over the polymeric membrane electrode was determined using a high impedance amplifier with internal resistance 10^{13} Ω (type 87F, Knick, Germany). The electrode signals were amplified 10 times with a laboratory-made amplifier and recorded by the data acquisition system.

2.5. Isolation of ambroxol from commercial tablets

The ambroxol tablet formulations (Surbronc®, Boehringer Ingelheim Pharma KG, Ingelheim, Germany, and Mucobron®, Polfa, Grodzisk Mazowiecki, Poland) were purchased from pharmacies in Belgium and Poland, respectively. Ten commercial tablets of each formulation were weighted and the mean weight of tablets was calculated. A single weighted tablet was finely powdered and a weight equivalent of 3.0 mg of ambroxol hydrochloride was transferred into a 10 ml volumetric flask containing 5 ml of mobile phase AcN–ethanol–perchloric acid (1.66 mM) (60:2:38, v/v/v) (pH* 2.45). The solution was ultrasonicated for about 3 min and then diluted to 10 ml with the same mobile phase. From this solution 1 ml was taken with volumetric sterile syringe (Terumo Europe NV, Leuven, Belgium) and filtered through 1.5 μm Millex AP (Millipore, Bedford, MA) borosilicate glass filter. The eluate was collected and subsequently filtered with use of 0.5 μm Millex LCR (Millipore) hydrofilized teflon filter. The eluate was collected into 2 ml RAM glass vials with TFE/butyl screw caps (Alltech Associates) and placed in the autosampler tray. From the eluate, 20 μl was injected into the cyano RP-HPLC system No. (4) employing the cyanosilica column.

An analogous procedure was used for isolation of clenbuterol and bromhexine. The standard addition method was used for the determination of the accuracy of potentiometric detection of these drugs in tablets.

2.6. Molecular modeling and statistical calculations

Molecular modeling of analytes and calculations of their molecular descriptors as dipole moment (μ), polarizability (P) and hydration energy (H_e) summarized in Table 1 were made with HyperChem v.6.0 (Hypercube, Inc., Guinesville, FL) software installed on an IBM-compatible PC. These calculations were performed on the in vacuo optimized geometry of the molecular structures of the solutes in neutral form after employing of MM^+ molecular mechanics mode with bond dipoles option. The Polak–Ribiere conjugate gradient procedure was used for optimization. The terminating gradient of 0.001 kcal mol Å^{-1} was employed in all cases.

The number of hydrogen bond acceptors (HBA) and the number of hydrogen bond donors (HBD) were calculated according to the method of Lipinski et al. [49] The values of n-octanol–water partition coefficient log P characterizing the lipophilicity of the analytes were calculated for their neutral forms by using neural network computing software developed by Parham et al. [50]. The values of the logarithm of the first global (macroscopic) acidic ionization constants pK_a of analytes

Table 2
Retention parameters of analytes in applied HPLC systems (injected concentration, 2.5×10^{-5} mol l^{-1}; injected volume, 20 μl)

No	HPLC system[a]	Clenbuterol	Ambroxol	Bromhexine
1	*Cation-exchange CEX*			
	t_R (min)	14.7	48.61	63.45
	log k	1.09	1.635	1.75
	α	–	3.49[b]	1.31[c]
	R_s	–	1.91[b]	2.41[c]
2	*Ion-interaction HAS*			
	t_R (min)	1.78	3.18	5.16
	log k	−0.127	0.324	0.608
	α	–	2.83[b]	1.92[c]
	R_s	–	1.00[b]	1.47[c]
3	*Reversed-phase RP18*			
	t_R (min)	1.63	2.46	4.55
	log k	−0.223	0.149	0.539
	α	–	2.36[b]	2.45[c]
	R_s	–	4.52[b]	1.15[c]
4	*Cyano RP*			
	t_R (min)	3.17	3.32	4.11
	log k	−0.336	−0.275	−0.048
	α	–	1.15[b]	1.68[c]
	R_s	–	1.16[b]	4.65[c]
	Micellar SDS			
	T_R (min)	4.27	9.64	15.83
	log k	0.486	0.912	1.148
	α	–	2.67[b]	1.72[c]
	R_s	–	1.06[b]	1.64[c]
6	*Hybrid RP*			
	t_R (min)	1.03	1.68	1.91
	log k	0.541	0.799	0.863
	α	–	1.81[b]	1.16[c]
	R_s	–	2.47[b]	1.08[c]

[a] See Section 2 for details.
[b] Calculated for peaks pair of ambroxol/clenbuterol.
[c] For peaks pair of bromhexine/ambroxol. Symbols: t_R—non-corrected retention time; log k—logarithm of capacity factor; α—separation factor; R_s—resolution.

were calculated with Pallas v.1.1 software (CompuDrug Chemistry, Budapest, Hungary). Using these values the pH-dependent log D values of the n-octanol-buffer distribution coefficient of analytes were calculated according to equation [51]:

$$\log D = \log P - \log(1 + 10^{pK_a - pH}) \quad (1)$$

Statistical calculations for interpretation of collected experimental data were performed with Statistica v.4.3 (Stat-Soft, Inc., Tulsa, OK) software.

