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Migration behaviour and separation of tramadol metabolites and diastereomeric separation of tramadol glucuronides by capillary electrophoresis

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Abstract

Capillary electrophoresis with UV detection was used to separate tramadol (TR), a centrally acting analgesic, and its five phase I (M1, M2, M3, M4, M5) and three phase II metabolites (glucuronides of M1, M4 and M5). Several factors were evaluated in optimisation of the separation: pH and composition of the background electrolyte and the influence of a micellar modifier, sodium dodecyl sulfate. Baseline separation of TR and all the analytes was obtained with use of 65 mM tetraborate electrolyte solution at pH 10.65. The lowest concentrations of the analytes that could be detected were below 1 μM for the *O*-methylated, below 2 μM for the phenolic and ca. 7 μM for the glucuronide metabolites. The suitability of the method for screening of real samples was tested with an authentic urine sample collected after a single oral dose (50 mg) of TR. After purification and five-fold concentration of the sample (solid-phase extraction with Oasis MCX cartridges), the parent drug TR and its metabolites M1, M1G, M5 and M5G were easily detected, in comparison with standards, in an interference-free area of the electropherogram. Diastereomeric separation of TR glucuronides in in vitro samples was achieved with 10 mM ammonium acetate–100 mM formic acid electrolyte solution at pH 2.75 and with basic micellar 25 mM tetraborate–70 mM SDS electrolyte solution at pH 10.45. Both separations showed that glucuronidation in vitro produces glucuronide diastereomers in different amounts. The authentic TR urine sample was also analysed by micellar method, but unambiguous identification of the glucuronide diastereomers was not achieved owing to many interferences.
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1. Introduction

Tramadol hydrochloride, 1*R**,2R*-2[(dimethylamino) methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride (TR), is a synthetic, centrally acting analgesic that is widely used for the treatment of moderate to severe pain [1]. Experimental data suggest that the analgesic effect of TR is partly mediated via opioid receptors (mainly μ-receptors), but nonopioid mechanisms (noradrenergic and serotonergic) are also involved [2]. TR is used in therapy as a racemate: both enantiomers contribute to the analgesic action [3]. Although TR possesses opioid agonist properties, the risk for dependence or abuse [4] as well as the incidence of opioid type side-effects are reported to be low [5,6].

TR is extensively metabolised by *N*- and *O*-demethylation [7–9]. In recent studies, 24 metabolites of TR were identified in rat and dog urines [8] and 23 metabolites in human urine [9]. Only one of the metabolites, *O*-demethylated TR (M1), is pharmacologically active [1,7]. The three phenolic phase I metabolites of TR (M1, M4 and M5) can further conjugate with glucuronic acid to form the corresponding glucuronides (M1G, M4G and M5G) [7–9]. Since each phase I metabolite comprises two enantiomers, conjugation results in the formation of a pair of diastereomeric glucuronides of M1, M4

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and M5. Besides these glucuronides, minor amounts of four other *O*-glucuronides of TR have recently been identified in rat, dog [8] and human [9] urine.

Analytical methods used in the determination of TR and its metabolites include thin-layer chromatography (TLC) [7], gas chromatography (GC) with nitrogen selective detection [10–12] or flame ionization detection [13], GC with mass spectrometric (MS) detection in electron ionization mode [14] and liquid chromatography (LC) with UV [15–18], fluorescence [16,19–24], electrochemical [25] or MS [26,27] detection. Additionally, capillary electrophoretic (CE) techniques, known for their high efficiency and good resolving power, have been successfully applied in enantioseparations of TR and its phase I metabolites [28–32,34]. The most common detection method in these studies has been UV absorption measurement [28–31], but also more structure-selective detections, laser-induced native fluorescence (LINF) [33,34] and MS [32], have been employed. Cationic isotachophoresis (ITP) with conductometric detection has been used to determine TR in pharmaceutical preparations [35].

So far, most of the published methods have been applied only to the determination of TR or its phase I metabolites, or both of these. As an exception, the three glucuronides of TR have been separated into their diastereomers by ion-pair chromatography with fluorescence detection [21]. Also, a CE method has been described for the simultaneous separation of TR, its phase I metabolites and the main phase II metabolite, the glucuronide of *O*-demethylated TR (M1G) [29]. Recently, Soetebeer et al. [34] separated the diastereomers of M1G by CE with LINF detection. To our knowledge, no report has been published of a simultaneous CE separation of TR, its phase I metabolites and all three glucuronides.

The aim of this work was to study the separation of TR and its main phase I and phase II metabolites by CE. Optimisation of the separation was pursued by investigating the factors that most affect the migration behaviour of the analytes and the selectivity of the separation, i.e. pH and composition and salt concentration of the background electrolyte. Finally, the suitability of the separation method to identify TR and its metabolites in an authentic urine sample was examined. During the course of the study we gained new information on the diastereoselective separation of TR glucuronides. Structures of the main metabolites of TR are shown in Fig. 1.

