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Separation of the glucuronides of entacapone and its (*Z*)-isomer in urine by micellar electrokinetic capillary chromatography

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Abstract

A micellar electrokinetic capillary chromatography (MECC) method was developed for the separation of the 3-*O*-glucuronides of entacapone and its (*Z*)-isomer, the two main urinary metabolites of entacapone in humans. Entacapone is a novel, potent inhibitor of catechol-*O*-methyltransferase (COMT) intended for use as an adjunct in the treatment of Parkinson's disease. Urine samples spiked with synthetic 3-*O*-glucuronides were used to study the effects of running buffer pH, composition and applied voltage on separation of the closely migrating glucuronides. The 3-*O*-glucuronide of nitecapone, was used as internal standard. The greatest improvement in separation was achieved by increasing the running buffer ionic concentration. Changes in pH had little effect on the separation, whereas increase in sodium dodecyl sulfate (SDS) concentration slightly improved resolution. Baseline separation and good selectivity relative to urine components were achieved by using a phosphate (25 m*M*)–borate (50 m*M*)–SDS (20 m*M*) running buffer, pH 7.0, in a 75 μm×60/67 cm fused-silica capillary at 15 kV and a 335 nm cut-off filter in the UV detector. The limits of detection (LOD) at a signal-to-noise ratio of 3 were about 0.25 μg/ml ($5.2 \cdot 10^{-7}$ *M*) (injection 0.5 p.s.i./8 s). The linear detection range was 2–100 μg/ml ($r^2 > 0.999$). Good repeatability of injection and relative migration times were obtained.

Keywords: Micellar electrokinetic capillary chromatography; Buffer composition; Entacapone; Glucuronides

1. Introduction

Entacapone –OR-611, (*E*)-2-cyano-*N*,*N*-diethyl-3-(3,4-dihydroxy-5-nitrophenyl)propenamide– is a novel nitrocatechol catechol–*O*-methyltransferase (COMT) inhibitor intended for use as an adjunct to levodopa in the treatment of Parkinson's disease [1,2]. Levodopa is widely used to counter the dopamine deficiency in Parkinsonian patients. The main metabolic pathway of levodopa is decarboxylation to dopamine, and decarboxylase inhibitors are normally coadministered to inhibit decarboxylation of levodopa in the peripheral tissues [3]. Levodopa is also metabolized by COMT to 3-*O*-methyldopa [3], and entacapone has been developed to prevent this and further improve the bioavailability of levodopa. Recent tests have demonstrated that entacapone can safely be added to levodopa/decarboxylase inhibitor therapy [4]. The metabolism of entacapone has been thoroughly investigated. In man the only major phase

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I metabolite of entacapone is its (*Z*)-isomer, and both entacapone and its (*Z*)-isomer are largely conjugated with glucuronic acid and excreted in urine as water-soluble glucuronides [5].

The use of capillary electrophoresis (CE) for analysis of drugs and their metabolites in biological samples has become increasingly widespread. Both capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) have been used to analyse glucuronide conjugates in urine. Phosphate, borate, or phosphate–borate buffer combinations with and without a surfactant have been employed in these separations. Separation of the estrogen conjugates estriol-3-glucuronide from amniotic fluid and estriol-16α-glucuronide from urine has been reported [6]. Morphine-3-glucuronide has been determined in pretreated urine samples [7], and dihydrocodeine-6-glucuronide has been measured in urine without prior extraction [8]. CE also allows a simultaneous analysis of phase I and phase II metabolites, as shown in recent work where 7-hydroxycoumarin and its glucuronide were determined in urine without sample clean-up [9]. The direct determination of glucuronides is often not possible because commercial reference compounds (i.e. glucuronides) are not available. normally either acidic or enzymatic hydrolysis is then required before determination of these conjugates.

The aim of the present work was to develop a CE method for the separation of the 3-*O*-glucuronides of entacapone (I) and its (*Z*)-isomer (II). The 3-*O*-glucuronide of another COMT inhibitor, nitecapone (III), was used as internal standard. The structural formulae of the three 3-*O*-glucuronides are shown in Fig. 1. To find a method that would provide acceptable separation and selectivity we carried out experiments investigating the composition of the running buffer (pH, ionic concentration, sodium dodecyl sulfate (SDS) concentration) and applied voltage.