3. Results and discussion

3.1. Retention in HPLC systems

For aliphatic amines, aminoalcohols and basic beta-adrenergic drugs separated in cation-exchange HPLC systems, the sensitivity of potentiometric detection was successfully indicated in previous studies [39,52]. The presently investigated compounds have a higher lipophilicity as expressed by the calculated n-octanol–water partition coefficient log P (see Table 1), and required another chromatographic separation approach. Application of previously reported RP-HPLC systems for the chromatographic determination of ambroxol and bromhexine [18–28,53] were excluded from our consideration as they used triethylamine, nonylamine [18,23–25] or ammonium acetate and diammonium phosphate, [23,25,53] as "silanol blockers". The use of such mobile phase components would lead to significantly decreased sensitivity and selectivity of potentiometric detection of the present analytes [38,39].

In Table 2 the retention characteristics of clenbuterol, ambroxol and bromhexine in six presently developed HPLC systems were summarized. The apparent pH* of the mobile phases in these HPLC systems was in the range 2.35–4.05 (cation-exchange HPLC–micellar HPLC). In all HPLC systems used, the observed retention sequence of the analytes was identical and in accordance with their increased lipophilicity, polarizability and dipole moment (see Table 1). Each of the proposed HPLC systems enables complete separation of the mixture of the analytes studied. However, the cation-exchange HPLC systems yield too high retention times for ambroxol and bromhexine (exceeding 40 min). Moreover, in cation-exchange-, ion-interaction- and micellar-HPLC systems, peak tailing of the highly lipophilic bromhexine was observed (see Fig. 3) despite increased concentrations of organic modifier. Short retention, satisfactory separation and sharp,