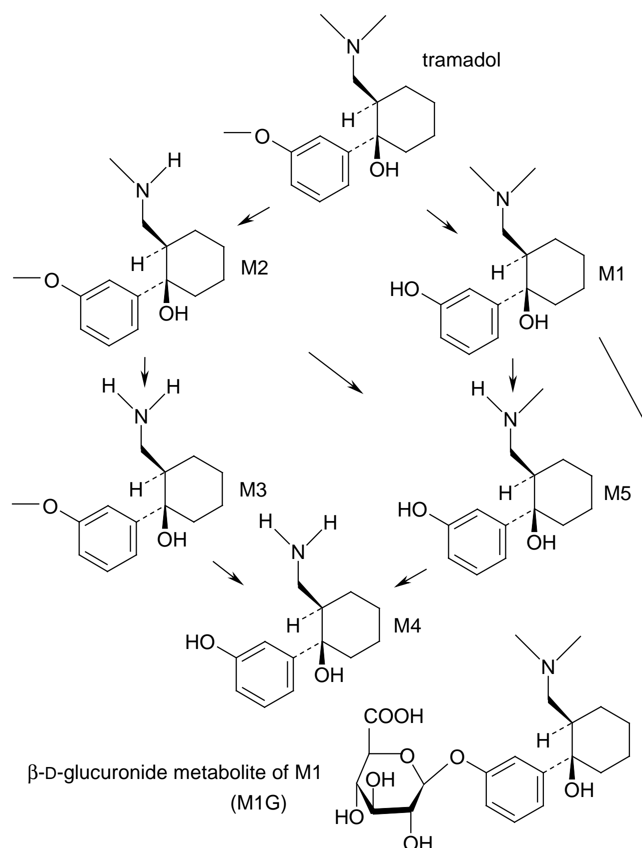

Fig. 1. Structures of tramadol and its main metabolites. Only one of the glucuronide metabolites (M1G) is presented. Corresponding glucuronides are formed from metabolites M4 and M5.

2. Experimental

2.1. Materials

TR and the phase I metabolites of TR—*O*-desmethyltramadol (M1), *N*-desmethyltramadol (M2), *N,N*-didesmethyltramadol (M3), *N,N,O*-tridesmethyltramadol (M4) and *N,O*-didesmethyltramadol (M5) hydrochloride salts—were obtained from the Department of Forensic Medicine, University of Helsinki. The glucuronides of TR (M1G, M4G and M5G) were enzymatically synthesised from the racemic phase I metabolites of TR (M1, M4 and M5) at the Department of Pharmacy, University of Helsinki by a modification of a method presented earlier [36]. All organic solvents and other chemicals were of analytical or chromatographic grade. Sodium hydroxide was purchased from Eka Nobel (Bohus, Sweden). Sodium dodecyl sulfate (SDS) was supplied by Serva (Heidelberg, Germany). (3-[Cyclohexylamino]-1-propanesulfonic acid), CAPS, was obtained from Sigma (St. Louis, MO, USA), while formic acid (of suprapur grade), ammonium acetate (CH_3COONH_4) and disodium tetraborate (borax, $Na_2B_4O_7{\cdot}10H_2O$) were from Merck (Darmstadt, Germany). Methanol and ammonium hydroxide (25%) were obtained from J.T. Baker (Deventer, The Netherlands) and hydrochloric acid (37%) was from Riedel-de Haën, (Seelze, Germany). Water was purified in a Milli-Q Plus water purification system (Millipore, Molsheim, France) (resistance 18 MΩ). Urine samples were obtained from a healthy volunteer. A control urine sample (0-U) was collected just before and the TR urine sample (TR-U) 12 h after TR (50 mg) intake. Urine samples were stored frozen at −20 °C until analysis.

2.2. Preparation of electrolyte and sample solutions

An electrolyte solution consisting of 10 mM ammonium acetate and 100 mM formic acid (pH 2.75) was prepared to study the migration behaviour of the analytes at low pH. CAPS and tetraborate electrolyte solutions were used to study the effect of pH on the mobility of the analytes at high pH. Six CAPS electrolytes, consisting of 150 mM CAPS and 24–86 mM of sodium hydroxide, were prepared by mixing CAPS stock solution (200 mM) with sodium hydroxide stock solution (1 M). The pH range of these solutions ranged from 9.8 to 10.58. Six tetraborate solutions, consisting of 40 mM disodium tetraborate and 50–105 mM sodium hydroxide, were prepared by mixing disodium tetraborate stock solution (60 mM) with sodium hydroxide stock solution (1 M). The pH range of these solutions ranged from 9.86 to 10.77. The effect of ionic concentration on the mobility of the analytes was studied in tetraborate electrolyte solutions prepared by mixing disodium tetraborate stock solution (75 mM) and sodium hydroxide stock solution (2 M). The five electrolytes that were prepared consisted of 30–65 mM of disodium tetraborate, but all had the same pH of 10.65 ± 0.02.

