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Capillary zone electrophoresis of cationic and anionic drugs in methanol

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Abstract

The actual mobilities and dissociation constants of acidic and basic pharmaceuticals were determined in methanol. Actual mobilities were derived from the dependence of the effective mobilities of the analytes on the pH of the methanolic background electrolyte solution (pH_{MeOH}). The pK_a values of the pharmaceuticals in methanol ($pK_{a,MeOH}$) were calculated by non-linear curve fitting to the measured mobility values. It was found that the shift in pK_a value (when compounds were transferred from water to methanol) increased with the acidity of the analyte. The average pK_a shift for compounds exhibiting acidic properties in water was ca. 5.5 units, and the shift for basic compounds about 2 units. As was shown for a mixture of β-blockers, the calculated actual mobilities and pK_a values can be utilised in the optimisation of pH conditions for separation. The practical value of the method was illustrated by the analysis of urine samples.

Keywords: Nonaqueous capillary zone electrophoresis; Methanol; β-Blockers; Catecholamines metabolites; Dissociation constants; Urine

1. Introduction

Electrophoretic mobilities and ionisation behaviour of analytes are the key factors driving separations in capillary zone electrophoresis (CZE). Knowledge of these basic physicochemical properties of analytes gives valuable information about their nature and makes it easier to choose appropriate experimental conditions for their separation. Optimal experimental conditions can then be found with less time and effort, and an estimate of the success of the separation can be made beforehand.

Most CZE separations have been carried out in aqueous media and the dissociation constants of a wide variety of analytes in water solutions are available in handbooks and scientific papers [1–5]. A relatively limited amount of data is available on dissociation constants and electrophoretic mobilities of analytes in nonaqueous media [6–8], since the methods to measure pH in nonaqueous solutions tend to be either time-consuming or prone to error [9,10].

CZE separation in organic solvents, i.e. nonaqueous capillary electrophoresis (NACE), has become increasingly popular over the last decade. The relative acid strengths in organic solvents are often different from those in water and enable the separation of compounds that are not easily separated in aqueous media. Organic solvents can also dissolve a wide variety of compounds that are insoluble or only poorly soluble in water, thereby expanding the field of CZE. The benefits of nonaqueous electrophoresis have been well covered in several papers and comprehensive NACE reviews, and readers are referred to these contributions [9–16].

Nonaqueous medium is also useful in providing stable spray and overall sensitivity for detection of analytes by electrospray ionization (ESI) mass spectrometry (MS). Low surface tension and high volatility favour stable formation of the spray. Because the spray itself carries a large portion of the current and currents are low in NACE, grounding of the capillary outlet at the ESI interface is not essential. Easy evaporation of organic solvents allows to lower the drying-gas flow-rate, increasing the number of ions in the MS. The advantages of nonaqueous over aqueous solvents in CZE-MS have been reported by several groups [17–20].

Methanol is one of the most widely used solvents in NACE. Although, like water, it is amphiprotic in nature, however, it dissolves numerous organic compounds that are poorly dissolved in aqueous media. Methanol has also proved to be a suitable separation medium for several compounds that cannot be separated in aqueous solutions. Often the

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dissociation constants for a particular set of compounds differ markedly in methanol while they overlap in aqueous solutions [21,22].

One clear drawback of NACE is the limited information available about protolysis, molecular interactions, and solvation phenomena occurring in organic solvents.

The aim of this research was to determine actual mobilities and dissociation constants of anionic metabolites of catecholamines and serotonin (5-HIAA) and cationic β-blockers in methanolic solutions and to use this information to find an optimum pH for NACE separation. The work was made by measuring the effective electrophoretic mobilities of analytes in methanolic solution of acetic acid–sodium acetate in a pH range between 8.7 and 11.4.

All the compounds studied are of clinical importance. Catecholamines serve as neurotransmitters in the central and peripheral nervous systems and as hormones in the systemic circulation, regulating heart rate and blood pressure. Monitoring the concentration of catecholamines, seratonins and their metabolites in body fluids is crucial for the proper diagnosis and treatment of certain potentially fatal catecholamine-producing tumours [23,24]. The β-blockers (beta-adrenoreceptor blocking drugs) are a group of drugs used in the treatment of cardiovascular disorders such as hypertension, cardiac arrhythmia and congestive heart failure, as well as in the treatment of migraines, essential tumour, high blood pressure and glaucoma [25–27]. Determination of β-blockers in biological fluids is required for many purposes, including doping control, forensic analysis, toxicology and pharmacokinetic studies [28,29].

Additional aims of our study were to obtain information about the suitability of methanol solutions for the separation of β-blockers and to demonstrate the usefulness of the developed method in the detection of drugs in urine samples.

2. Experimental

2.1. Chemicals

The metabolites of catecholamines and serotonin were homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid, HVA), 4-hydroxy-3-methoxymandelic acid (vanillic mandelic acid, VMA), DL-3-hydroxy-4-methoxymandelic acid (isovanillic mandelic acid, V'MA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-3-indoleacetic acid (5-HIAA). All were from Sigma-Aldrich (St. Louis, Mo, USA, or Steinheim, Germany).