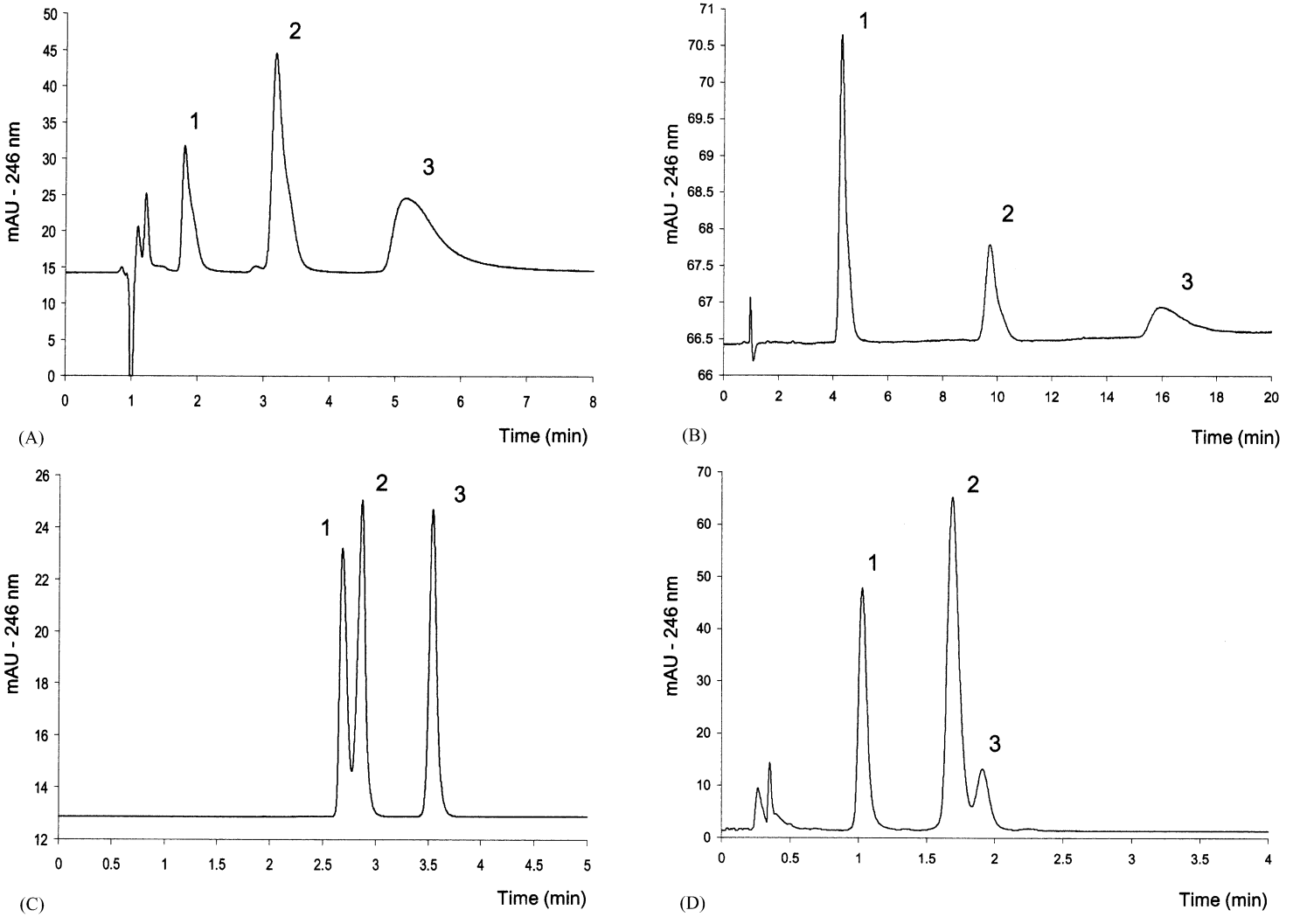

Fig. 3. Isocratic separation of (1) clenbuterol, (2) ambroxol and (3) bromhexine in (A) ion-interaction-, (B) micellar-, (C) cyano RP- and (D) hybrid RP-HPLC systems with UV detection at 246 nm. Injected volume 20 μl, injected concentration of analytes 2.0×10^{-5} mol l^{-1}. Compare Section 2 for details of measurements.

Table 3
Detection limits at $S/N = 3$ (injected concentration, mol l^{-1}) for potentiometric detection of mucolytic drugs in diversified HPLC systems

No	HPLC system[a]	Solute	BOR[a] (mol l^{-1})	TOPA[a] (mol l^{-1})	OCD[a] (mol l^{-1})	UV, $\lambda = 246$ nm (mol l^{-1})
1	Cation-exchange CEX	Clenbuterol	6.7×10^{-7}	3.9×10^{-7}	1.1×10^{-7}	2.0×10^{-7}
		Ambroxol	3.7×10^{-7}	2.2×10^{-7}	8.6×10^{-8}	1.4×10^{-7}
		Bromhexine	1.3×10^{-7}	7.7×10^{-8}	2.5×10^{-8}	1.2×10^{-7}
2	Ion-interaction HSA	Clenbuterol	2.2×10^{-6}	1.8×10^{-6}	9.5×10^{-7}	2.1×10^{-7}
		Ambroxol	1.3×10^{-6}	8.9×10^{-7}	7.5×10^{-7}	1.5×10^{-7}
		Bromhexine	9.1×10^{-7}	6.3×10^{-7}	4.5×10^{-7}	1.3×10^{-7}
3	Reversed-phase RP18	Clenbuterol	4.3×10^{-8}	3.8×10^{-8}	3.2×10^{-8}	1.9×10^{-7}
		Ambroxol	7.7×10^{-9}	5.1×10^{-9}	6.5×10^{-9}	1.3×10^{-7}
		Bromhexine	5.2×10^{-10}	3.7×10^{-10}	4.5×10^{-10}	1.1×10^{-7}
4	Cyano RP	Clenbuterol	1.8×10^{-7}	1.6×10^{-8}	4.3×10^{-9}	2.1×10^{-7}
		Ambroxol	7.2×10^{-8}	3.7×10^{-9}	4.1×10^{-9}	1.4×10^{-7}
		Bromhexine	4.5×10^{-8}	2.6×10^{-10}	3.5×10^{-9}	1.2×10^{-7}
5	Micellar SDS	Clenbuterol	1.1×10^{-6}	8.7×10^{-7}	6.5×10^{-7}	2.2×10^{-7}
		Ambroxol	8.5×10^{-7}	5.3×10^{-7}	2.1×10^{-7}	1.5×10^{-7}
		Bromhexine	6.1×10^{-7}	4.1×10^{-7}	1.2×10^{-7}	1.4×10^{-7}
6	Hybrid RP	Clenbuterol	1.3×10^{-7}	1.1×10^{-8}	2.9×10^{-9}	1.2×10^{-7}
		Ambroxol	4.3×10^{-8}	2.1×10^{-9}	2.6×10^{-9}	6.7×10^{-8}
		Bromhexine	2.4×10^{-8}	1.4×10^{-10}	1.7×10^{-9}	5.3×10^{-8}