The effect of SDS on the mobility of the analytes was studied in tetraborate−SDS electrolytes. The solutions were prepared by mixing disodium tetraborate stock solution (60 mM), sodium hydroxide stock solution (1 M) and SDS stock solution (200 mM) to obtain electrolytes containing disodium tetraborate (25 mM), sodium hydroxide (60 mM) and SDS (0–90 mM), pH 10.45.

Before every buffer preparation the pH meter (WTW inolab pH Level 1) equipped with a pH combination electrode (WTW pH-Electrode SenTix 81, WTW, Weilheim, Germany) was calibrated with commercial buffer standards pH 7.000 and 4.005 or pH 7.000 and 10.012 (Radiometer, Copenhagen, Denmark). All solutions and water were passed through 0.45-μm membrane filters (Tracer) and degassed in an ultrasonic bath for 8 min before analysis.

2.2.1. Preparation of sample solutions

Stock solutions of TR and metabolites M1-M5 were prepared by dissolving the substances in methanol−water (50:50, v/v) to a concentration of 0.1 mg/ml. Stock solutions of the glucuronides (1 mg/ml) were prepared in water. The stock solutions were further diluted with water before use to obtain working solutions at appropriate concentrations. All sample solutions were stored at 4 °C.

2.2.2. Preparation of urine samples

0-U and TR-U samples were purified in the same way by solid-phase extraction (SPE) with Oasis MCX, 30 mg 1 ml cartridges (Waters, Milford, MA, USA). The cartridges were conditioned with 1 ml of methanol and 1 ml of water. The urine samples (1 ml) were acidified with 100 μl of hydrochloric acid (1 M), whereafter they were slowly loaded onto the cartridges and washed with 1 ml of hydrochloric acid (0.1 M) and 1 ml of methanol. Elution was performed with 1 ml of a solution that contained 0.25% of ammonium hydroxide in water–methanol (5:95 v/v). The extracts were evaporated to dryness under a gentle stream of nitrogen. Residues were reconstituted in 200 μl of water (a five-fold concentration of the original sample) and passed through 0.45 μm filters (Millex-HV, Millipore, Japan) before analysis.

2.3. CE instrumentation and conditions

The experiments were performed with a P/ACE System 2200 CE instrument (Beckman Coulter, Fullerton, CA, USA) controlled with a Beckman P/ACE Station Version 1.21. The UV detector was set at 200 nm. The capillary temperature was set at 25 °C and controlled through liquid cooling. Uncoated fused-silica capillaries [57 cm (50 cm to the detector) or 47 cm (40 cm to the detector) × 50 μm i.d. × 375 μm o.d.] were from Composite Metal Services (Hallow, UK). Capillaries with a total length of 87 cm (80 cm to the detector) × 75 μm i.d. × 375 μm o.d. were used in the method development. Samples were injected at the anodic end of the capillary at 0.5 p.s.i. (3447.38 Pa) for 3–10 s. For work at low pH, the sample injection was followed by an electrolyte injection of 1 s. Spectral information about the glucuronide diastereomers was gathered with an HP^{3D} CE system (Agilent Technologies, Waldbronn, Germany) equipped with a photodiode array detector. A Chemstation software package (HP) was used for instrument control, data acquisition and data handling. Separations were carried out in 75 μm i.d. capillaries (described above) with a total length of 64.5 cm (effective length 56 cm). The capillary was thermostated at 25 °C with air cooling. UV spectra were recorded in the wavelength region 190–300 nm.

New capillaries were conditioned before use by rinsing with 1 M NaOH for 25 min, with water for 5 min and with electrolyte for 10 min. Each day capillaries were flushed with 0.1 M NaOH for 10 min, with water for 5 min and with electrolyte for 5 min. In work at low pH, new capillaries were flushed before use only with the electrolyte, for 30 min, and at the beginning of each day all capillaries were flushed with the electrolyte for 10 min. Before analyses the capillary was flushed with the electrolyte solution for 3–5 min. The capillaries were stored in purified water.

2.4. Calculations

The electrophoretic mobilities of the analytes were calculated from the apparent migration times with the equations $\mu_a = L_D L_T / tV$; $\mu_a = \mu_e + \mu_{eof}$, where μ_a is the apparent mobility, μ_e the effective mobility of the analyte, μ_{eof} the electroosmotic mobility, L_D the effective length of the capillary (length to the detector), L_T the total length of the capillary, t the migration time (in seconds) measured directly from the electropherogram and V is the applied voltage (in volts). In the absence of electroosmotic flow (EOF), the correlation $\mu_a = \mu_e$ is valid. The disturbance

in the electropherogram baseline (from sample water or methanol−water) was used as an EOF marker.