The β-blockers were acebutolol (*N*-[3-acetyl-4-{2-hydroxy-3-[(1-methylethyl)amino]propoxy}phenyl]butanamide, ACE), alprenolol {1-[(1-methylethyl)amino]-3-[2-(2-propenyl)phenoxy]-2-propanol, ALP}, atenolol (4-{2-hydroxy-3-[(1-methylethyl)amino}propoxy]benzeneacetamide, ATE), labetalol (2-hydroxy-5-{1-hydroxy-2[(1-methyl-3-phenylpropyl)amino]ethyl}salicylamide, LAB), metoprolol {1-[4-(2-methoxyethyl)-phenoxy]-3-[(1-methylethyl)amino]-2-propanol, MET}, nadolol (5-{3-[(1,1-dimethylethyl)-amino]-2-hydroxypropoxy}-1,2,3,4-tetrahydro-2,3-naphtalenediol, NAD), oxprenolol {1-[(1-methylethyl)amino]-3-[2-(2-propenyloxy)phenoxy]-2-propanol, OXP}, pindolol {1-(1H-indol-4-yloxy)-3-[(1-methylethyl)amino]-2-propanol, PIN} and timolol {(2S)-1-[(1,1-dimethylethyl)-amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2-propanol, TIM} from Sigma-Aldrich (St. Louis, MO, USA); bopindolol {1-[(1,1-dimethylethyl)amino]-3-[(2-methyl-1H-indol-4-yl)oxy]-2-propanol benzoate ester, BOP} from Sandoz Pharma (Basel, Schwitzerland); practolol (*N*-[4-{2-hydroxy-3-[(1-methylethyl)amino]propoxy}phenyl]acetamide, PRA) from Orion-Pharma (Espoo, Finland); propranolol {1-[(1-methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol, PRO} from Medipolar (Oulu, Finland); and sotalol (*N*-[4-{1-hydroxy-2-[(1-methyethyl)amino]ethlyl}phenyl]methanesulfonamide, SOT) from Lääke (Salo, Finland). The structures of the analytes are depicted in Figs. 1 and 2.

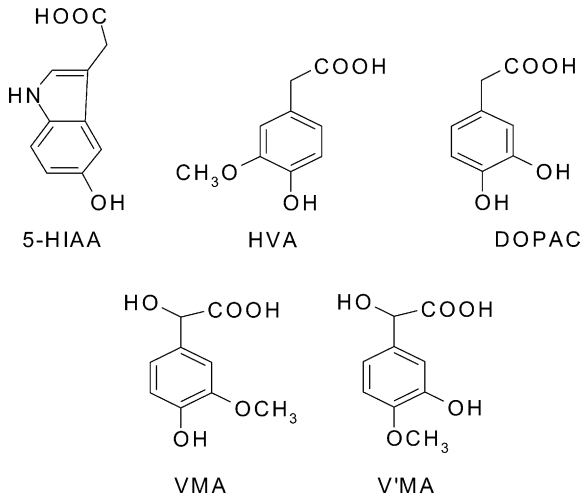

Fig. 1. Molecular structures of the meatabolites of catecholamines analysed and 5-HIAA. For explanation of abbreviations, see Table 1.

Methanol and dimethyl sulfoxide (DMSO) were HPLC grade and they were from Lab-Scan (Dublin, Ireland). Glacial acetic acid was from J.T.Baker (Deventer, The Netherlands). Sodium hydroxide was from Merck (Darmstadt, Germany). Disodium hydrogenphosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$) from Merck (Darmstadt, Germany) and sodium dihydrogenphosphate monohydrate ($NaHPO_4{\cdot}H_2O$) from J.T.Baker were used for the sample pre-treatment.

The β-blocker tablets taken by patients were 100 mg of alprenolol (Apin N, Hässle), 25 mg of propranolol (Propral, Medipolar), 40 mg of oxprenolol (Trasicor, Ciba-Geigy) and 50 mg of atenolol (Tenoblock, Leiras). The tablets were administered one to each volunteer and no other drug was used before the urine was demonstrated to be drug-free. The β-blockers were administrated after overnight fasting. The urine was collected in periods of 0–4, 4–8, 8–12 and 12–24 h and stored at −20 °C. The 4–8 h fraction of each sample was used in the study.

