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Determination of itraconazole and hydroxyitraconazole in human serum and plasma by micellar electrokinetic chromatography

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Abstract

The electrokinetic separation of the hydrophobic antimycotic drug itraconazole (ITC) and its major metabolite, hydroxyitraconazole (HITC), by a binary aqueous–organic solvent medium containing sodium dodecylsulfate, by microemulsion electrokinetic chromatography (MEEKC) and by micellar electrokinetic chromatography (MEKC) was studied. The results suggest that the first approach is difficult to apply and that there is no substantial difference between separations performed using MEEKC and MEKC modified with *n*-butanol. The simpler MEKC method is more than adequate and was thus employed for the analysis of ITC and HITC in human serum and plasma. Separation was achieved in plain fused-silica capillaries having a low-pH buffer (pH 2.2) with sodium dodecyl sulfate micelles and reversed polarity. The addition of 2-propanol and *n*-butanol enhanced analyte solubility and altered the selectivity of the separation by influencing the magnitude of the electrophoretic component in the separation mechanism. Under optimised conditions and using head-column field-amplified sample stacking, an internal standard, ITC and two forms of HITC could be separated in under 9 min, with detection limits less than 0.01 μg/mL. Analysis of samples from patients currently prescribed ITC revealed a different HITC peak area ratio to that of the standards, suggesting a stereoselective component of ITC metabolisation. Comparison of MEKC data with those of a HPLC method employed on a routine basis showed excellent agreement, indicating the potential of this approach for therapeutic drug monitoring of ITC.

Keywords: Microemulsion electrokinetic chromatography; Electrokinetic chromatography; Itraconazole; Hydroxyitraconazole

1. Introduction

Itraconazole (ITC; for chemical structure, see Fig. 1) is an orally administered antifungal drug (tradename Sporanox) recommended for the treatment of various fungi in both immunocompetent and immunocompromised adult patients [1]. It has both in vitro and in vivo activity against yeasts (*Candida* and *Cryptococcus*), a number of dimorphic fungi and several molds, including *Aspergillus* sp., and is one of the few orally administered drugs to target both *Candida* sp. and *Aspergillus* sp., the two most common fungal pathogens. It has relatively mild side-effects, the most common being dizziness, nausea, diarrhoea and vomiting, and is better tolerated than most antifungal agents [2].

ITC is highly lipophilic (estimated $\log P_{oct} = 6.2$

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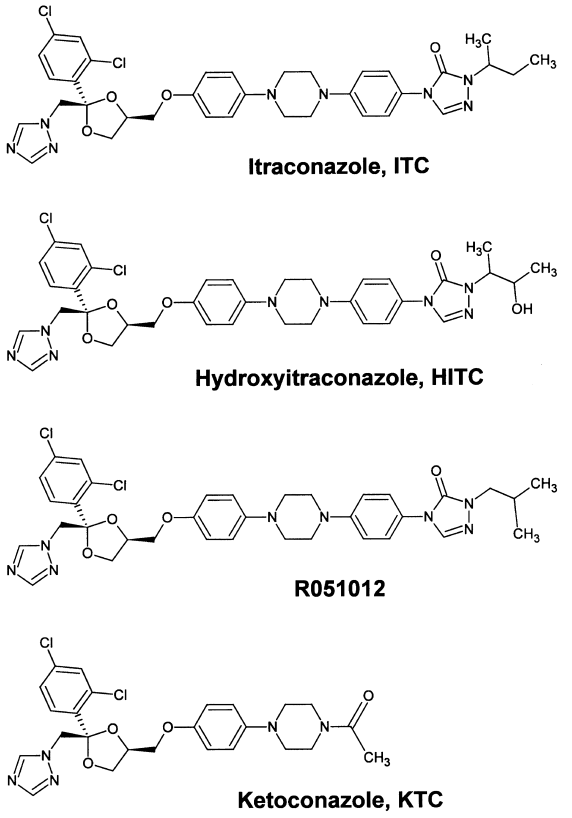

Fig. 1. Molecular structures of ITC, HITC, R051012 and KTC.

[3]) and virtually insoluble in water at neutral pH (<1 ng/mL). However, at pH<pK_a (estimated to be between pH 3.7 and 4.0) solubility increases markedly (~4 mg/mL) and it is highly absorbed in an acidic intragastric environment, with uptake maximised in the presence of food or an acidic beverage such as cola. It undergoes extensive metabolisation in the liver by the cytochrome P450 isoenzymes to give over 30 different metabolites, however most of these are inactive and constitute less than 5% of the parent compound. The exception is hydroxyitraconazole (HITC; for chemical structure, see Fig. 1), which is highly active and can reach levels two to three times higher than that of the parent compound. Significant intra-individual variations in plasma ITC levels have been reported [1,2] and the dosage must be individually adjusted to give an active trough plasma ITC concentration of 0.250 μg/mL, or an ‘‘active level’’ of combined ITC and HITC of 1.0 μg/mL [4].

Several high-performance liquid chromatography (HPLC) methods have been developed to quantitate both ITC and HITC in serum and plasma [2,4–9] and also in tissue [10]. In all cases, separation is achieved using a C_{18} column, a low-pH electrolyte (typically pH 2–3) and moderate levels of organic solvents (e.g. ~50–60% acetonitrile). To prevent analyte tailing due to silanolphillic interactions, a competing amine (typically triethylamine) is added to the mobile phase or a base-deactivated column is employed. Analysis times can be as low as 3 min with the use of highly selective MS detection [11,12], but when more general UV or fluorescence detection is employed, slightly longer analysis times of 10–20 min result. In all cases, sample pretreatment employing solid-phase extraction, liquid–liquid extraction or protein precipitation is employed to free the protein-bound drug and metabolites and to remove matrix components.

Capillary electrophoresis (CE) is a complementary technique to HPLC, often providing substantially different selectivity due to the different separation mechanism. It can also provide higher separation efficiencies, lower sample and reagent requirements and quicker analysis times, and as such has been used extensively to separate drugs and their metabolites [13]. To date, there have been three reports on the separation of ITC by CE, the first of which was by Zhang et al. [14] where ITC and a mixture of other hydrophobic positively chargeable drugs (dextromethorphan, methadone, ketoconazole, amiodarone and desethylamiodarone) were separated in various aqueous and mixed aqueous–organic media by capillary zone electrophoresis (CZE) and by using ion-pair interactions or, in some cases, micellar electrokinetic chromatography (MEKC). It was found that the use of organic solvents in conjunction with a neutral complexing agent could be effectively employed for their separation. Crego et al. [15] examined formate and phosphate buffers (pH 2.15 and 2.30, respectively) for the separation of seven antifungal compounds (ketoconazole, clotrimazole, terbinafine, verapamil, fluconazole, itraconazole and voriconazole) by CZE, with complete resolution achieved in both buffer systems, although sensitivity and analysis times were different. These

buffer systems were later used to analyse solid-phase extracts from in vitro liver microsomal studies of these drugs [16]. Metabolites for clotrimazole and verapamil were observed in the electrophoretic separation, but only the un-metabolised parent drug was observed for the remainder, although it is unclear whether this was due to inadequate metabolisation of the drugs, or an inability to adequately separate the metabolites. As yet, there has been no published method for the separation of ITC and its main metabolite, HITC, by any form of CE.