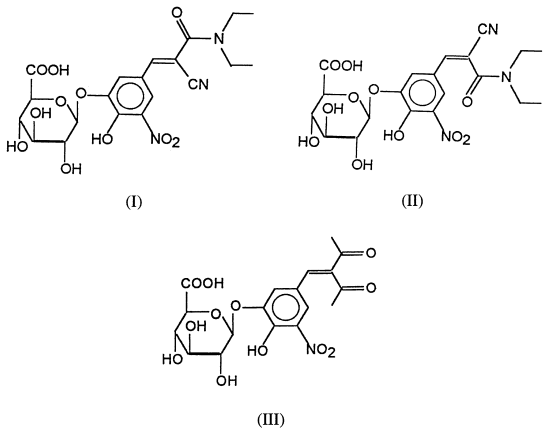

Fig. 1. Structural formulae of the 3-*O*-glucuronides of entacapone (I), its (*Z*)-isomer (II) and nitecapone (III) (internal standard).

2. Experimental

2.1. Materials

The 3-*O*-glucuronides of entacapone, its (*Z*)-isomer and nitecapone were synthesised at the Division of Pharmaceutical Chemistry, Department of Pharmacy, University of Helsinki, Finland [10]. All organic solvents and other chemicals were of analytical or chromatographic grade. Acetone was purchased from Riedel-de Haën (Seelze, Germany), sodium dodecyl sulfate (SDS) was supplied by Serva Feinbiochemica (Heidelberg, Germany), while boric acid (H_3BO_3), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2\ H_2O$) (extra pure), sodium hydroxide (NaOH), and disodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) were obtained from Merck (Darmstadt, Germany). Water was purified in a Milli-Q plus water purification system (Millipore, Molsheim, France). Blank urine was obtained from a healthy volunteer.

2.2. Spiked urine samples

For purposes of method development, the glucuronides of entacapone, its (*Z*)-isomer and nitecapone (internal standard) were dissolved in water. One volume of blank urine was spiked with one volume of aqueous glucuronide solution so that the final concentration of all the glucuronides was 25 μg/ml. The urine samples were stored at −20°C before analysis, and before injection the samples were filtered (Millipore HV, 0.45 μm, Nihon Millipore, Yonezawa, Japan) and sonicated.

The resolution between the glucuronide of entacapone and its (*Z*)-isomer was calculated according to the equation $R_s = 2\ (t_2 - t_1) \cdot (w_1 + w_2)^{-1}$ where t_1 and t_2 are the migration times of the glucuronides of

entacapone and its (*Z*)-isomer and w_1 and w_2 are the respective baseline peak widths [11].

2.3. CE instrumentation and conditions

The experiments were performed with a P/ACE 2200 CE instrument controlled with System Gold software (Beckman, Fullerton, CA, USA). Temperature was controlled through liquid cooling. A UV absorbance detector with cut-off filter (335 nm) was used. Separations were carried out in a 75 μm I.D. fused-silica capillary (Composite Metal Services, Hallow, UK), where the total length was 67 cm and the distance to the detector was 60 cm. The different conditions (ionic concentration and pH of the running buffer, SDS concentration, and applied voltages) tested in the development of the separation method are given in Table 1. The running buffer solutions (**A–C**) were prepared and adjusted to the desired pH value (PHM83 Autocal pH meter, Radiometer, Copenhagen, Denmark) by mixing NaH_2PO_4–H_3BO_3–SDS buffer (pH appr. 4.7) with NaH_2PO_4–$Na_2B_4O_7$–SDS buffer (pH appr. 8.3). Phosphate buffer (20 m*M*) used during the preliminary studies was prepared by mixing 20 m*M* NaH_2PO_4 solution with 20 m*M* Na_2HPO_4 solution. All samples were injected at the anode end using pressure injection 0.5 p.s.i. (3447.38 Pa) for 8 s. The operating temperature was 25°C throughout the method development.

Acetone (3% v/v aqueous solution) was used as a neutral marker to monitor the electro-osmotic flow (EOF). The electro-osmotic breakthrough time (t_0) (detected as a negative peak) was used as the reference peak when relative migration times were determined. Acetone solution was injected for 4 s.

New capillaries were conditioned before use by rinsing with 0.1 *M* sodium hydroxide solution for 30 min, with water for 5 min and with running buffer for 10 min. Three to five test runs (1) with the running buffer and (2) with the analytes were performed as a check on new capillaries. At the beginning of each working day the capillary was rinsed with 0.1 *M* sodium hydroxide solution for 10 min, with water for 5 min and with running buffer for 5 min. A separate buffer vial was used for the washings. Before each run the capillary was purged for 2 min with the running buffer. At the end of each day the capillary was flushed with 0.1 *M* sodium hydroxide solution and water. All solutions and water were filtered (Gelman Nylon Bulk Acrodisc 13, 0.45 μm) and sonicated before injection.