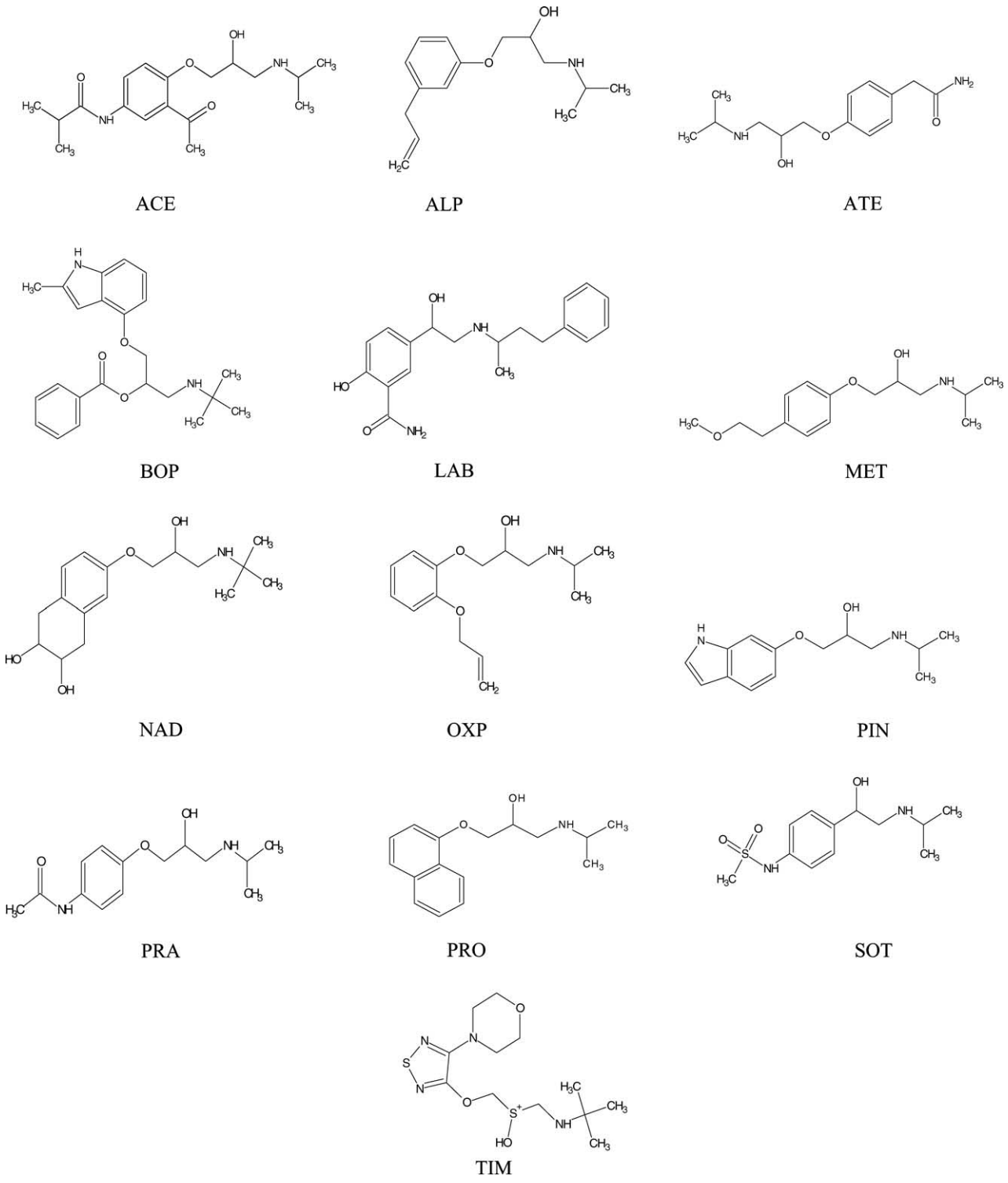

Fig. 2. Molecular structures of β-blockers analysed. For explanation of acronyms, see Table 3.

2.2. Instrumentation and operating conditions

Capillary electrophoresis experiments were carried out with an HP ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany) equipped with a photodiode array detector and an air cooling unit for the capillary. The instrument control and data analyses were performed with HP Chemstation software (Hewlett-Packard, Version 6.03). Uncoated fused silica capillaries (Composite Metal Services, The Chase, Worchester, UK) were of 50 μm i.d., 375 μm o.d. and total length 58.5 cm; there was a detection window at 50.0 cm. The capillary cassette temperature was maintained at 25.0 °C with

air cooling, and the autosampler temperature was adjusted to 25.0 ± 0.5 °C with an external water bath (Heto, Birkeröd, Denmark). Running voltages were +10 and +30 kV and the resulting electric currents were 0.6–10 μA. Sample injection was made at 50 mbar pressure for 2.0 s. The instrument was placed in a special room with automated temperature and humidity control.

Identification of alprenolol, atenolol, oxprenolol and propranolol and their hydroxy-substituted metabolites was performed with a GC-electron impact ionisation (EI) MS system comprising a Hewlett-Packard (Avondale, PA, USA) Model 5890A gas chromatograph and a Hewlett-Packard Model 5989A single-stage quadruple mass spectrometer. Analyses were performed with a NB-30 capillary column (8.5 m × 0.32 mm, 0.25 μm) and a retention gap column (2 m, i.d. 0.32 mm, DPTMS) using a temperature gradient from 30 to 300 °C and rates between 5 and 15 °C/min, varying with the resolution of the matrix compound and the analyte in the chromatogram. Drugs in urine samples were identified with total ion current (TIC) and selected-ion monitoring (SIM) modes (*m*/*z* 200, 158, 140, 98, 72 and 56; in addition *m*/*z* 204 for hydroxy metabolites of oxprenolol).

In solid-phase extraction (SPE), Oasis HLB copolymer (M-divinylbenzene and *N*-vinylbenzene copolymer, 1 ml, Waters, Milford, MA, USA) cartridges were used. The extraction was performed with a Supelco Visiprep solid-phase extractor. A model PHM95 reference pH meter (Radiometer, Copenhagen, Denmark) was used in adjusting the pH of the SPE buffer.

2.3. Procedures

2.3.1. Conditioning of the capillary

The fused silica capillaries were conditioned before use with 0.1 M NaOH in methanol for 10 min, methanol for 10 min and with the background electrolyte (BGE) solution used in the analysis for 30 min. Between analyses the capillary was rinsed with the BGE solution for 2 min. After each sequence the capillary was washed with methanol for 10 min and finally it was dried with air for 5 min. The capillary was stored dry.