[a] See Section 2 for details.

symmetrical peaks of all analytes were achieved in RP-, cyano RP- and hybrid RP-HPLC systems (see Fig. 3) employing, respectively, trifluoroacetic, perchloric or phosphoric acid as acidic additives and slightly decreased concentrations of organic modifiers (60–70%). As it was reported earlier [54], compared with classical RP-HPLC mode with octadecylsilica columns, the hybrid RP-HPLC XTerra™ RP18 column with polymer–silica packing the concentration of organic modifier can be substantially reduced (to 25%) without loss of resolution and enhanced peak tailing of basic analytes.

3.2. Performance of potentiometric detection

The TOPA (see Fig. 2) originally developed as the selective ionophore for batch potentiometric determination of barium ions [55–57] was successfully applied in the construction of coated-wire liquid membrane microelectrodes for sensitive potentiometric detection of ammonium, alkaline earth and alkali metal ions separated by capillary electrophoresis [58–61]. Recently, Katsu et al. [62,63] described the use of TOPA in PVC-based liquid membrane electrodes for selective potentiometric batch assay of organic ions like hexylammonium [62], appetite suppressant phentermine, amphetamine and phenethylamine in various pharmaceutical and biochemical samples [63].

The highly lipophilic mono- and polyfunctionalized α-, β- and γ-cyclodextrins are used as oriented monolayers in chemosensors [64] and as the cation-sensing (or chiral) ionophores of polymeric membrane electrodes developed for highly selective batch quantitation of alkyl- and arylammonium ions, acetyl choline, local anesthetics, tricyclic antidepressants as well as ephedrine and propranolol enantiomers [65]. More recently we observed [52] that use of octylated α-cyclodextrin as the component of liquid membrane electrode improved its response during potentiometric detection of a series of cationic beta-adrenergic drugs in cation-exchange HPLC conditions. In the present study, we focused on comparison of neutral ionophores with podant (acyclic, as TOPA: see

Fig. 2) and macrocyclic structure (as OCD) as components of PVC-based liquid membrane electrodes used in dynamic HPLC conditions. The properties of these electrodes were compared with the properties of electrodes incorporating exclusively a typical lipophilic cation-exchanger such as potassium BOR.

Table 3 shows the detection limits determined at signal-to-noise ratios of three ($S/N = 3$) for potentiometric detection of clenbuterol, ambroxol and bromhexine by three different liquid membrane electrodes in HPLC. These results indicate that in comparison to UV detection at 246 nm, and independent on the type of electrode used, potentiometric detection leads to improved detection limits for all analytes in the RP-, cyano RP- and hybrid RP-HPLC systems. Such improvement was not observed in the ion-interaction HPLC and micellar HPLC systems, probably because of the presence of sodium ions in the applied mobile phases which contain, respectively, the heptasulfonic- and the dodecylsulfate sodium salt. In the cation-exchange HPLC system, the lowering of the detection limit was observed for all three analytes by using an OCD liquid membrane electrode. Only in case of bromhexine the TOPA liquid membrane electrode gives improved detection in cation-exchange HPLC as compared with UV detection.

Inspection of detection limit values indicates that, compared with the BOR electrode, both ionophore containing electrodes, TOPA and OCD, give improvement of detection limits of all analytes independent of the type of HPLC system used. However, the lowest detection limit at 2.6×10^{-10} mol l^{-1} was observed for a TOPA liquid membrane electrode in case of potentiometric detection of bromhexine eluted in the cyano RP-HPLC system. Fig. 4 shows the comparison of potentiometric detection of standard solutions of all three analytes by each type of liquid membrane electrode in this mentioned HPLC system. Including contribution of reduced column diameter this detection limit was lowered to the value of 1.4×10^{-10} mol l^{-1} in the conditions of hybrid RP-HPLC system. Similarly, the TOPA electrode gives the lowest detection limit for ambroxol at 2.1×10^{-9} mol l^{-1} in the same hybrid RP-HPLC system. Surprisingly, the OCD electrode, contain-