Described herein are (i) the separation of ITC and HITC by a binary aqueous–organic solvent medium containing sodium dodecylsulfate (SDS), by microemulsion electrokinetic chromatography (MEEKC) and by MEKC, and (ii) a validated MEKC method for the analysis of ITC and its main metabolite HITC in human serum and plasma samples by CE. A solvent-modified MEKC buffer is employed to resolve ITC, I.S. (R051012) and two diastereomeric forms of HITC in under 9 min. Sample pretreatment involves liquid–liquid extraction with diethyl ether. No preconcentration during the extraction step is required to reach the sensitivity for therapeutic drug monitoring.

2. Materials and methods

2.1. Drugs, chemicals and origin of samples

ITC, HITC, R051012 and ketoconazole (KTC; for chemical structure, see Fig. 1) were kindly obtained from Janssen Pharmaceuticals (Beerse, Belgium). SDS was from Bioprobe (Basel, Switzerland). HPLC-grade 2-propanol (2-PrOH), *n*-butanol (*n*-BuOH) and ethanol, and analytical-grade H_3PO_4 and $NaH_2PO_4 \cdot H_2O$ were obtained from Merck (Darmstadt, Germany) and acetonitrile (ACN) was purchased from Biosolve (Walkenswaard, Netherlands). HPLC-grade heptane and diethyl ether were obtained from Fluka (Buchs, Switzerland) and tetrahydrofuran was from Hänseler (Herisau, Switzerland). Patient plasma and serum samples were obtained from the departmental drug assay laboratory where they were received for therapeutic drug monitoring of ITC using HPLC. Bovine plasma was obtained from the local slaughter house and was used for the preparation of calibration and control samples.

2.2. Solutions

Phosphate buffer (25 m*M*) was prepared using 12.5 m*M* NaH_2PO_4 and titrated to pH 2.2 with H_3PO_4 to give approximately 25 m*M* total phosphate. MEEKC and MEKC electrolytes were prepared daily by weighing the appropriate amounts of reagents and then placing them in an ultrasound to create a homogeneous solution. Unless otherwise stated, the MEKC electrolyte contained 3.2% (w/w) SDS, 20.0% (w/w) 2-PrOH, 7.0% (w/w) *n*-BuOH and 69.8% (w/w) phosphate buffer. For convenience, the pH values given for all systems used in this paper are the values of an aqueous buffer having the same phosphate composition.

2.3. Capillary electrophoresis

For the initial experiments in aqueous and binary media, an air-cooled 270A-HT CE system (Applied Biosystems, San Jose, CA, USA) equipped with a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D. was employed. The capillary length was 58.7 cm (37.7 cm to the detector), the air temperature was set to 30 °C, the applied voltage was +30 kV and the detection wavelength was 263 nm. Injection was performed via vacuum (at 5 in.Hg; 1 in.Hg = 338.638 Pa) for 0.5 s. All other CE experiments were performed on a Beckman 5510 CE system (Beckman, Fullerton, USA) with a Beckman P/ACE station version 1.1 used for data acquisition. Fused-silica capillaries (Polymicro Technologies) of 27.0 cm (20.0 cm to detector), 50 μm I.D. and a voltage of −20 kV were used for all separations unless stated otherwise. Detection was performed using UV absorbance at 214 nm with the capillary temperature set at 25 °C. Injection was performed via pressure for 5 s for standards during method development and electrokinetically at −10 kV for 20 s for serum extracts unless otherwise noted. When analysing serum samples, a small amount of sample matrix (a 5 s hydrodynamic injection) was introduced into the capillary prior to electrokinetic injection to improve

the reproducibility and longevity of the capillary [17].

2.4. Standard solutions and extraction procedure

Stock solutions of 200–300 μg/mL of ITC, HITC, KTC and R051012 were prepared in methanol and diluted in water/background electrolyte for method development studies. For quantitation of ITC and HITC in serum and plasma extracts, standard solutions containing 0, 0.1, 0.5, 1.0, 2.0 and 4.0 μg/mL of ITC and HITC were prepared by dilution in bovine plasma, and stored at −20 °C until required. A solution of 2 μg/mL R051012 in 200 m*M* Na_2CO_3 was prepared and added to serum samples as an internal standard for migration time and peak area normalisation. Serum or plasma samples (100 μL), internal standard solution (50 μL) and diethyl ether (700 μL) were mixed for 10 min in a 1.5 mL plastic tube using a horizontal shaking apparatus. After centrifugation (9000 *g* for 180 s), 600 μL of the upper organic phase was transferred to a new 1.5 mL plastic tube and evaporated to dryness in a water bath at 35 °C under continuous airflow. The residue was reconstituted in 100 μL of 5% background electrolyte and transferred to a 0.5 mL plastic tube that could be placed in the autosampler. Quantification was performed using time corrected peak areas normalised to that of the internal standard (R051012).

2.5. HPLC routine assay

A HPLC method similar to that described by Cox et al. [10] was employed. The system consisted of a Model 501 HPLC pump (Waters Associated, Milford, MA, USA), a Model 712 WISP autosampler (Waters) and a Model LC304 fluorescence detector (Linear Instruments, Reno, NV, USA). Chromatography was performed at ambient temperature using a C_{18} LiChrochart guard column (Merck, Darmstadt, Germany) and a 250/8/4 mm Nucleosil 7 μm C_6H_5 column (Macherey–Nagel, Oensingen, Switzerland). The mobile phase was composed of water–acetonitrile (48:52, v/v) containing 2 ml of diethylamine per liter and its pH was adjusted to 3 with H_3PO_4. The flow-rate was 1.1 mL/min and detection occurred with excitation and emission wavelengths set to 260 and 366 nm, respectively. Chromatograms were registered and evaluated with a HP ChemStation Rev. A.06.01 (Agilent Technologies, Basel, Switzerland). The assay is based upon deproteination of the sample with acetonitrile. One hundred and twenty five μL of sample (patient plasma or serum) or a calibrator/control solution (125 μL bovine plasma fortified with appropriate aliquots of 100 μg/mL methanolic ITC and HITC solutions) and 250 μL of acetonitrile containing 2 μg/mL I.S. were vortex-mixed for about 15 s and centrifuged at 10 000 *g* for 8 min. Then, the clear supernatant was decanted and dried at 45 °C under a gentle stream of air. The residue was reconstituted in 200 μL mobile phase and 80 μL was injected. R051012 (Fig. 1) was employed as internal standard and quantitation of ITC and HITC was based upon multilevel internal calibration using peak areas (calibration range for both compounds: 0.1 to 4 μg/mL). Intra-day imprecision values ($n=6$) for both compounds and assessed for drug levels of 0.2, 1.0 and 3.5 μg/mL were determined to be <3.7, <3.4 and <1.2%, respectively. Corresponding inter-day values ($n=6$) were <7.1, <5.2 and <4.4%, respectively. Typical chromatograms are presented in Fig. 2.