2.3.2. Solutions in measurement of electrophoretic mobilities

The effective electrophoretic mobilities (μ_{eff}) of the analytes used in $pK_{a,MeOH}$ calculations were measured in methanolic solutions of acetic acid and sodium acetate. Where sodium acetate was 5.0 (β-blockers) or 7.9 (metabolites of catecholamines and 5-HIAA) mM. The acetic acid/sodium acetate ratios were 10:1, 3:1, 1:3, 1:10 and 1:50 (mol:mol) and the pH_{MeOH} values of the solution calculated according to the Henderson-Hasselbalch equation were 8.7, 9.2, 10.2, 10.7 and 11. 4, respectively. The pK_a value for acetic acid in methanol is 9.7 [7].

2.3.3. Solutions used in separations

Stock solutions of analytes were prepared at concentration of 1000 μg/ml in methanol. Mixtures of 1–30 μg/ml were used for spiking in urine analyses. In pK_a studies, the concentration of 5-HIAA and metabolites of catecholamines was 50 μg/ml and the concentration of β-blockers 70 μg/ml, except for timolol and labetalol whose concentrations were 140 and 200 μg/ml, respectively. The concentration of the neutral marker, DMSO, was 35 mM in methanol.

Separation of the mixture of β-blockers was carried out in a methanolic solution of 5 mM sodium acetate and 30 mM acetic acid (pH of the solution was 8.9).

2.3.4. Pre-treatment of the urine samples

The urine samples were analysed after solid-phase extraction of filtered samples (0.45 μm, PTFE membranes, Millipore). Oasis HLB copolymer SPE cartridges were regenerated with methanol and distilled, deionised water, and then rinsed with 30 mM phosphate buffer (pH 7.0) prepared from 30 mM $Na_2HPO_4{\cdot}2H_2O$ and 30 mM $NaH_2PO_4{\cdot}H_2O$. Aliquots of 500 μl of sample urine were pipetted on the sorbent. After the analyte adsorption, 1 ml of methanol–water (5:95, v/v) was added. Finally, the analyte sorption was performed with 1 ml of methanol. The SPE eluate was evaporated to dryness at 40 °C under nitrogen flow. For analysis; the precipitate was diluted into 500 μl of methanol. When stored, it was placed into a vacuum desiccator.

The GC–MS identification of analytes was carried out on derivatised samples according to ref. [30]. The methanolic SPE eluent containing the analytes was evaporated under nitrogen atmosphere at 60 °C and the β-blockers and their metabolites were acetylated with a mixture of acetanhydride–pyridine (3:2, v/v). The reaction time was 1 h at 80 °C. After the reaction the solution was evaporated to dryness under nitrogen, and the precipitate was dissolved into 200 μl of dichloromethane–methanol (95:5, v/v). The analytes were not derivatised for nonaqueous capillary electrophoresis separations.

The β-blockers in the patient urine samples were identified by spiking the samples with 1–30 μg/ml solutions. The drug metabolites were monitored by viewing the sample profile against the blank urine profile.

2.3.5. Technique used in CE

The effective mobilities were determined with a capillary electrophoresis instrument by the method of Williams and Vigh [31] in methanol solutions of different pH. The Williams and Vigh method was particularly suitable for our study because it eliminates the variations in electroosmotic flow.

Because the electroosmotic flow was weak, all the electrophoretic mobilities were measured by first injecting an analyte and the electroosmotic flow marker, DMSO, and moving them to the thermostatted region of the capillary with injection pressure for 2 min. Running voltage was then applied, and the analyte and DMSO were allowed to separate for 5 min. The separation was carried out inside the

thermostatically controlled region of the capillary. A second DMSO zone was then injected to the capillary, and finally all three zones were moved under the applied voltage to the detector with aid of the injection pressure.

The electrophoretic mobility of each analyte was calculated using the distance between the analyte peak and the peak of the first injected DMSO zone. Pressure delay time (ca. 0.05 min) and pressure ramp-time (0.17 min) were considered in the calculations. The HP ^{3D}CE instrument was modified as described by Porras [32] to minimise the pressure fluctuations.

UV absorbances of the analytes and DMSO were detected at wavelength 200 nm, except when mobilities were so low that the peaks of the analyte and DMSO could not be differentiated. In those cases the analytes were detected at 254 nm (DMSO does not absorb UV light at that wavelength).

2.3.6. Calculations

Dissociation coefficients, $pK_{a,MeOH}$, were calculated by using the effective mobility values of the analytes, μ_{eff}, in methanol. The effective mobilities were plotted as a function of pH_{MeOH} with a commercial Origin50 calculation programme (Microcal Software, Inc.).

3. Results and discussion

The success of CE separations depends on the values and differences of the electrophoretic mobilities of the analytes. The electrophoretic mobility of an analyte is directly proportional to its effective charge and inversely proportional to its solvation radius [33]. Both of these properties are affected by the solvent that is selected. The dissociation of an analyte (and thus its effective charge) is influenced by the acid–base properties of the solvent. If the degree of dissociation of the analyte mostly depends on proteolysis, then the actual mobility and dissociation constant of the analyte are the main factors determining the behaviour of the analyte in a given medium.

The present work expands the applications of an earlier approach developed by Porras et al. [34–36] to measure and calculate actual mobilities and dissociation constants of analytes in organic solvents. The selected solvent is methanol, which is among of the most used nonaqueous solvents in CE [10]. In this work the above-mentioned physicochemical properties were calculated for two groups of pharmaceuticals: the metabolites of catecholamines and β-blockers. The analytes were selected so that both anionic and cationic species would be present under the conditions employed. We demonstrate how knowledge of actual and effective mobilities, as well as of dissociation constants, can be utilised in the characterisation of analytes and in the prediction of their separation. Finally the NACE method is tested in the analysis of β-blockers in urine samples.