3. Results and discussion

3.1. Preliminary studies

Preliminary studies with different buffer solutions showed that the glucuronides of entacapone and its (*Z*)-isomer could rather easily be separated by CZE with use of a 20 m*M* phosphate buffer at pH 7.0. This was somewhat surprising since separation by CZE is generally considered to be based on differences in molecular charge and mass. In this case both the charge and the mass of the glucuronides were the same. However, shape also contributes to the separation process [12,13]. Thus the most prob-

Table 1
Conditions tested for CE separation of the 3-*O*-glucuronides of entacapone and its (*Z*)-isomer

Parameter	Electrolytes (m*M*)	SDS (m*M*)	pH[a]	Voltage (kV)	Current (μA)
pH	**A** Phosphate (15)–Borate (36)	20	5–8	13	17–36
	B Phosphate (20)–Borate (40)	20	5–8	13	20–45
	C Phosphate (25)–Borate (50)	20	5–8	13	23–46
Ionic conc.	**A** Phosphate (15)–Borate (36)	20	7	13	25–29
	B Phosphate (20)–Borate (40)	20	7	13	29–34
	C Phosphate (25)–Borate (50)	20	7	13	33–38
SDS conc.	**C** Phosphate (25)–Borate (50)	20–60	7	13	33–47
Voltage	**C** Phosphate (25)–Borate (50)	20	7	7–25	17–79

[a] pH was adjusted by titrating ''acidic'' NaH_2PO_4–H_3BO_3–SDS buffer (pH appr. 4.7) with ''basic'' NaH_2PO_4–$Na_2B_4O_7$–SDS buffer (pH approximately 8.3).

able explanation for the separation of the glucuronides of entacapone and its (*Z*)-isomer by CZE is their different molecular shapes.

A review of the literature revealed the wide applicability of MECC, a CE technique that relies on a micellar running buffer solution to separate compounds especially in biological fluids. A good MECC separation of the glucuronides of entacapone and its (*Z*)-isomer was initially achieved by using a phosphate (15 m*M*)–borate (36 m*M*) buffer that contained 23 m*M* of SDS at pH 7.0. Moreover a clear resolution of the analytes and the matrix components of urine was obtained in MECC with a phosphate (20 m*M*)–borate (40 m*M*) buffer that contained 20 m*M* of SDS at pH 7.0. Because we required a separation method selective for the three glucuronides in urine, the MECC mode was accordingly chosen for further development. The running buffer pH and the amounts of the running buffer components were modified to improve the separation and selectivity in urine analysis.

3.2. Choice of internal standard

Use of an internal standard is recommended in quantitative CE analysis to compensate for differences in injection volumes and to improve peak area precision [14–16]. The internal standard should be of similar mobility to the analytes, because the EOF may change during the run and affect compound mobilities [17,18]. The effects will be minor if the compounds migrate close to each other [18]. Over 20 water-soluble compounds that were charged at pH 7.0, several glucuronides included, were evaluated as internal standards. The 3-*O*-glucuronide of nitecapone was chosen as internal standard because it met the set requirements (structural similarity, water-solubility, charged at the same pH range as the analytes) and its migration behaviour was similar to that of the analytes. Additionally, the UV maximum of this glucuronide, 370 nm, was suitable for the high analytical wavelength selected.

3.3. Effect of buffer pH

pH is one of the most important parameters for improving selectivity in CE because it affects both the charge of the analytes and the magnitude of the EOF [19–21]. The effect of running buffer pH was studied for three different phosphate–borate concentrations, see Fig. 2, (SDS concentration was 20 m*M*) in the pH range 5.0–8.0 at a voltage of 13 kV and temperature of 25°C. The running buffer solutions (**A–C**) were prepared so that only pH was varied, while the concentrations of phosphate, borate and SDS were kept constant. pH was adjusted between 5.0 and 8.0 in steps of 1.0 pH unit, except in the pH range 6.0–7.0 where the steps were 0.2 units. In the present glucuronides the pK_a value of the carboxylic acid group of glucuronic acid is 3–4 [22], while that of the free phenolic hydroxyl group *ortho* to the nitro group is 4.1–4.6 [1]. Hence all three glucuronides existed mainly as doubly charged anions since both the carboxylic acid moiety and the free phenolic hydroxyl group were ionized in the pH range investigated. The glucuronides thus migrated powerfully against the EOF, but were still carried by the stronger EOF to the cathode end, where they were detected. They were well separated in the pH range 6.0–8.0 in all three phosphate–borate buffers.