3. Results and discussion

3.1. Selection of CE separation mode

Initial attempts to separate ITC and HITC by CE based on differences in their electrophoretic mobilities proved difficult, with no separation obtained in numerous aqueous and binary aqueous/non-aqueous electrolytes. In all cases, ITC and HITC migrated as a single peak, which is unsurprising given their similar molecular structures (Fig. 1). The addition of hydroxypropyl-β-cyclodextrin had little effect on the separation, although it did provide partial separation of enantiomeric forms of both ITC and HITC. The addition of low concentrations of SDS was found to provide some separation of ITC and HITC, indicating that a CE method exploiting the hydrophobic properties of the analytes may be more appropriate than one based on differences in electrophoretic mobilities (see below). Nevertheless, complete sepa-

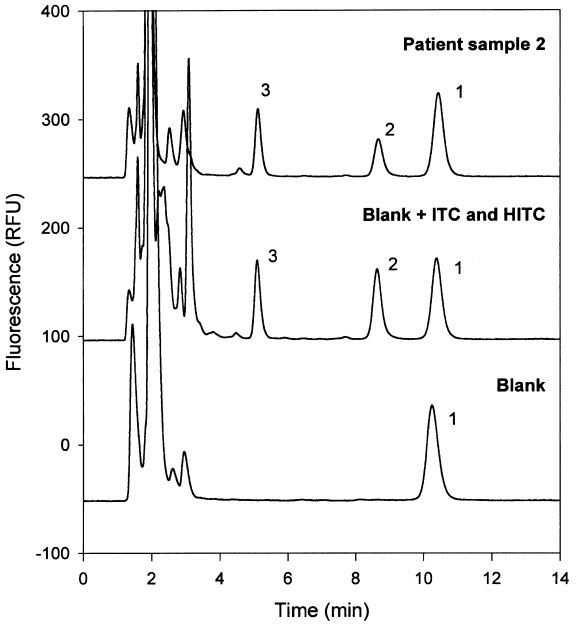

Fig. 2. Representative HPLC separations of ITC and HITC from a serum sample of an adult patient being treated with ITC and from blank and spiked blank plasma. Concentrations of ITC and HITC are: patient 2, 0.98 μg/mL ITC and 1.68 μg/mL HITC. Spiked blank plasma has 2.0 μg/mL ITC and HITC added. The concentration of the I.S. (R051012) is 4 μg/mL in all cases. Other conditions as described in the Materials and methods. Peaks: 1 = R051012, 2 = ITC, 3 = HITC.

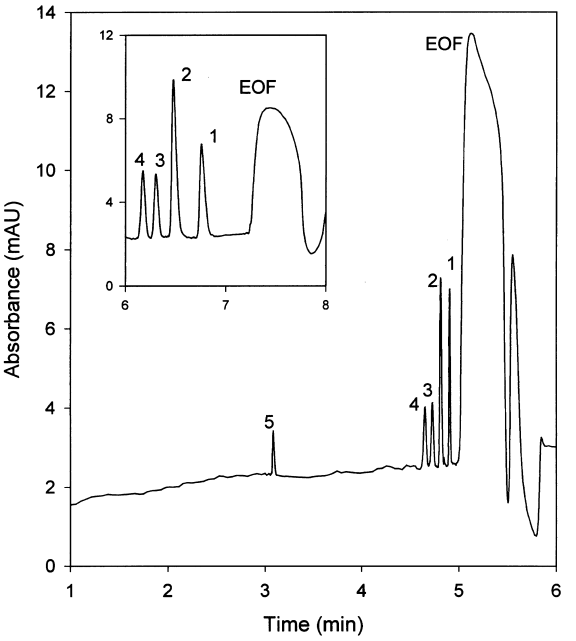

Fig. 3. Cationic separation of ITC, HITC, R051012 and KTC in a running buffer composed of 8 m*M* H_3PO_4, 12 m*M* NaH_2PO_4, 16 m*M* SDS and 30% acetonitrile using the air-cooled ABI 270A-HT instrument. The applied voltage was +30 kV (current 85 μA). Sample is 40–50 μg/mL of each compound prepared in methanol. The insert depicts the separation of a similar sample dissolved in acetonitrile that was analysed in a buffer composed of 10 m*M* H_3PO_4, 10 m*M* NaH_2PO_4, 16 m*M* SDS and 30% acetonitrile (voltage +30 kV, current 65 μA). Peaks: 1 = R051012, 2 = ITC, 3,4 = HITC, 5 = KTC. EOF, electroosmotic flow.

ration was obtained using a low-pH binary medium with acetonitrile containing SDS. The data presented in Fig. 3 illustrate the separation of ITC, HITC and potential I.S.s (KTC and R051012) in a 20 m*M* phosphate buffer containing 16 m*M* SDS and 30% acetonitrile (pH about 2.2; the pH value given here and those throughout this paper correspond to the value of an aqueous buffer having the same phosphate composition). In this configuration, normal polarity was applied, solutes were detected as cations and HITC was found to produce two peaks of comparable magnitude. The experiment was performed in an air-cooled capillary of 75 μm I.D. at a power level of 4.5 W/m and the electroosmotic mobility was determined to be about $2.2 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}. The net electrophoretic mobility of KTC was calculated to be $1.49 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} and KTC was found to be well separated from the other compounds, whereas the mobilities of HITC, ITC and R051012 ranged between $0.26 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} (firstly detected HITC peak) and $0.13 \cdot 10^{-8}$ m^2 V^{-1} s^{-1} (R051012 peak) and were thus detected rather close to the solvent peak. The optimised separation of HITC, ITC and R051012 was obtained by lowering the pH to 2.15 (see inset). As the resolution between the latter group of peaks was found to be insufficient and effective sample injection difficult (particularly without polarity switching), no further efforts were undertaken in pursuing this method for the therapeutic drug monitoring of ITC.

Separations in CE based on hydrophobicity are generally achieved by MEKC, however the separation of highly hydrophobic analytes is often difficult due to complete association with the micelles. Recently, MEEKC has emerged as a better method than MEKC for the separation of highly hydrophobic

analytes due to the higher solubilising power of lipophilic substances [18]. They are created by stabilising an immiscible lipophilic organic solvent (typically *n*-octane or *n*-heptane) in an aqueous solution by the use of charged surfactants (typically SDS) and short-chain alcohols (typically *n*-butanol), the latter functioning as a co-surfactant, reducing the surface tension between the two phases. When highly hydrophobic analytes ($\log P_{oct} > 5$) are to be analysed, the addition of 2-PrOH has been found to improve the separation by increasing the solubility of the compounds in the aqueous or non-microemulsion phase [19,20]. Because a high concentration of surfactant is required to stabilise the microemulsion (typically 3–6%, w/w) the field strengths must be kept reasonably low to avoid joule heating. This prolongs the already lengthy analysis time caused by a slow electroosmotic flow (EOF) due to the high ionic strength of the separation electrolyte. While numerous strategies have been developed to provide more rapid separations, the most promising is the use of low pH to eliminate the EOF, creating a system in which the microemulsion carries the analytes to the detector by association. In this case, analytes migrate in order of decreasing interaction with the emulsion [20,21].