3.1. Determination of effective mobilities

The dissociation constant of an analyte can be determined if the actual mobility and at least one effective mobility of the analyte are known. The term *actual mobility* denotes the mobility of a fully charged analyte at a given ionic strength, whereas *effective mobility* is the actual mobility weighted by the degree of ionisation. In other words, effective mobility is measured at a pH value where the analyte is partly dissociated. To obtain conditions where an analyte is fully charged in a solution (to measure its actual mobility) is not always trivial. In many cases it requires the use of extreme pH values. Furthermore, the BGE counter ion for actual mobility measurements should be the same as for effective mobility measurements in order that the effect of ion-pairing can be ignored [35,37]. In our work, instead of measuring the actual mobility and one value for effective mobility, we measured the effective mobilities of the analytes at four to five different pH_{MeOH} values. Every measurement was made in four times.

An appropriate pH range of the methanolic BGE solutions was established with the help of the Henderson–Hasselbalch equation, i.e. by taking correct amounts of the selected acid and its salt. It is clear that the acid must be weak also in methanol, and its pK_a value in methanol must be known. We chose for the purpose acetic acid, for which these requirements are fulfilled: it is a weak acid in methanol with pK_a of 9.7 at 25 °C [7]. By varying the ratio of acetic acid and its sodium salt, we established a pH range from 8.7 to 11.4 in methanol.

The analytes chosen for our work are fairly heterogeneous, in both medical and chemical terms (see the chemical structures in Figs. 1 and 2). From the point of view of CE, charged analytes can always be divided into two groups: anionic and cationic species. This is also how we have categorised our analytes (in addition to the medical classification). According to this classification, the metabolites of catecholamines and 5-HIAA we studied are anionic (and exhibit acidic properties in aqueous media), while the β-blockers are cationic (with basic properties in water).

3.1.1. The metabolites of catecholamines and 5-HIAA

In order to calculate the desired parameters for the anionic metabolites of catecholamines and 5-HIAA (i.e. the actual mobilities and dissociation constants), we measured the effective mobilities of all analytes at four different pH values in methanol. As seen from Table 1, the effective mobilities are increased for all the analytes with increasing pH, however at different rate. The analytes like VMA and V′MA, which exhibit stronger acidic properties in water and have pK_a values around 2–3 (Table 2), already have relatively high mobilities at pH 8.7, and the values level up at higher pH values (see Table 1). The other three analytes have weaker acidic properties in water and exhibit a continuous increase in effective mobilities (Table 1). It is clear that the applied pH range does not cover the pH where the less acidic metabolites would be

Table 1
Measured effective mobilities, μ_{eff}, of the metabolites of catecholamines and 5-HIAA in methanol at 25 °C. Mobilities are given as 10^{-9} m^2 V^{-1} s^{-1}

Analyte	Abbreviation	pH 8.7	pH 9.2	pH 10.2	pH 10.7
Homovanillic acid	HVA	−3.7	−9.5	−21.8	−24.4
5-Hydroxy-3-indole acetic acid	5-HIAA	−2.1	−5.6	−16.8	−21. 9
4-Hydroxy-3-methoxy mandelic acid	VMA	−15.0	−21.1	−25.0	−25.6
DL-3-Hydroxy-4-methoxymandelic acid	V'MA	−14.0	−20.7	−25.3	−25.7
3,4-Dihydroxyphenylacetic acid	DOPAC	−2.9	−7.8	−20.0	−23.3

The values in the table are the average of four parallel measurements. The average R.S.D. is less than 1%.; The pH_{MeOH} values are calculated with the Henderson–Hasselbalch equation.

Table 2
Actual mobilities (μ_{act}) and dissociation constants (pK_a) of the metabolites of catecholamines and 5-HIAA

Analyte	μ_{act} (10^{-9} m^2 V^{-1} s^{-1})	pK_a (H_2O)[a]	pK_a (MeOH)	ΔpK_a
HVA	−30.03 (3.20)	4.4	9.4 (0.14)	5.0
5-HIAA	−29.50 (3.80)	4.5	9.7 (0.14)	5.2
VMA	−26.56 (0.28)	2.3	8.6 (0.02)	6.3
V'MA	−26.66 (0.41)	3.1	8.7 (0.03)	5.6
DOPAC	−29.53 (2.20)	4.4	9.5 (0.14)	5.1

The dissociation constants in methanol are derived from curve fitting according to Eq. (1). Standard deviations (of four parallel experiments) are given in parenthesis; For identification of abbreviations see Table 1.
[a] The pK_a values in water were calculated with Pallas software.

fully ionised. The actual mobilities of the analytes are thus calculated from the effective mobility as a function of pH in methanol according to the well-known relation

$$\mu_{eff} = \frac{\mu_{act}}{1 + 10^{pK_a - pH}} \quad (1)$$

by applying non-linear curve fitting to the measured effective mobility values (μ_{act} is the absolute mobility of the analyte, pH is the pH of the methanolic background electrolyte solution and pK_a is the dissociation constant of the analyte in methanol). For more acidic analytes the actual mobilities are only slightly different from the effective mobilities at pH 10.7, as can be seen from Tables 1 and 2 (the effective mobilities at pH 10.7 in Table 1 and the actual mobilities calculated from non-linear curve fitting in Table 2).