Resolution of the analytes was calculated with the equation $R_s = 2(t_2\text{-}t_1)/(w_{b1} + w_{b2})$, where t_1 and t_2 are the migration times of two adjacent compounds and w_{b1} and w_{b2} their peak widths at baseline.

The pK_a values of TR and its metabolites were estimated with the Pallas 1.2 program (CompuDrug Chemistry, Budapest, Hungary).

3. Results and discussion

3.1. Optimisation of CE conditions: effect of pH and ionic concentration

The electrolyte pH is the most important factor affecting the selectivity of CE separations. It determines both the degree of ionisation of the analytes and the magnitude of the EOF. The suitable pH is largely determined by the pK_a values of the functional groups of the analytes.

In our case, three different functional groups of the analytes determine the choice of pH of the background electrolyte (BGE) and make the separation of TR and its metabolites particularly challenging. All the analytes (Fig. 1) possess a primary, secondary or tertiary amino group. Metabolites M1, M4 and M5 also possess a phenolic hydroxyl group, and the glucuronides M1G, M4G and M5G have a carboxyl group in the glucuronic acid moiety. pK_a values of 8.3 [37] and 9.44 [35] are reported for the tertiary amine of TR, but no pK_a values were available for the amine metabolites. The computationally predicted pK_a values are 10.70, 10.96 and 9.79 for the primary, secondary and tertiary amino groups, respectively (see Section 2.4). The pK_a value of glucuronic acid is 3.2 [38] and pK_a values of the glucuronides are reported to be of the same order of magnitude [39]. The phenolic hydroxyl group has a pK_a of approximately 10 (see Section 2.4). Thus, the glucuronides are mainly zwitterions in the pH range of 3–10 and, with a net charge near zero, they are unable to migrate under capillary zone electrophoresis (CZE) conditions. Accordingly, the pH range suitable for a single run separation of TR and its metabolites with conventional CZE is limited to pH values below 3 or above 10. Our study was accordingly focused on separations with low pH (pH < 3) and high pH (pH > 10) BGEs.

Results of the experiments in ammonium acetate−formic acid electrolyte (pH 2.75) are presented in Fig. 2A–G. In both *O*-methylated and phenolic metabolite groups, the migration order corresponds to the molecular size, with primary amines migrating first. Baseline separation of *O*-methylated (R_s for M3-M2 2.4 and for M2-TR 1.0, Fig. 2A) and phenolic (R_s for M4-M5 2.2 and for M5-M1 1.0, Fig. 2B) analytes was easily obtained but separation of all six metabolites could not be achieved due to comigration of the primary, secondary and tertiary amines (Fig. 2C).

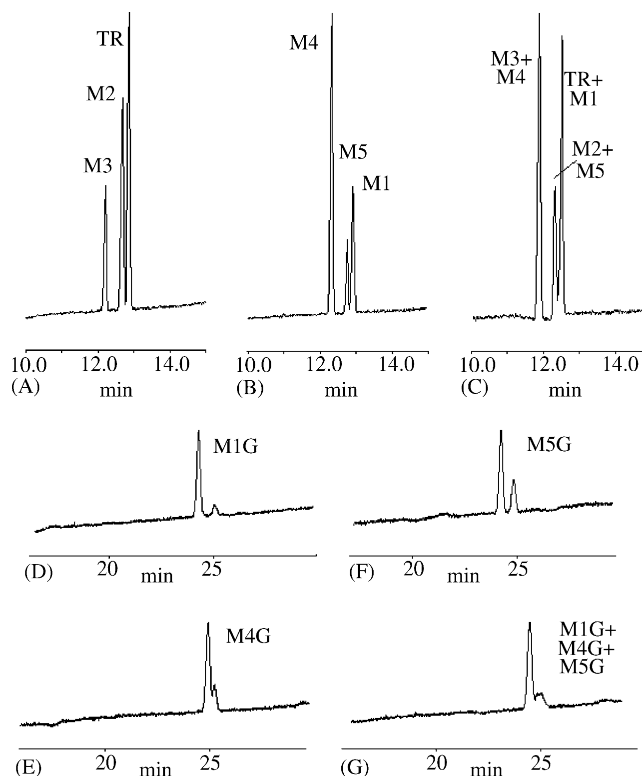

Fig. 2. Separation of TR and its metabolites in 10 mM ammonium acetate−100 mM formic acid electrolyte, pH 2.75. Panels—*O*-methylated metabolites (A); phenolic metabolites (B); *O*-methylated and phenolic metabolites (C); diastereomeric separation of M1G (D); M4G (E); M5G (F); a mixture of the three glucuronides (G). The first migrating diastereomer is the 1*S*,2*S* form. Other conditions—capillary: 57 cm (50 cm to detector) × 50 μm i.d., 17 kV, 12.4 ± 0.2 μA; 25 °C. Detection at 200 nm. Sample injection 5 s followed by electrolyte injection 1 s.