To examine the ability of MEEKC to separate ITC, HITC and potential I.S.s (KTC and R051012), separations were performed in low- and high-pH MEEKC buffers containing 20.0% (w/w) of 2-PrOH. Additional components in the emulsion buffers were 3.0% (w/w) SDS, 6.6% (w/w) 1-BuOH, 0.8% (w/w) heptane, and 69.6% (w/w) of either 10 m*M* borate buffer (pH 9.2) or 25 m*M* phosphate buffer (pH 2.2). Upon examination of the separations at low and high pH, shown in Fig. 4, several features are immediately apparent. Firstly, two peaks were obtained for HITC in both the low-pH and high-pH MEEKC electrolytes, although resolution is poor in both cases. The two HITC peaks were also observed in the binary system containing SDS (Fig. 3). This was quite unexpected given that only one peak is obtained for the same analyte when separated by HPLC (Fig. 2). Split peaks have been observed in MEKC by Crabtree et al. [22] and more recently by Ràfols et al. [23] when the sample is dissolved in pure organic solvent (such as acetonitrile), which will disrupt the micelle structure. However, in the

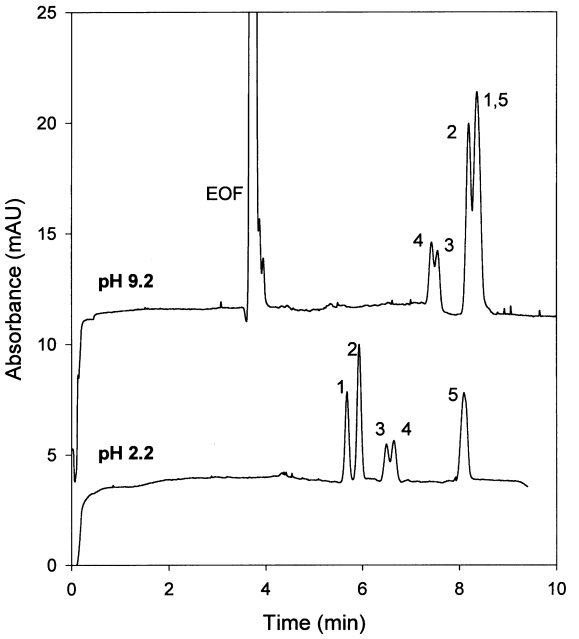

Fig. 4. Separation of ITC, HITC, R051012 and KTC by MEEKC buffered at pH 9.2 (top trace) and pH 2.2 (bottom trace). MEEKC electrolyte: 3.0% (w/w) SDS, 6.6% (w/w) 1-BuOH, 0.8% (w/w) heptane, 20.0% (w/w) 2-PrOH, and 69.6% (w/w) of either 10 m*M* borate buffer (pH 9.2) or 25 m*M* phosphate buffer (pH 2.2). Conditions: 27.0 cm (20.0 cm to detector)×50 μm I.D., $V = +20$ kV for high pH and -20 kV for low pH, $T = 25$ °C, $\lambda = 214$ nm. Sample is 30 μg/mL of each compound prepared in 100% MEEKC electrolyte and injected for 5 s by pressure. Peaks: 1 = R051012, 2 = ITC, 3,4 = HITC, 5 = KTC. EOF, electroosmotic flow.

MEEKC separations shown, the analytes were dissolved in 100% electrolyte, thus it is unlikely that this is the reason for the two peaks. Examination of the area ratio of the two peaks for patient samples (discussed later) showed a different ratio to that obtained using standards, suggesting that stereoselective metabolisation is occurring and that the peaks are different diastereoisomeric forms of HITC. This will be dealt with in a forthcoming publication.

The second apparent difference between the two separations is the separation selectivity, with KTC comigrating with R051012 in the high-pH buffer, but migrating well after HITC in the low-pH buffer. If hydrophobicity were the only mechanism responsible for migration of the analytes, then the selectivity at low pH would be the exact reverse of that at high pH. This is clearly not the case, with KTC changing

from last in the low-pH buffer to equal last in the high-pH buffer. Since the analytes are charged at low pH (pK_{a1} estimated to be between 3.5 and 4 for all compounds) the separation mechanism will be a combination of hydrophobic interaction with the microemulsion core, electrostatic interaction with the negatively charged surface and an electrophoretic mobility component in the opposite direction to the migration of the emulsion. Given that the mobility of KTC ($1.49 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}) is much higher than those of R051012, ITC and HITC (all approximately $0.2 \cdot 10^{-8}$ m^2 V^{-1} s^{-1}), the migration of KTC after HITC in the low-pH buffer is most likely due to its high electrophoretic mobility when not interacting with the microemulsion phase. At a high pH, this component is lost in the separation mechanism, and separation is based solely on differences in hydrophobicity.

Finally, comparison of both separations indicates that the low-pH buffer is slightly better for the separation of these compounds. While the resolution between the two HITC peaks and the overall analysis times are similar, the separation of the remainder of the analytes is superior at low pH, and was selected for further optimisation.

3.2. Optimisation of the separation conditions

To improve the separation of the selected analytes, the effect of varying the composition of the low-pH MEEKC electrolyte on the separation was examined. Due to the high currents generated by the addition of relatively high concentrations of SDS (typically 3%, w/w), the concentration and pH of the phosphate buffer were kept constant at 25 m*M* at pH 2.20. Studies undertaken by Pedersen-Bjergaard et al. [24,25] have demonstrated that variation of the type and concentration of the lipophilic solvent has only a minor influence on the migration of neutral analytes in MEEKC. Indeed, the presence of the lipophilic solvent has been shown to be unnecessary in the separation of vitamins [25] and only provides superior efficiency and analysis times for the separation of hydrophobic polymer additives [19]. However, removal of the co-surfactant resulted in significant changes in selectivity, a loss in resolution and prolonged analysis times, suggesting that it is the function of the co-surfactant rather than the organic solvent that is responsible for the separation selectivity. The need for the lipophilic solvent and co-solvent for the separation of ITC, HITC, KTC and R051012 was evaluated by a series of three buffers: MEEKC electrolyte (top trace in Fig. 5), MEEKC electrolyte minus the lipophilic solvent heptane (middle trace), and MEEKC electrolyte minus the lipophilic solvent and the co-solvent *n*-BuOH (bottom trace). It can be seen quite clearly from the separations that there is no significant difference in the separation when the oil-phase heptane is removed from the MEEKC buffer, with only a slight lengthening of analysis time resulting. Removal of the co-surfactant, however, has a significant effect on the separation, with resolution significantly decreased and the position of KTC changed relative to that of the other analytes, so that it now migrates between R051012 and ITC. This change is again related to the electrophoretic component in the separation