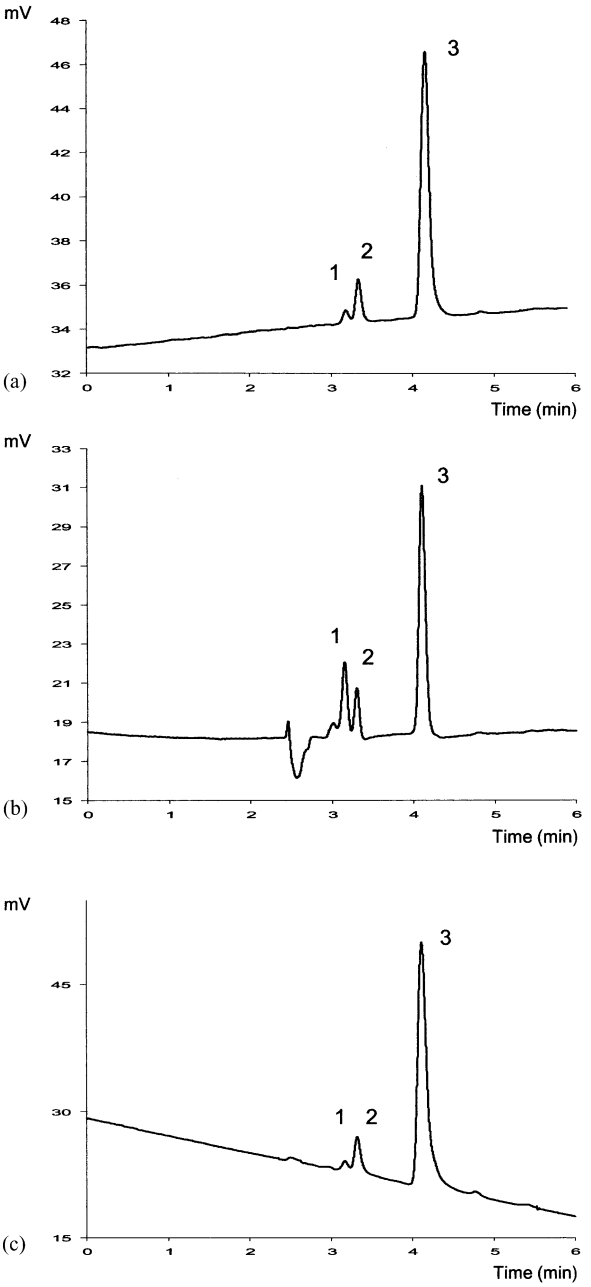

Fig. 4. Isocratic separation of (1) clenbuterol (injected concentration: 7.5×10^{-7} mol l^{-1}), (2) ambroxol (7.5×10^{-7} mol l^{-1}) and (3) bromhexine (2.5×10^{-6} mol l^{-1}) by cyano RP-HPLC system with potentiometric detection using (a) BOR, (b) OCD, (c) TOPA liquid membrane electrodes. Injected volume 20 μl. Compare Section 2 for details of measurements.

ing a macrocyclic ionophore especially sensitive to host–guest interactions with hydrophobic organic cations [52,65], gives slightly worse detection limits for all separated analytes as compare to the TOPA electrode containing an acyclic, helically shaped ionophore. However, it should be stressed that irrespective on the kind of used HPLC system the OCD-modified electrodes indicated the lowest detection limits up to 2.9×10^{-9} mol l^{-1} in the conditions of hybrid RP-HPLC system (see Table 3).

The values of the detection limit for all analytes studied can be related to increased concentrations of organic modifier (AcN or methanol) in the mobile phase applied in HPLC system. Indeed, as shown in Fig. 5, the peak area of clenbuterol detected by an OCD electrode in the range 0.1–100 nmol l^{-1} injected to hybrid-RP HPLC system increased with increasing concentration of AcN in the mobile phase. This effect can be explained by a significant decrease of the dielectric constant ε and decreased excess viscosity η^{E} of water–polar organic modifier mixture [66]. Such changes increase the basicity of the analytes [67], increase the diffusion rates of lipophilic analytes in liquid polymeric membranes [46,68] and enhance the free energy of supramolecular interactions between analytes and neutral ionophores [65,69].

In our previous report [52], we postulated that the logarithm of the detection limit (log DL) of the BOR or OCD liquid membrane electrode for a series of beta-adrenergic drugs in cation-exchange HPLC systems is mostly dependent on changes in lipophilicity (expressed by log P) and polarizability (P_o) of such drugs. This relation calculated for eighteen diversified beta-adrenergic drugs is presented in Fig. 6. This function predicts that the lowest detection limit on the BOR and OCD electrodes should be located in the bottom left-hand corner of the approximation surface calculated by the least square method, i.e. for more lipophilic cationic drugs such as tertatolol, propranolol, bevantolol, alprenolol and clenbuterol, and possessing moderate polarizability. In the present studies, a similar relationship was confirmed as a detection limit of clenbuterol, ambroxol and bromhexine, characterized by near similar polarizability (see Table 1), on the BOR, OCD and TOPA electrodes decreased along with increased lipophilicity of these drugs despite of the type of used HPLC system.