Under the same conditions, a diastereomeric separation of all three glucuronide metabolites was observed (Fig. 2D–F). The R_s values were 1.4 for M1G diastereomers and 1.2 for M5G diastereomers. Only partial separation of the M4G diastereomers was obtained (R_s 0.7). As noted above, a corresponding diastereomeric separation of M1G has been reported [34]. The individual glucuronides were, however, inseparable under acidic conditions (Fig. 2G). At pH 2.75 the EOF is negligible and the electrophoretic mobilities of the cationic analytes are the only contributors to the separation. This results in inadequate selectivity of separation in cases where the positively charged analytes have only minor structural differences (Fig. 1).

During our preliminary experiments, diastereomeric separation of TR glucuronides was obtained in many low pH BGEs (pH < 3). All these findings support the assumption that natural mobility differences exist between diastereomers, and the separation of glucuronide diastereomers in acidic media can be explained by subtle, conformation-induced differences in charge distribution and pK_a values [40]. Carboxymethyl-β-cyclodextrin (CM-β-CD) was tested as a BGE additive during the preliminary experiments at pH < 3, but no improvement was achieved

in the resolution of the glucuronide diastereomers. This is understandable since inclusion of the hydrophilic glucuronides into the hydrophobic cavity of the CD ring is unlikely.

A second set of experiments was conducted in basic solvent systems, pH > 9.8. Disodium tetraborate (pK_a 9.14) and CAPS (pK_a 10.4) electrolytes, which according to the literature have useful pH ranges between pH 9.2 and 11.4 [41], were tested. To find the optimum pH for separation, mobilities of TR metabolites were measured in 150 mM CAPS and 40 mM tetraborate electrolyte solutions in pH range 9.8–10.77. The results indicated that net charge of the phenolic metabolites changes from positive to negative as the pH increases. They also showed that the glucuronides have a negative net charge while the *O*-methylated compounds are cations. In the pH range studied, the mobility differences of analytes seemed to be sufficient for separation only at pH 10.65. The final selection of BGE was not unambiguous: good buffering capacity and low current favoured selection of CAPS. However, the clearly sharper analyte zones obtained in tetraborate electrolyte were regarded as necessary where the differences in analyte mobilities were small. Hence tetraborate was chosen as BGE for further experiments.

The effect of ionic concentration on the mobilities of the phenolic and glucuronide metabolites was studied in 30−65 mM tetraborate solution, pH 10.65. As assumed, the mobilities of analytes decreased with increasing ionic concentration of the BGE. The most notable finding, however, was that separation of the analytes M5G-M4 improved when tetraborate concentration was at least 60 mM.

A successful separation of TR metabolites (R_s > 1.1) was obtained with tetraborate solution 65 mM (pH 10.65) as the BGE. High ionic concentration was chosen to ensure sufficient buffer capacity. Separation of the *O*-methylated, phenolic and glucuronide metabolites of TR in the method conditions is shown in Fig. 3. The electropherogram reveals that the migration order does not follow the order of molecular size but is determined by the pK_a value of the amine moiety and the net charge of the compound. In all three metabolite groups the migration order is secondary amines, primary amines and tertiary amines. Hence, the metabolites with the highest amine pK_a value (pK_a ca. 11; M2, M5 and M5G) reach the detector first, while the analytes with the lowest amine pK_a values (pK_a < 10; TR, M1 and M1G) are detected last. The negative charge of the phenolic metabolite is smaller than that of the glucuronide. This explains why the phenolic metabolite always migrates before the respective glucuronide. Although complex formation between borate ions and vicinal hydroxyls of glucuronides is possible at alkaline pH its role in the present separation is difficult to demonstrate.

Because of the basic nature of the amino group, interactions with the capillary wall cannot be avoided and peak tailing occurs, most with the primary amine metabolites (M3, M4, M4G; A_s 1.3–1.4) and least with the tertiary amine