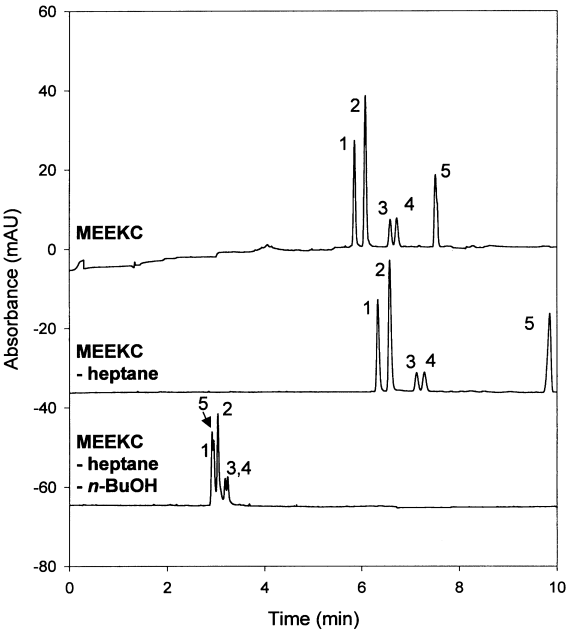

Fig. 5. Separation in MEEKC (top trace) electrolyte, MEEKC electrolyte minus the lipophilic solvent heptane (middle trace), and MEEKC electrolyte minus lipophilic solvent and co-solvent *n*-BuOH (bottom trace). MEEKC electrolyte contains 3.0% (w/w) SDS, 20.0% (w/w) 2-PrOH, 6.6% (w/w) *n*-BuOH, 0.8% (w/w) heptane and 69.6% (w/w) 25 m*M* phosphate buffer, pH 2.2. When each buffer component is removed, it is replaced with additional phosphate buffer. All other conditions as in Fig. 4.

mechanism of KTC and the relative time of association with the micelle/emulsion phase, with removal of *n*-BuOH decreasing the solubility of the analytes in the non-micellar/emulsion phase and promoting micelle/emulsion interaction. These results suggest that there is little benefit in the addition of the lipophilic organic solvent, and that the simpler MEKC method is more than adequate.

The presence of *n*-BuOH was found to be critical to provide sufficient separation between the analytes. However, there may be other organic solvents that may provide a similar or better enhancement in resolution. Hansen et al. [25] examined the effect of eight different organic solvents on separations in MEKC and MEEKC. Significant changes in migration were observed with different solvents in both systems and were related to the relative hydrophobicity of the solvents and their ability to penetrate the micelles/emulsion. Fig. 6 shows separations of ITC, HITC, KTC and R051012 in MEKC systems with the addition of 6.6% (w/w) ethanol, tetrahydrofuran or *n*-BuOH, solvents demonstrated to have different effects on MEKC separations, and without co-surfactant for comparison. In all cases, 20% (w/w) 2-PrOH and 3.2% (w/w) SDS were present in the background electrolyte. Again, selectivity changes were apparent, induced by the movement of KTC, similar to what has already been previously discussed. The progression from no co-surfactant, through ethanol, tetrahydrofuran and finally *n*-BuOH illustrates nicely the change in position of KTC, and follows the log *P* values for these solvents (−0.16, 0.46 and 0.84, respectively [25]). This supports the notion that this movement is due to an increased electrophoretic component in the separation mechanism, as KTC spends more time in the non-micellar phase with the addition of the more lipophilic co-surfactant. It could be anticipated that the addition of a highly lipophilic solvent, such as the oil-phase added to the microemulsion, would have an even more pronounced effect on the separation. However, practically the concentration of the oil phase that can be added to the micelle electrolyte is limited to approximately 1% (w/w), which is much lower than that added here. In terms of quality, the separation using *n*-BuOH is superior due to the better detector response and higher separation efficiency of the critical pair of HITC peaks.

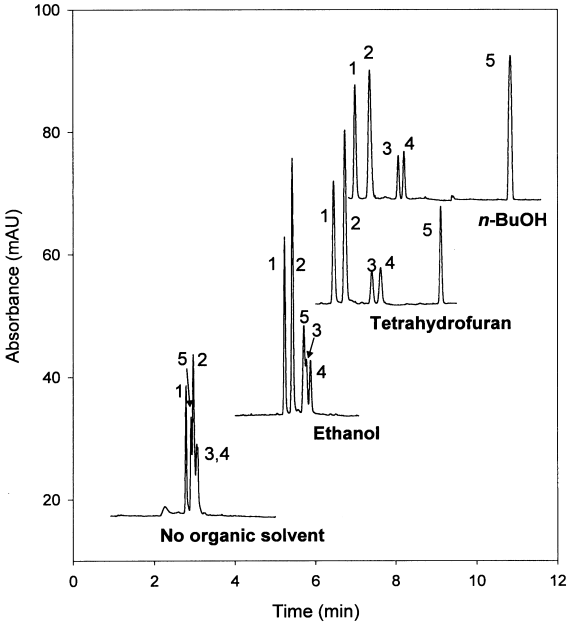

Fig. 6. Influence of various organic solvents on the separation of ITC, HITC, R051012 and KTC with various organic solvents added to the MEKC electrolyte. Addition of 6.6% (w/w) of no solvent, ethanol, tetrahydrofuran, and *n*-BuOH. All other conditions are as in Fig. 5 (center trace). The time axis applies to all electropherograms.

Having determined that the best electrolyte contains 2-PrOH, *n*-BuOH and SDS, studies were undertaken to examine the optimal concentration of each added component. In all cases, two parameters were held constant [at 20% (w/w) 2-PrOH, 8.0% (w/w) *n*-BuOH and 3.0% (w/w) SDS], while the amount of the other parameter was varied. Fig. 7A shows the impact of varying the *n*-BuOH concentration, while Fig. 7B shows that for 2-PrOH. It can be seen in both cases that the higher concentrations of both *n*-BuOH and 2-PrOH provided the best separations, with quality judged by the height, efficiency and resolution of the two HITC peaks. Additions of solvent above 8% (w/w) *n*-BuOH and 20% (w/w) 2-PrOH provided an unstable current and could not be adequately evaluated. Separations at the maximum level of both alcohols produced a system that was stable for approximately an hour, before destabilising, with current drifts and a reduction in separation efficiency resulting. A reduction in *n*-BuOH concentration to 7.0% (w/w) was found to