3.3. Application to pharmaceutical formulations

From the results of the studies described above, one can conclude that the cyano RP-HPLC system

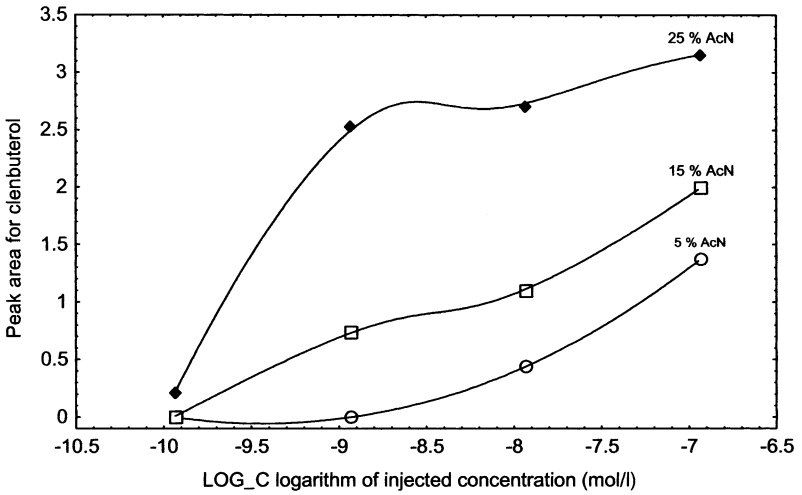

Fig. 5. Influence of AcN concentration in mobile phase on response of OCD liquid membrane electrode for clenbuterol in the range 0.1–100 nmol l^{-1} injected. Lines were fitted by the least-squares method. The hybrid RP-HPLC system was used in these measurements.

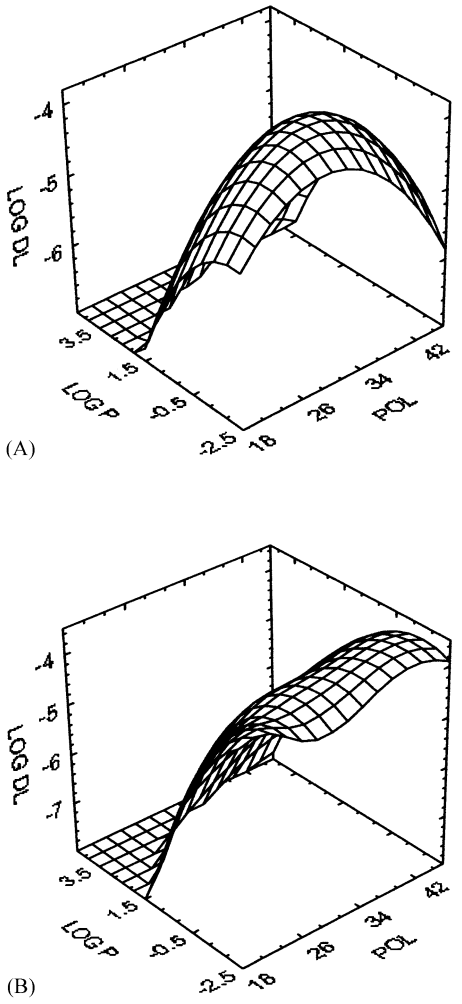

Fig. 6. Dependence of the logarithm of the detection limits (log DL) of (A) BOR and (B) OCD liquid membrane electrode determined for eighteen β-adrenergic drugs on their lipophilicity (expressed by log P) and polarizability (POL). Approximation surfaces were fitted by the least-squares method. Experimental points were omitted for clarity. The cation-exchange HPLC system was used in these measurements (compare Section 2).

employing a cyanosilica column and AcN–ethanol–perchloric acid (1.66 mM) (60:2:38, v/v/v) (pH* 2.45) as the mobile phase is most convenient to separate all analytes and to detect them sensitively with a TOPA electrode. Fig. 4 gives examples of such separation and detection. Alternatively, the hybrid RP-HPLC system can be used to enhance the sample throughput and down-scaling sensitivity during analysis of mixtures of these drugs.

The highly lipophilic character of clenbuterol, ambroxol and bromhexine gives the reason for very low concentrations of these drugs in human plasma, e.g. in range 66–580 nmol l^{-1} for ambroxol [24], 13–850 nmol l^{-1} for bromhexine [16] and 5–20 nmol l^{-1} for clenbuterol in lactating cows plasma [11,70]. Thus, we tested the linearity of calibration lines in similar concentration ranges for the most sensitive TOPA and OCD modified liquid membrane electrodes used for potentiometric detection of the mentioned drugs in cyano RP-HPLC conditions. A good linear relationship was obtained between the logarithm of concentration injected ($\log c$) and corrected peak area (y) for each component of calibration solutions of the analyzed drugs. The calculated linear regression parameters of calibration lines are given in Table 4. We found that both electrodes give satisfactory linear response over the range of injected concentrations (18–500 nmol l^{-1}). As it was pointed earlier, the TOPA-modified electrode was more sensitive to the more lipophilic bromhexine and gives slightly better linearity as compared with an OCD electrode.