3.1.2. β-Blockers

All studied β-blockers are cationic over the pH range investigated. Their effective mobilities increase with decreasing pH (Table 3). As can be seen from Table 3, the ionisation behaviour of the β-blockers is closely similar. The fully ionised state (actual mobility) for the β-blockers is not reached and again is calculated with Eq. (1), now slightly modified: the power of 10 in the denominator is replaced by ($pH - pK_a$). The actual mobilities of the β-blockers are presented in Table 4.

3.2. Dissociation constants

One of the tasks of this work was to estimate the pK_a values for the studied analytes in methanol. This was done according to Eq. (1) by applying non-linear curve fitting to the data

Table 3
Measured effective mobilities of β-blockers in methanol at 25 °C

Analyte	Abbreviation	pH 8.7	pH 9.2	pH 10.2	pH 10.7	pH 11.4
Acebutolol	ACE	21.8	21.6	18.9	13.8	8.6
Alprenolol	ALP	26.8	26.8	23.8	17.2	9.8
Atenolol	ATE	22.5	22.3	19.9	14.5	8.2
Labetalol	LAB	18.8	18.0	12.3	7.9	2.5
Metoprolol	MET	24.9	24.3	22.1	17.7	12.1
Nadolol	NAD	21.3	20.9	20.6	18.2	12.1
Oxprenolol	OXP	27.0	26.2	24.5	21.5	12.6
Pindolol	PIN	25.8	24.3	22.7	20.0	11.8
Timolol	TIM	23.5	23.5	20.8	15.3	9.4
Bopindolol	BOP	24.3	24.1	22.9	18.9	12.7
Practolol	PRA	22.5	22.7	20.3	16.3	10.0
Propanolol	PRO	25.8	26.1	23.2	17.9	10.5
Sotalol	SOT	23.3	23.4	21.1	16.5	10.0

Mobilities are given as 10^{-9} m^2 V^{-1} s^{-1}. The values in the table are the average of four parallel measurements. The average R.S.D. is less than 1.5%; The pH_{MeOH} values are calculated with the Henderson–Hasselbalch equation.

Table 4
Actual mobilities (μ_{act}) and dissociation constants (pK_a) of β-blockers

Analyte	μ_{act} (10^{-9} m^2 V^{-1} s^{-1})	pK_a (H_2O)[a]	pK_a (MeOH)	ΔpK_a
ACE	21.6 (0.06)	9.2	11.1 (0.09)	1.9
ALP	26.81 (0.06)	9.6	11.1 (0.06)	1.5
ATE	21.95 (0.06)	9.3	11.1 (0.07)	1.8
LAB	18.82 (0.08)	9.4	10.5 (0.06)	1.1
MET	24.11 (0.06)	9.2	11.3 (0.07)	2.1
NAD	21.24 (0.01)	9.4	11.5 (0.02)	2.1
OXP	26.55 (0.02)	9.3	11.4 (0.02)	2.1
PIN	24.89 (0.4)	9.5	11.3 (0.04)	1.8
TIM	23.36 (0.05)	9.2	11.1 (0.07)	1.9
BOP	24.06 (0.09)	8.6	11.4 (0.08)	2.8
PRA	21.89 (0.05)	9.2	11.3 (0.07)	2.1
PRO	25.55 (0.05)	9.2	11.2 (0.06)	2.0
SOT	23.24 (0.04)	9.2	11.2 (0.06)	2.0

The dissociation constants in methanol are derived from curve fitting according to Eq. (1). Standard deviations of four parallel experiments are given in parenthesis.; For identification of acronyms, see Table 3.
[a] The pK_a values in water are from ref. [23].

set (four to five measured effective mobilities). For cationic analytes, the power of ten in the denominator is replaced by (pH − pK_a).

Non-linear curve fittings for selected analytes are presented in Fig. 3. For VMA and V′MA, the actual mobilities are nearly reached within the pH range applied; for others, like DOPAC and 5-HIAA, they are not yet reached. The pK_a values for studied compounds in water and methanol are presented in Table 2 together with the corresponding ΔpK_a (p$K_{methanol}$ − pK_{water}) values. The average ΔpK_a is about 5.5 units.

It has to be mentioned here that the metabolites of catecholamines and 5-HIAA have rather complex structure and the pK_a values calculated here can only serve as estimates. The determined pK_a values are attributed to carboxylic group of the compound. The shift of pK_a values between aqueous

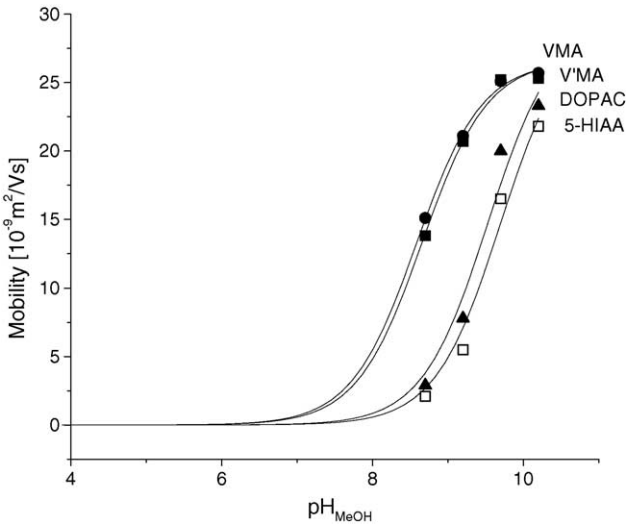

Fig. 3. Mobilities of the metabolites of catecholamines vs. pH$_{MeOH}$. Curve fittings are according to Eq. (1). For explanation of abbreviations, see Table 1.