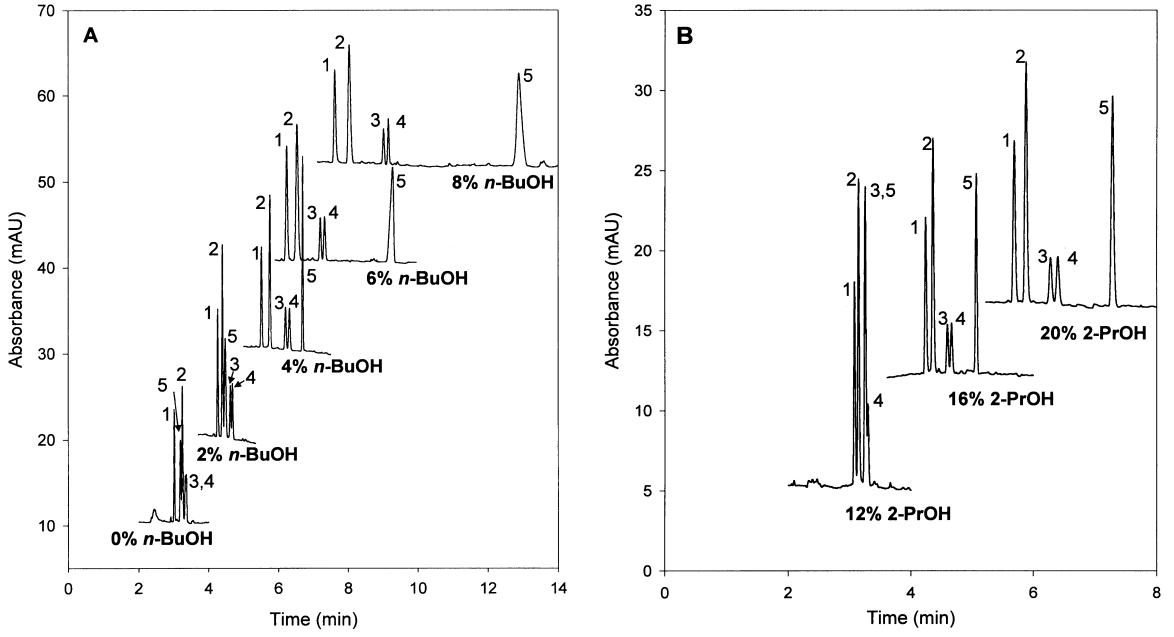

Fig. 7. (A) Influence of varying the concentration of *n*-BuOH on the separation of ITC, HITC, R051012 and KTC in MEKC. The concentration of 2-PrOH was kept constant at 20.0% (w/w) and the concentration of SDS was kept at 3.0% (w/w). (B) Influence of varying the concentration of 2-PrOH on the separation of the same four compounds in MEKC. The concentration of *n*-BuOH was kept constant at 8.0% (w/w) and the concentration of SDS was kept at 3.0% (w/w). Removed *n*-BuOH and 2-PrOH was replaced with the same addition of phosphate buffer. Other conditions as in Fig. 5 (center trace). The time axis applies to all electropherograms.

provide a very stable system with no current problems while still providing adequate separation of the analytes in question. Variation of the SDS concentration from 1.6 to 4.0% (w/w) (data not shown) provided little change in the selectivity, although it did produce significant changes in current, analysis time and detector response. Concentrations higher than 3.2% (w/w) provided rapid separations with a high current (~120 μA) and better sensitivity when using electrokinetic injection [the sample was prepared in 0.1% (w/w) SDS, 0.2% (w/w) 2-PrOH and 0.1% (w/w) *n*-BuOH]. Concentrations of SDS lower than 3.2% (w/w) produced longer analysis times (a factor of three times longer going from 3.2 to 1.6%, w/w) and the sensitivity became worse due to a combination of a loss in efficiency and less analytes injected via electrokinetic injection (similar matrix to above). A concentration of 3.2% (w/w) was found to be the best compromise, providing moderate currents (75 μA), reasonable analysis times and adequate sensitivity.

The optimum buffer conditions providing the best separation were found to be 3.2% (w/w) SDS, 20% (w/w) 2-PrOH, 7.0% (w/w) *n*-BuOH and 69.8% (w/w) 25 m*M* phosphate buffer at a pH of 2.20. The I.S. selected was R051012 as the movement of this compound was not as susceptible to changes in electrolyte conditions as was KTC. Furthermore, when applied to human serum samples, a series of peaks in the region of migration of KTC was observed (see below), reducing its ability to function as a suitable I.S. At the applied voltage of −20 kV (current 75 μA), the power level was calculated to be 5.56 W/m.

3.3. MEKC determination of ITC and HITC in serum and plasma

3.3.1. Sensitivity optimisation

Human serum and plasma samples are routinely analysed for ITC and HITC at concentration ranges between 0.1 and 4.0 μg/mL. These levels could only be reached by preparing the sample in diluted background electrolyte and using head-column field-

amplified sample stacking as described by Zhang and Thormann [17]. Fig. 8A shows the variation in the peak area of standards of ITC, HITC and R051012 (I.S.) as the amount of background electrolyte in the sample is decreased from 33.3% (v/v) to 1.0% (v/v) when using a −10 kV, 10 s injection. As expected, a lower concentration of background electrolyte in the sample produces larger peaks due to an increase in the field strength over the sample during electrokinetic injection. This trend continued down to 0.1% (v/v) of background electrolyte (BGE) in the sample (not shown), which provided the highest amount of analyte injected. This was surprising given this equates to ~0.003% (w/v) (or 0.1 m*M*) SDS present in the sample, which is much lower than the cmc of SDS in water (6–8 m*M*). While the addition of 2-PrOH and *n*-BuOH is known to reduce the cmc of SDS, the effective concentration in the sample would be 0.2% (w/v) 2-PrOH and 0.007% (w/v) *n*-BuOH, which is lower than has typically been examined. Work is clearly needed to examine the ability of these solvents to lower the cmc to enable more efficient stacking when using MEKC systems. Lower concentrations of SDS were not examined as it was impractical when applied to serum samples, as will be discussed below.

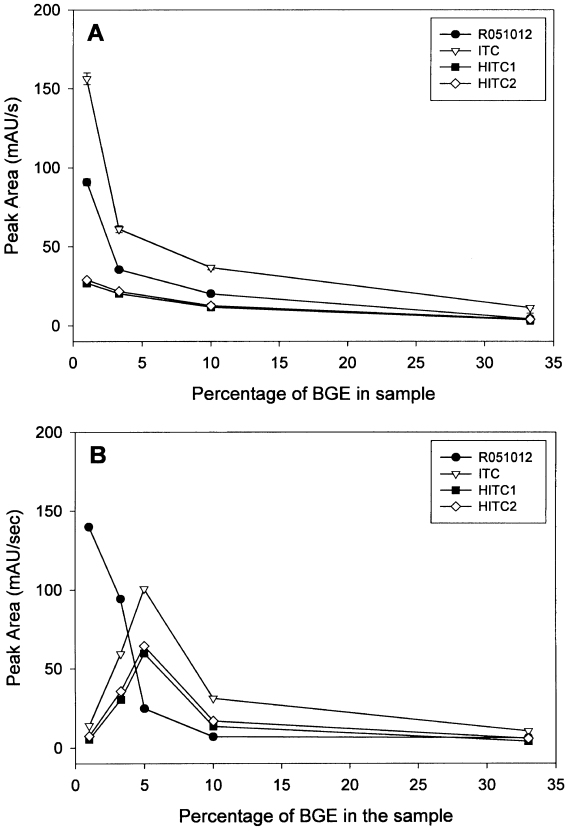

Fig. 8. Influence of sample composition on peak area response for selected analytes in (A) standard solutions and (B) reconstituted liquid–liquid extractions of spiked bovine plasma samples. Background electrolyte: 3.2% (w/w) SDS, 20.0% (w/w) 2-PrOH, 7.0% (w/w) *n*-BuOH and 69.8% (w/w) 25 m*M* phosphate buffer, pH 2.2. Injection: −10 kV for 10 s after a 5 s injection of sample matrix. Samples contained 3 μg/mL of ITC and HITC in 1–33% background electrolyte or 3 μg/mL ITC and HITC in bovine plasma and treated by liquid–liquid extraction as described in Materials and methods. I.S. (R051012) at 3 μg/mL was added during reconstitution of the dried extract with background electrolyte solution.