The results of four replicate intra-day determinations of 2.0×10^{-7} mol l^{-1} clenbuterol, ambroxol or bromhexine showed relative standard deviation (RSD = 100 × S.D./mean) of 4.5–8.5% for TOPA and OCD electrodes, thus indicating good repeatability of the developed potentiometric detection procedure for such drugs. Inter-day (day-to-day) reproducibility of the calibration lines as evaluated by the RSD of the slope and intercept data was in the range 18.1–25.2%. The decreasing day-to-day precision is probably due to increased leaking of the ionophores from the PVC matrix [38,39,52], and to increased PVC membrane swelling [68] in the mobile phase containing high concentrations of polar organic modifier. In practice, the working time of TOPA or OCD liquid membrane electrodes should not exceed 3 hours per day and these electrodes should not be used longer than 3 days.

The potentiometric detection procedure employing TOPA or OCD modified liquid membrane electrodes and normal-phase HPLC systems was

Table 4
Linear regression calibration parameters[s] for TOPA and OCD electrode in range 7–500 nmol l^{-1} injected analytes in cyano RP-HPLC (20 μl injection)

Electrode	Compound	*a*	*b*	*r*	*N*
TOPA	Clenbuterol[p]	0.9695 ± 0.0689	58.7791 ± 1.4963	0.9901	6
	Ambroxol	1.3907 ± 0.0769	115.6073 ± 1.3139	0.9834	6
	Bromhexine	1.6622 ± 0.0875	123.3585 ± 1.0085	0.9887	6
OCD	Clenbuterol	1.0559 ± 0.0865	72.4152 ± 1.3868	0.9878	6
	Ambroxol	1.3023 ± 0.0922	90.2354 ± 1.3334	0.9845	6
	Bromhexine	1.4293 ± 0.0716	105.1527 ± 1.4731	0.9932	6

[s] $y = a \log c + b$, where y = peak area of analyte determined cyano RP-HPLC system; c = analyte concentration (mol l^{-1}); a = slope; b = intercept; r = correlation coefficient; n = number of calibration points (each of the three calibration solution was measured in triplicate).
[p] In range 18–500 nmol l^{-1} injected.

used for the determination of ambroxol as the single active component in two different branches of marketed tablets. Typical tablets with mean weight approximately 250 mg contain 30 mg of ambroxol as the pharmacologically active substance [12]. The tablets combining clenbuterol and ambroxol contain, respectively, 0.020 and 30 mg of mentioned drugs per tablet [9]. For comparison, the single tablet combining of salbutamol sulfate, another beta2-adrenergic bronchodilator, and bromhexine contains, respectively, 2 and 8 mg of these drugs [17].

Fig. 7 demonstrates typical chromatograms of ambroxol extracted from both types of analyzed tablets and detected by using a TOPA electrode. The results of the performed analyses, including repeatability data ($n = 4$), are summarized in Table 5 and indicate good agreement between the claimed and determined values. However, the increased content of an unknown cationic substance (probably used as an excipient in the production process of the pharmaceuticals) isolated from Mucobron® tablets was registered by potentiometric detection (see Fig. 7). The results presented in Table 5 were obtained during a 1-day experimental session of TOPA and OCD liquid membrane electrodes.

According to the remarks of Pospisilová et al. [30], the method of standard addition was used for examinations of accuracy of the developed HPLC potentiometric detection method of clenbuterol, ambroxol and bromhexine in the analyzed tablets. As it is seen from Table 6, the recoveries of all added drugs ranged between 96.1 and 99.2% indicating that intra-day accuracy of the proposed cyano RP-HPLC assay of the mentioned drugs with potentiometric detection is acceptable.

4. Conclusions

The use of the podant-type TOPA ionophore as a constituent of PVC-based electrodes in the potentiometric detection system hyphenated with

Table 5
Results of ambroxol determinations in pharmaceutical preparations by cyano RP-HPLC[a] with potentiometric detection

Marketed drug	Declared content	Determined content ± RSD[b] (%)	
		OCD electrode[a]	TOPA electrode[a]
Surbronc® (Belgium)	60 mg/tablet	98.5 ± 5.25	99.1 ± 5.10
Mucobron® (Poland)	30 mg/tablet	96.9 ± 5.82	97.5 ± 5.56

[a] See Section 2 for details.
[b] Calculated for $n = 4$.