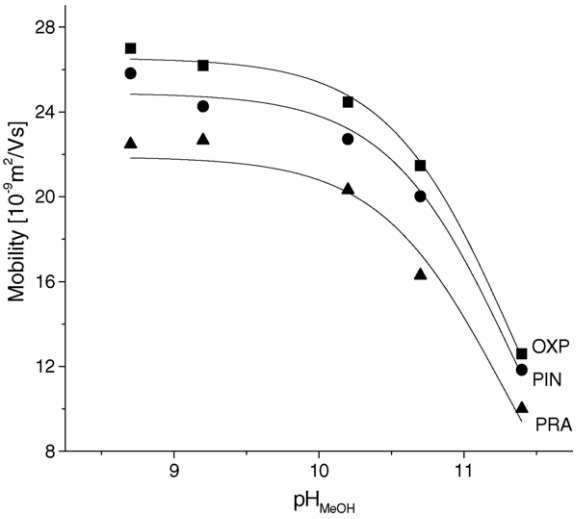

Fig. 4. Mobilities of selected β-blockers vs. pH$_{MeOH}$. Curve fittings are according to Eq. (1). For explanation of acronyms, see Table 3.

and methanolic solutions for phenol groups (the next group to dissociate) is around 4–4.5 units [8,22]. The pK_a values for the phenol group of the studied compounds (except for HVA) are higher than 9 (values calculated by Pallas programme) and the corresponding pK_a values in methanol are not expected to be lower than 13. Therefore the dissociation of phenol groups (pK_a − 2 units) was not expected in experimental pH range (the highest experimental pH for the metabolites of catecholamines was 10.7). For HVA the pK_a value of phenol group in water is 7.9 (calculated by Pallas programme) and the pK_a value in methanol is expected to be around 12–13. Therefore, HVA may start to dissociate at the highest experimental pK_a values (around pH 10). However, even then the contribution of the phenol group would still be very small.

Typical curves for the β-blockers are presented in Fig. 4. The inflection point is reached for the majority of the analytes and in some cases even exceeded. This is in agreement with the pK_a values presented in Table 4—most of the pK_a values in methanol are equal to or below the highest experimental pH value (11.4) applied. The only exception is NAD, whose calculated pK_a value in methanol is 11.5.

One feature is characteristic of all the compounds studied—the value of ΔpK_a increases with the acidity as demonstrated in Fig. 5. The correlation between the measured data points and the trendline is greater than 0.98. The mean ΔpK_a value for the metabolites of catecholamines and 5-HIAA, which are acidic in water, is 5.5 (ranging from 5.0 to 6.3), in agreement with earlier studies of HA-type acids in methanol [8,38]. The mean ΔpK_a value for β-blockers was about 2 units (ranging from 1.1 to 2.8), which again is similar to previous values for cation acids in methanol [2,6].

3.3. Separation of analytes

As can be seen from Tables 1 and 3, the differences between the effective mobilities of the analytes change with pH.

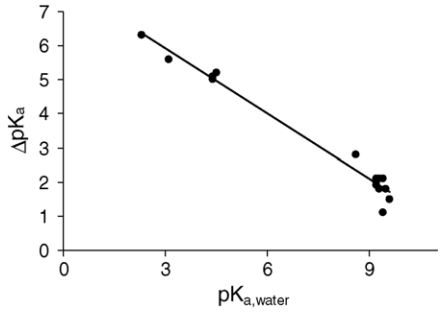

Fig. 5. Difference between pK_a values of analytes in methanol and water (ΔpK_a) as a function of pK_a in water. Linear correlation coefficient is 0.98.

In order to confirm that the prediction based on known mobilities (and pK_a values) is correct, we carried out the NACE separation of a model mixture of β-blockers.

The mixture of β-blockers consisted of ten analytes: OXP, ALP, PRO, PIN, MET, BOP, SOT, PRA, NAD and LAB. Separation of these analytes in aqueous CZE is troublesome because their pK_a values in water are closely similar (see Table 4). In fact, for four of the analytes—PRO, MET, SOT and PRA—the pK_a values in water are overlapping. The same is true for NAD and LAB. The pK_a values in methanol (Table 4) are more promising: MET and PRA are separated from PRO and SOT. However, knowledge of pK_a values is not sufficient for a prediction that all ten analytes can be separated at a pH value close to the pK_a values of the analytes in methanol.

The values of actual mobilities of the β-blockers (Table 4) are different, however, Table 3 shows that at pH value 11.4 the mobilities of PRO and SOT, as well as those of MET and NAD, coincide. But it also shows that the differences in mobility between neighbouring analytes increase with decreasing pH so that the most suitable separation pH for most of the analytes should be 8.7. This pH was also the most desirable in terms of separation time, as the mobilities were increased at lower pH values. Interestingly enough, at pH 8.7 the effective mobilities of PRO and PIN coincide, while at pH 9.2 they are different. For these reasons, pH 8.9 (a pH value between 8.7 and 9.2) was selected.