In contrast to the results obtained using standards, when applied to serum samples, a minimum amount of SDS was required to resolubilise material from the liquid–liquid extraction procedure. This is illustrated in Fig. 8B, where the change in peak area of ITC and HITC is shown as a function of background electrolyte in which the sample is reconstituted. R051012 was added as an I.S. with the reconstitution solution, with the peak area response for this analyte increasing similar to that observed in the standards. However, for ITC and HITC, which were extracted from spiked serum, the peak area decreased when less than 5% (v/v) background electrolyte was present in the sample, indicating that most of the material was not resolubilised. This is not surprising given the extra material observed in the separation of serum extracts which will reduce the amount of SDS available for resolubilisation of the drugs. Therefore, serum extracts were reconstituted in 5% (v/v) background electrolyte prior to electrokinetic injection.

Optimisation of the electrokinetic injection time revealed that the peak height showed an almost linear response ($r^2 = 0.97$) as the injection time was increased from 5 to 40 s, with only a minor loss in resolution between the two HITC peaks (R_s changed from 1.41 to 1.28). Injection times longer than 40 s resulted in substantial broadening of the peaks and a significant loss of resolution. However, satisfactory

sensitivity could be achieved by using a 20 s injection, and was used for all further experiments.

3.3.2. Calibration data, detection limits, repeatability and accuracy

Analysis of blank bovine plasma fortified with 0.1 to 4 μg/ml ITC and HITC (see Section 2.4) provided linear calibrations graphs with r^2 values between 0.9999 and 0.9967 for ITC (mean 0.9986) and between 0.9965 and 0.9995 for HITC (mean 0.9979). For ITC, mean values (RSD) for slopes and *y*-intercepts were determined to be 1.325 (5.15%, $n=6$) and −0.022 (212%, $n=6$), respectively. Corresponding values for HITC were 1.234 (6.96%, $n=6$) and −0.016 (120%, $n=6$), respectively. Analysis of blank plasma spiked with 0.1 μg/mL of ITC and HITC established the detection limit ($S/N=3$) as 0.005 and 0.010 μg/mL, while the limits of quantitation (LOQs) ($S/N=10$) were 0.015 and 0.033 μg/mL, respectively. Further sensitivity could be obtained by increasing the injection time (from 20 to 40 s with minimal loss in resolution) or by performing a preconcentration during the liquid–liquid extraction procedure. In this work, we used 100 μL of plasma for each extraction and reconstituted in a final volume of 100 μL of 5% (v/v) background electrolyte. It would be relatively simple to obtain a five- to 10-fold increase in sensitivity by combining the above approaches, which would easily provide detection limits less than 10 ng/mL. This level is suitable for most pharmacokinetic studies, and approaches the 4–1000 ng/mL range obtained by HPLC–MS used recently by Carrier and Parent [12], although at the expense of selectivity and sample volume (20 μL of serum was used for HPLC–MS).

The repeatability of the CE method was evaluated by analysing a series of bovine plasma samples spiked with ITC and HITC prepared independently to that of the calibration graph. Intra-day ($n=6$) and inter-day ($n=6$) variation of normalised migration times was less than 1% RSD for all peaks in both cases. Normalised peak heights and areas were less than 3% RSD for samples spiked with 4.00 μg/mL ITC and HITC, and less than 8% for samples spiked with 0.35 μg/mL ITC and HITC. Accuracy and precision data for these spiked samples are shown in Table 1, where all precision data are within about 6% RSD, while accuracy values are less than 5%.

3.3.3. Application to patient samples

Sample pretreatment of serum samples for HPLC analysis have involved either liquid–liquid extraction using diethyl ether [4,8] or heptane–isoamyl alcohol (90:10) [6] or by performing protein precipitation by the addition of organic solvent [10,26]. Protein precipitation was found to be unsuitable as it does not remove the inorganic salts present in the sample and enrichment using electrokinetic injection does not occur. Liquid–liquid extraction using both diethyl ether and heptane–isoamyl alcohol (90:10) provided similar recoveries (between 70 and 80% of

Table 1
Repeatability data for extraction of ITC and HITC from spiked bovine serum

Duration	Sample	ITC				HITC			
		Added (μg/mL)	Found (μg/mL)	RSD (%)	Accuracy (%)	Added (μg/mL)	Found (μg/mL)	RSD (%)	Accuracy (%)
Intra-day ($n=6$)	C1	4.00	3.87±0.10	2.64	−3.25	4.00	4.02±0.09	2.08	0.25
	C5	0.35	0.36±0.01	3.00	2.86	0.35	0.35±0.01	3.04	0
Inter-day ($n=6$)	C1	4.00	3.81±0.22	5.69	−4.75	4.00	3.99±0.14	3.53	−0.25
	C2	3.50	3.58±0.12	3.36	2.29	3.50	3.56±0.13	3.65	4.28
	C3	2.40	2.36±0.15	6.35	−1.67	2.40	2.39±0.12	5.04	−0.42
	C4	1.00	1.03±0.04	3.88	3.00	1.00	1.04±0.06	6.05	4.00
	C5	0.35	0.35±0.01	4.26	0	0.35	0.33±0.14	3.53	−5.71
	C6	0.20	0.20±0.01	6.03	0	0.20	0.20±0.01	1.87	0

ITC, HITC and R051012 recovered), however extractions performed using heptane–isoamyl alcohol were found to provide intermittent current problems with prolonged extraction times (>5 min). Although the exact cause is unknown, we believe it may be due to the formation of small microemulsion droplets of the heptane–isoamyl alcohol during extraction, which transferred significant matrix components, producing instabilities during separation. Extractions with diethyl ether were found to provide few, if any, disruptions to the current during separation.

Representative electropherograms obtained with samples from patients being treated with ITC and fortified bovine plasma are shown in Fig. 9. Analysis of the separations shows the presence of additional peaks between 3 and 5 min, which are well separated from the target analytes. Separations performed using blank serum or plasma from individuals not being treated with ITC showed no additional peaks, indicating that there were no interferences for this method. Further examination of the separations reveals that there is considerable disparity between the areas and heights of the two HITC peaks, particularly when compared with blank plasma or serum spiked with standards. The area ratio of the first to second HITC peaks was typically between 1.5 and 3 (Fig. 9), in contrast to the ratio being between 0.9 and 1.0 for standards. Analysing 96 patient samples produced a mean±SD peak area ratio for the first to second HITC peaks of 1.74±0.49 (median, 1.68; range, 0.71–3.54) with a ratio <1 in two samples only. This strongly suggests stereoselective metabolisation (either formation or elimination or both) of ITC and/or HITC and that the two peaks for HITC are diastereoisomers of HITC. Confirmation of this hypothesis will be discussed in a forthcoming publication.