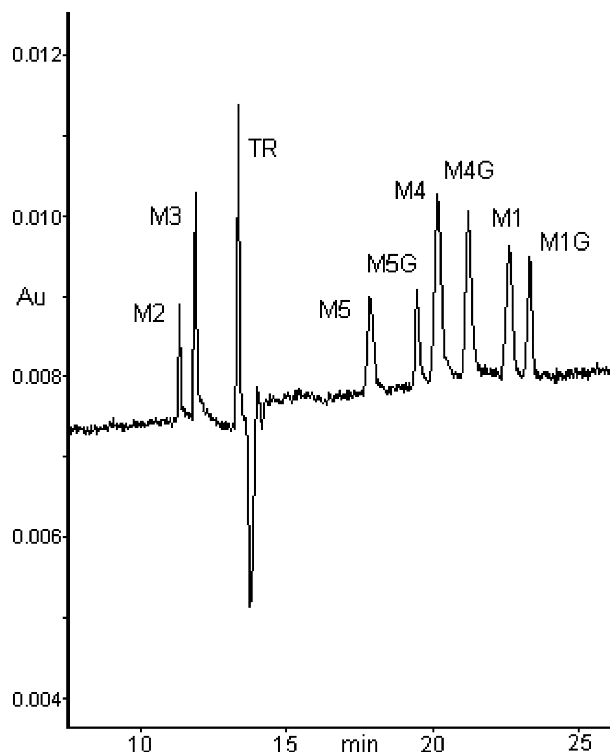

Fig. 3. CZE separation of TR and its phase I (M1-M5) and phase II (M1G, M4G, M5G) metabolites. Conditions—electrolyte disodium tetraborate 65 mM, NaOH 162.5 mM (pH 10.65); capillary 47 cm (40 cm to detector) × 50 μm i.d., 9 kV; electric field 192 V/cm, current 74 ± 1 μA; 25 °C. Detection at 200 nm. Sample—TR, M1 and M5 4 μg/ml, M2 3 μg/ml, M3 and M4 5 μg/ml, M1G and M5G 20 μg/ml, M4G 35 μg/ml. Sample injection 3 s.

metabolites (TR, M1, M1G; A_s 0.9–1.2) where the positive charge is sterically best protected.

3.2. Effect of SDS

In an attempt to improve the separation of phenolic and glucuronide metabolites of TR we tested the more selective form of CE, micellar electrokinetic chromatography (MEKC). The mobilities of these metabolites were examined by adding different concentrations (0–90 mM) of SDS to the 25 mM tetraborate solution (pH 10.45). To ensure buffer capacity a somewhat lower pH of the BGE was used. The addition of SDS was found to affect the migration behaviour of analytes considerably. The hydrophilic glucuronide metabolites became well separated from the more hydrophobic phenolic metabolites, which owing to partitioning with the micelles migrated slowly. As well, a reversal in migration order of the analytes occurred in both metabolite groups, and diastereomeric separation of the glucuronide metabolites was observed. The selectivity of the glucuronide separation improved (M5G > M4G > M1G) with increasing SDS concentration. For complete separation of all six glucuronide diastereomers SDS concentration of 70 mM was considered optimal. Simultaneously, the

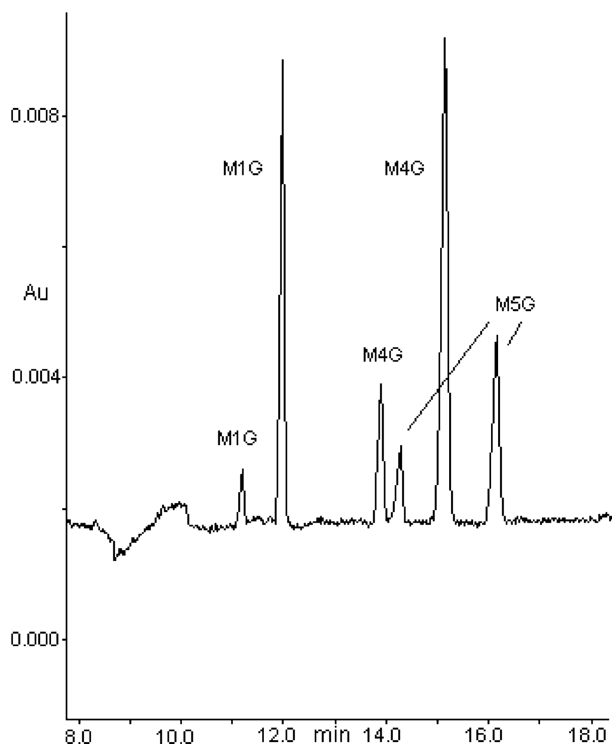

Fig. 4. MEKC separation of TR glucuronides in 25 mM disodium tetraborate-70 mM SDS electrolyte, pH 10.45. The first migrating diastereomer is in each case the 1*R*,2*R* form. Other conditions—capillary: 47 cm (40 cm to detector) × 50 μm i.d., 13 kV, 61 ± 1 μA; 25 °C. Detection at 200 nm. Sample concentration M1G 30, M4G 50 and M5G 20 μg/ml, sample injection 10 s.

migration times of the phenolic metabolites increased to about 50 min, so their analysis was no longer reasonable. Accordingly, we decided to use the MEKC method only for separation of glucuronide diastereomers (Fig. 4).