As can be seen from Fig. 6, all analytes except PRO and PIN are baseline separated at pH 8.9. (Though not baseline separated, PRO and PIN are nevertheless clearly distinguished from each other.) The migration order of the analytes (Fig. 6) follows strictly the order of effective mobilities at pH 8.7 (Table 3). In the case of PRO and PIN, PRO migrates first, as expected from the mobility data for pH 9.2 (Table 3). The analysis time was less than 5 min.

As shown by the example, knowledge of the actual and effective mobilities of compounds in a given medium can help in selecting the experimental conditions and shorten the time required for optimisation.

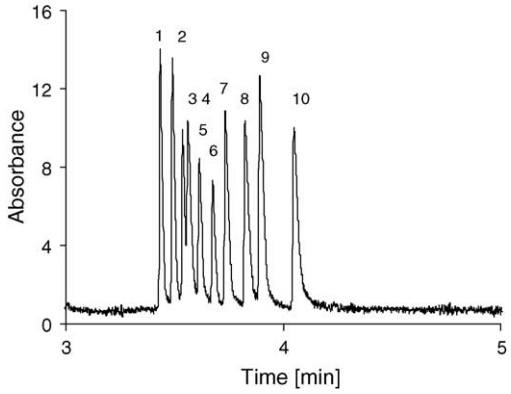

Fig. 6. Separation of β-blockers in methanol. BGE solution: 5 mM sodium acetate + 30 mM acetic acid in methanol (pH 8.9, UV detection 200 nm, voltage +30 kV, injection 25 mbar s, uncoated capillary 58.5 cm (with effective length 50 cm). DMSO was used as EOF marker. Concentration of analytes is 10 ppm. Peaks: (1) OXP, (2) ALP, (3) PRO, (4) PIN, (5) MET, (6) BOP, (7) SOT, (8) PRA, (9) NAD, (10) LAB. For identification of acronyms, see Table 3.

3.4. Analysis of urine samples

The NACE method that was developed was tested in the analysis of β-blockers in patient urine samples. The sample

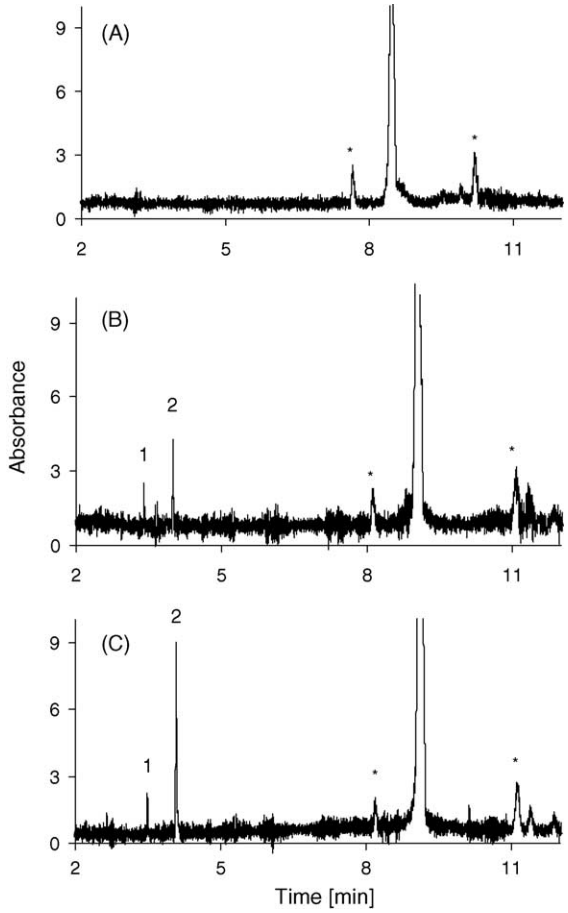

Fig. 7. Analysis of urine samples. A, blank urine sample, B, patient urine sample after ingestion of ATE, C, patient urine sample spiked with ATE. Peaks: (*) endogenous compounds, (1) a possible metabolite of ATE, (2) ATE. Experimental conditions as in Fig. 6.

collection and pre-treatment have been described in the experimental section. The urine samples were collected from four patients, each of whom was fed one tablet of the following pharmaceuticals: ALP, ATE, OXP and PRO. In all these samples, the parent compounds were detected in less than 5 min. Fig. 7 shows the analysis of a patient sample containing ATE. As can be seen, there are no disturbing or overlapping peaks present in the blank urine (Fig. 7A and B). Spiking with ATE standard confirmed the identity of the parent drug peak (Fig. 7C) but the metabolite could not be identified due to the lack of reference material. The results were further confirmed by analysing the samples by (GC–MS). GC–MS analysis proved the presence of the respective drugs and their metabolites in the samples. The NACE analysis was about three times as fast as the GC–MS analysis.

4. Conclusions

Actual mobilities and dissociation constants were determined for anionic and cationic pharmaceuticals in methanol. It was found that relative to aqueous solutions methanol increases the pK_a values of anionic analytes exhibiting acidic properties in water (pK_{water} below 7) by about 5–6 units. For cationic analytes with basic properties in water (pK_{water} above 7), the change in pK_a values was considerably less: the average pK_a shift for the cationic analytes was 1.9 units.

An experiment carried out with a model analyte mixture (β-blockers) demonstrated that knowledge of the dissociation constants and actual mobilities can be exploited in the optimisation of separations.

The method was also applied to the identification of β-blockers in patient urine samples. The parent compounds and their possible metabolites of interest were detected in less than 5 min.

Acknowledgement

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