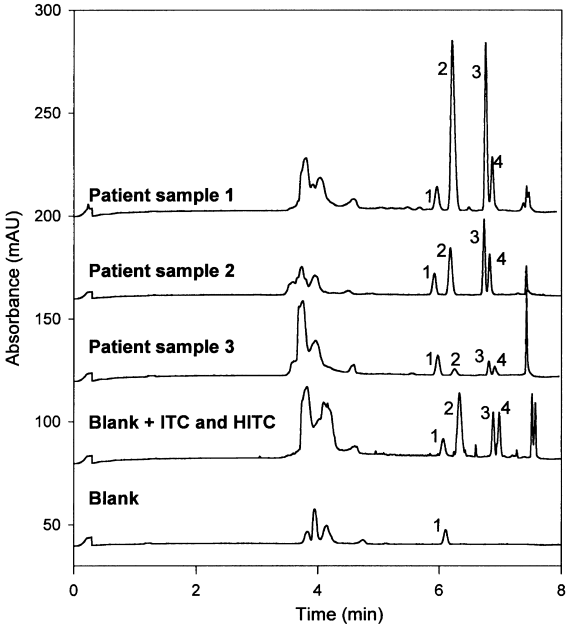

Fig. 9. Representative separations of ITC and HITC extracted from samples of adult patients being treated with ITC, from fortified blank plasma and from blank plasma. Concentrations of ITC and HITC are: patient 1, 4.88 μg/mL ITC, 3.89 μg/mL HITC with the first HITC peak being 2.76 times larger than the second; patient 2, 0.98 μg/mL ITC, 1.68 μg/mL HITC with the first HITC peak being 1.73 times larger than the second; patient 3, 0.24 μg/mL ITC, 0.51 μg/mL HITC with the first HITC peak being 1.31 times larger than the second. Spiked blank bovine plasma, 1.5 μg/mL ITC, 1.5 μg/mL HITC with the first peak being 0.95 times larger than the second. Sample extraction performed as described in Section 2.4 and injected at −10 kV for 20 s after a 5 s hydrodynamic injection of sample matrix. The applied voltage was −20 kV (current 75 μA). Other conditions as in Fig. 8B.

Comparison with an existing HPLC method employed for the routine analysis of ITC and HITC in patient serum samples establishes excellent agreement between the two methods (Fig. 10) for both ITC and HITC. Plotting the difference of the two values against the mean, shown in Fig. 10C and D for ITC and HITC, respectively, again shows excellent consistency between the two methods. The mean of the differences was negligible (less than −0.05 for both ITC and HITC), but the standard deviation was slightly lower for ITC than HITC (0.18 and 0.33, respectively). While this is relatively insignificant, we believe that it can be simply explained by the fact that two peaks are obtained for HITC, and thus error is increased due to integration and quantification based on two peaks and not one.

4. Conclusions

A low-pH, reversed-polarity MEKC method for the separation of ITC and HITC in human serum and plasma samples was developed. There was no substantial difference between separations performed using MEEKC and MEKC modified with *n*-BuOH.

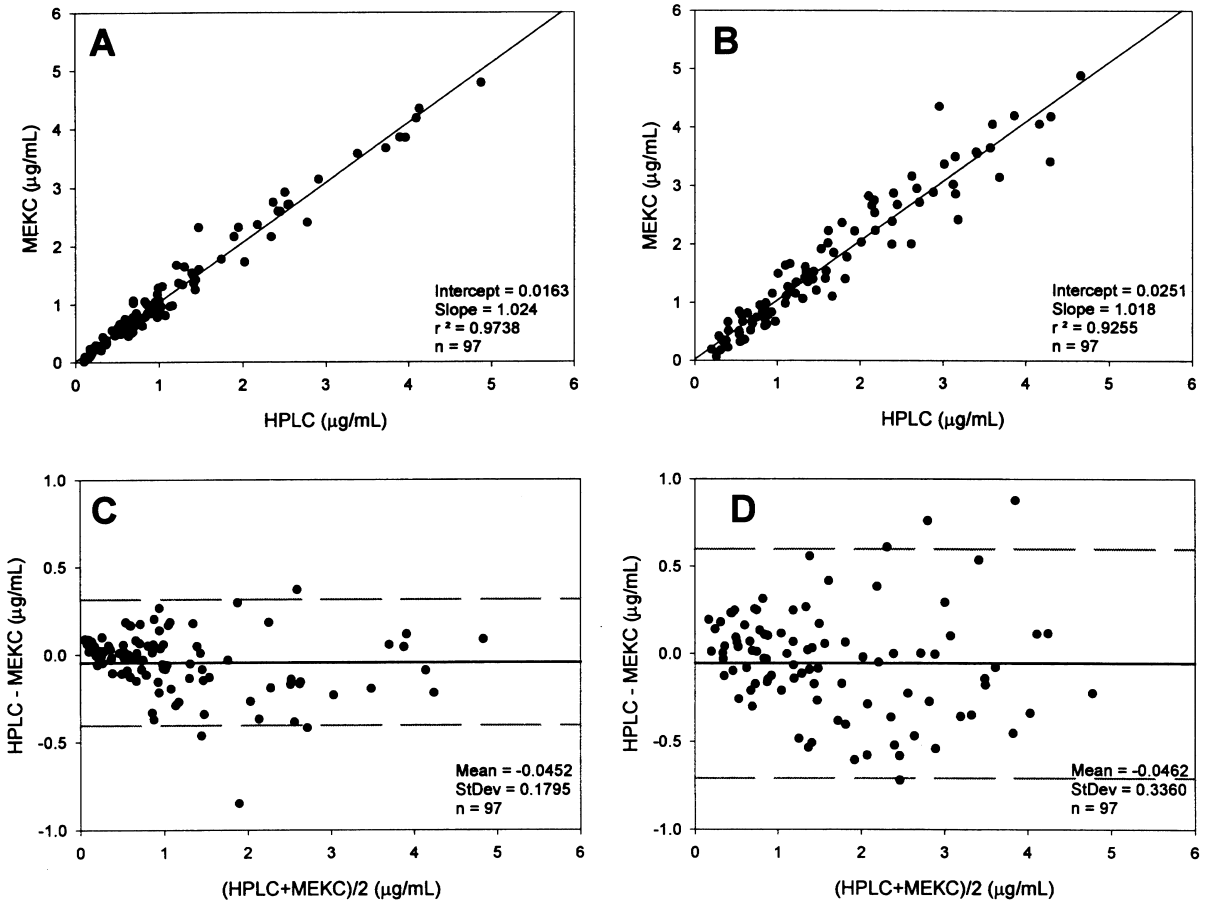

Fig. 10. Comparison of ITC (left) and HITC (right) concentrations in patient's samples determined by CE and HPLC. Correlation plots (A and B) and difference vs. mean of each data pair (C and D) for ITC and HITC, respectively.

Variation of the type and concentration of organic solvent produced pronounced effects on the separation selectivity, attributed to a change in the relative contribution of electrophoretic migration and micellar association to the overall migration of the analytes. Optimisation of the electrolyte components enabled R051012 (internal standard), ITC, and two different forms of HITC to be separated in under 9 min. Comparison of the peak area ratios of the two forms of HITC showed different values between patient samples and standards, suggesting that there is a stereoselective component in the metabolisation of ITC. Agreement between HPLC and MEKC for the analysis of 97 patient samples was excellent, indicating the suitability of this method for therapeutic drug monitoring of ITC.

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