Another interesting finding in the diastereomeric separation of TR glucuronides was that in both acidic and basic micellar media the diastereomers separated into two zones of different size (Figs. 2 and 4). However, the preliminary experiments showed that the phenolic aglycons M1, M4 and M5, the substrates of enzyme-assisted synthesis, were racemic mixtures of two enantiomers, which in CM-β-CD modified phosphate-Tris buffer (pH < 3) (method, ref. [31]) migrated in two zones of equal size. Information obtained with photodiode array detector monitoring confirmed that the difference observed in the responses of glucuronide diastereomers was not due to differences in absorption maxima. Thus these findings confirm earlier results of Overbeck and Blaschke [21], who demonstrated with an LC method that in vitro glucuronidation with rat liver microsomes produces glucuronide diastereomers in different amounts. As pure glucuronide diastereomer standards were not available, we were unable to confirm the stereochemistry of the diastereomers. However, the data for M1G diastereomers presented by Soetebeer et al. [34] allowed us to assume that in our case the 1*S*,2*S*-diastereomer was present in diastereomeric excess. Furthermore, as shown by the electropherograms (Figs. 2D–F and 4), peak area ratios differ for the individual glucuronides. The ratio of 8.3 was obtained for the M1G diastereomers, 4 for the M4G diastereomers and 2.6 for the M5G diastereomers. This means that the amine structure somehow influences the glucuronidation process.

To evaluate the suitability of the CZE method to analyse TR and all its metabolites, we determined the limit of detection (LOD) for these analytes, as well as the repeatability of the method (precision of relative migration times and peak areas). LODs were also determined for the diastereomeric glucuronides to evaluate the suitability of the MEKC separation.

3.3. Limit of detection

LODs of the analytes were detemined so as to correspond with a signal-to-noise ratio (S/N) of 3 calculated on the peak heights. Results obtained with the CZE and MEKC methods are given in Table 1. The lowest concentrations that could be detected by CZE were 1.5–2 μM for the phenolic metabolites and 6–7 μM for the glucuronide metabolites. The lowest LOD value, 0.7 μM, was obtained for the *O*-methylated metabolites.

Table 1
LODs for the analytes with CZE and MEKC methods, and repeatability (precision) of the CZE method (relative standard deviations, R.S.D.s, of relative migration times and peak areas)

Method	TR	M1	M2	M3	M4	M5	M1G[c]	M4G[c]	M5G[c]
CZE LOD[a] (μM)	0.7	1.8	0.7	0.7	1.5	1.8	6.0	7.0	7.0
MEKC LOD[b] (μM)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	15[1] (2.5)	11[1] (2.7)	8.5[1] (3.3)
Repeatability CZE method									
Realtive migration time R.S.D. (%)	0.23	1.97	0.53	0.49	1.99	1.53	1.38	1.49	4.6
Peak area R.S.D. (%)	6.7	4.3	6.5	6.5	3.1	5.4	6.3	6.3	

CZE method; separation conditions as in Fig. 3. Sample injection 3 s. MEKC method; separation conditions as in Fig. 4. BGE: disodium tetraborate 25 mM, NaOH 60 mM and SDS 70 mM (pH 10.45). Sample injection 6 s. n.d. = not determined.

[a] $n = 6$.

[b] $n = 3$.

[c] The first migrating diastereomer is designated with index[1].

3.4. Repeatability of the CZE method

The repeatability of the system (precision of the relative migration times and peak areas) was determined with six consecutive injections of the sample that contained low concentrations of all the analytes (TR and M1-M5 3–5 μg/ml and glucuronides 20–35 μg/ml). Results are given in Table 1. The repeatability of the relative migration times was acceptable; relative standard deviations (R.S.D.s) were 0.23–1.99%. The best repeatability was obtained for the analytes TR, M2 and M3, which had a single chargeable group. The variation in peak areas (3.1–6.7%) could be partly explained by the low concentrations of analytes in the sample solution, short-injection time (3 s) and inaccurate integration of tailing peaks (especially M2, M3 and M4G).

3.5. Applicability of the methods

The applicability of the CE methods in identification of TR metabolites was tested with an authentic urine sample collected for 12 h after administration of a single dose (50 mg) of TR. A SPE procedure was optimised for purification and concentration of the samples. Data obtained for urine extracts (0-U, TR-U and 0-U spiked with the analytes) with the CZE method are presented in Fig. 5. As can be seen, the 0-U sample (graph c) was virtually free of interference and allowed the separation of TR metabolites. Comparison of the TR-U sample (graph b) with the spiked sample (graph a) reveals a TR peak and four other peaks, which, in migration order, can be assumed to represent TR metabolites M5, M5G, M1 and M1G, respectively. In a ten-fold concentrated TR-U sample, small peaks that are suggested to represent the metabolites M2 and M3 were detected as well, but again there was no sign of metabolites M4 or M4G (data not shown). The results are largely in agreement with those of earlier studies: unchanged TR and the metabolites M1, M2 and M5 together with the conjugates of M1 and M5 were present as main metabolites in human urine [7,9]. The metabolites M3 and M4 and conjugates of M4 were detected only in trace amounts [7]. More sensitive and structure selective detection methods are needed, however, to be able to detect all metabolites present in trace quantities.