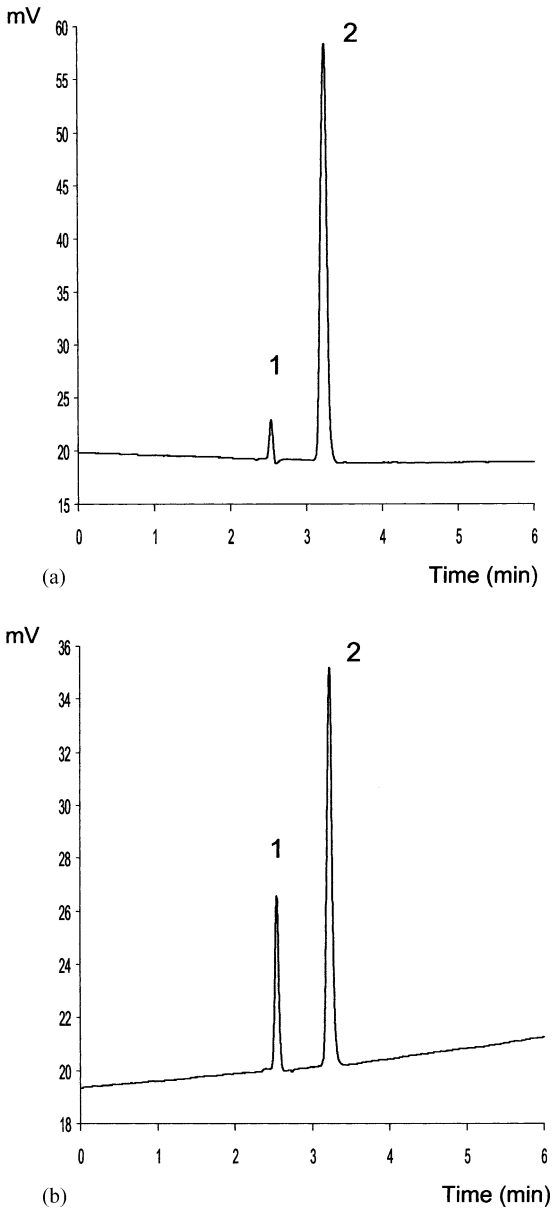

Fig. 7. Potentiometric detection with a TOPA electrode of (1) unknown cationic substance and (2) ambroxol isolated from (a) Surbronc® and (b) Mucobron® pharmaceutical tablet formulations and separated by a cyano RP-HPLC system. Compare Section 2 for details of measurements.

Table 6
Results of recovery analysis of drugs added to pharmaceutical formulations as determined by cyano RP-HPLC[a] with potentiometric detection—TOPA electrode[a]

Formulation	Clenbuterol			Ambroxol			Bromhexine		
	Added	Found ± RSD[b] (%)	Recovery[c] (%)	Added	Found ± RSD[b] (%)	Recovery[c] (%)	Added	Found ± RSD[b] (%)	Recovery[c] (%)
Surbronc®	40 μg/tablet	39.2 ± 5.4	98.1	5 mg/tablet	4.96 ± 4.8	99.2	0.50 mg/tablet	0.48 ± 3.7	96.1
Mucobron®	20 μg/tablet	19.4 ± 6.9	97.2	3 mg/tablet	2.91 ± 5.2	97.0	0.30 mg/tablet	0.29 ± 4.8	96.7

[a] See Section 2 for details.
[b] Determined for $n = 4$.
[c] Recovery (%) = Found × 100/added

cyano RP-HPLC or hybrid RP-HPLC modes gives superior sensitivity for qualitative and quantitative determination of clenbuterol, bromhexine and ambroxol. The PVC-based liquid membrane electrodes can be applied successfully in HPLC systems employing high concentrations (60–75%, v/v) of AcN and methanol in the mobile phase. Increasing concentration of organic modifier (AcN or MeOH) in mobile phase significantly improved detection limits of hydrophobic-charged analytes by the potentiometric detector employing PVC-based liquid membrane electrodes with TOPA or OCD ionophores. The potentiometric detection of clenbuterol, bromhexine and ambroxol offers a promising alternative for determination of these cationic drugs by HPLC in view of increased sensitivity and specificity, with good linearity, precision and accuracy. Miniaturization of the electrodes is envisaged for in vivo monitoring of ambroxol and bromhexine as the radical scavengers in living cells and for pharmacokinetic determinations of these drugs in body fluids.

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