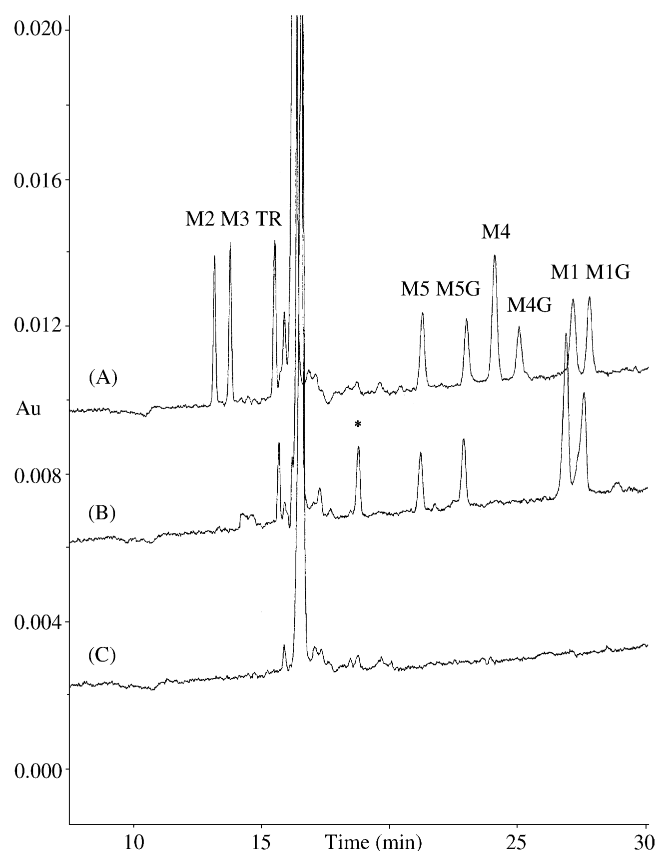

Fig. 5. CZE electropherograms of a spiked urine sample (A), TR urine sample (B) and 0-urine sample (C). The urine samples were five-fold concentrated. Sample concentration in the spiked sample: TR and the phase I metabolites 4 μg/ml and M5G and M1G 30 μg/ml and M4G 25 μg/ml. The peak marked with an asterisk refers to an unknown compound or metabolite. Data are depicted with an y-axis offset of 0.0035 AU. Experimental conditions as in Fig. 3.

We had hoped to be able to identify the glucuronide diastereomers of M5G and M1G in the TR-U sample with the MEKC method. However, the electropherogram obtained from the 0-U sample (in Fig. 5) in the presence of the micelles showed that the migration window between 7 and 25 min was full of peaks and would not allow unambiguous identification of the glucuronide diastereomers (data not shown).

4. Conclusions

Separation of TR and its *O*-methylated, phenolic and glucuronide metabolites in a single run represents a considerable analytical challenge. The main challenge is the limited pH area in which separation is possible. In low pH media (pH < 3) and in the absence of EOF, only part of the metabolites could be separated with the CZE mode. An unexpected finding was that the three glucuronides separated into diastereomers. The separation was carried out with use of volatile BGE, which means that the use of more selective MS detection may be possible in future. The method could then be applied in enzyme kinetic monitoring of in vitro glucuronidation of individual glucuronides. Separation under high pH conditions (pH > 10) was complicated by the presence of two functional groups in each of the six analytes—an amino group and either a phenol group or a carboxyl group—all involved in the mobilities.

Baseline separation of TR and the above-mentioned metabolites M1, M2, M3, M4, M5, M1G, M4G and M5G was achieved in basic media using 65 mM tetraborate (pH 10.65). Working close to the pK_a values of the analytes was demanding, however, and required frequent renewal of the electrolyte. The experiments also revealed that highly

selective diastereomeric separations of TR glucuronides could be achieved in 25 mM tetraborate solution (pH 10.45) by adding SDS as micellar modifier.

The suitability of the CZE method for screening purposes was tested with an authentic urine sample after TR administration. With a five-fold sample concentration, clearly detectable peaks of TR and the presumed metabolites M1, M1G, M5 and M5G were found in an interference-free area of the electropherogram. On this basis, we suggest that the present simple and cost-effective CZE method can suitably be applied in metabolism research and therapeutic monitoring of TR and its main metabolites in urine when more sophisticated methods are not available.

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