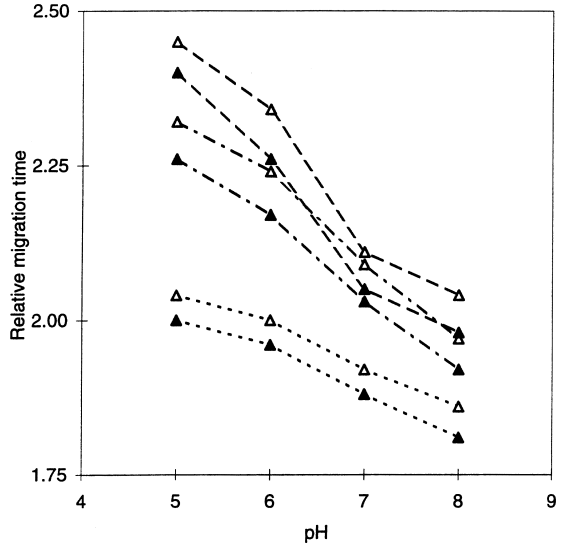

Fig. 2. Effect of pH on the relative migration times of the 3-*O*-glucuronides of entacapone and its (Z)-isomer. ▲: glucuronide of entacapone; △: glucuronide of (*Z*)-isomer of entacapone; · · ·: running buffer **A**; phosphate (15 m*M*)–borate (36 m*M*)–SDS (20 m*M*); –·–: running buffer **B**; phosphate (20 m*M*)–borate (40 m*M*)–SDS (20 m*M*); - - -: running buffer **C**; phosphate (25 m*M*)–borate (50 m*M*)–SDS (20 m*M*). Capillary: fused-silica 60/67 cm, 75 μm I.D. Voltage and temperature: 13 kV, 25°C

Separation was also achieved at pH 5.0, but the peaks were then broad, probably due to diffusive effects and reduced EOF [23,24]. The influence of pH on the relative migration times of the glucuronides of entacapone and its (*Z*)-isomer is shown in Fig. 2 (data not shown for pH values 6.2, 6.4, 6.6 and 6.8). Because of the good buffering capacity of phosphate at pH 7.0, this was selected as running buffer pH in subsequent experiments.

3.4. Effect of running buffer ionic concentration

In this set of experiments the running buffer pH was fixed at 7.0 and the concentrations of phosphate and borate were varied (SDS concentration 20 m*M*). Other conditions were kept constant (13 kV, 25°C). The effect of running buffer ionic concentration on the separation and migration times of the glucuronides at pH 7.0 is shown in Fig. 3. Of the three phosphate–borate concentrations investigated, the one with the highest concentrations (**C**) gave the best resolution for the glucuronides. The corresponding R_s values obtained for running buffers **A, B** and **C** were 0.865, 1.141 and 1.263, respectively, indicating a gradual improvement in the resolution. This is consistent with several publications that emphasize the use of running buffer solutions with high ionic concentration to improve resolution and peak shape [13,19,25,26] and also to give more consistent migration times [21]. High ionic concentration buffers increase current generation and Joule heating may result [26]. However, the current generated in this work with the highest ionic concentration buffer (**C**) was only 33–38 μA. This beneficial effect is largely attributed to borate [27], and borate was therefore used as major component of the running buffer.

3.5. Effect of SDS concentration

In these experiments, separation of the glucuronides was studied with a phosphate (25 m*M*)– borate (50 m*M*) buffer, pH 7.0, while the concentration of the anionic surfactant, SDS, was varied (20, 40 and 60 m*M*). Other conditions were kept constant (13 kV, 25°C). Slight improvement in the resolution of the analytes was observed when the SDS concentration was increased from 20 m*M* to 60 m*M*; the R_s values were 1.236, 1.263 and 1.353. As the concentration of SDS was increased, both the migration times of the glucuronides and the current also increased slightly. The increase in current was probably due to the increase in the sodium ion concentration originating from the SDS.

Surfactants above their critical micelle concentration (CMC) are normally employed when enhanced selectivity of CE separations is needed [28]. Although the separation of the glucuronides was not essentially improved when SDS was added to the running buffer, the analytes became well separated from the matrix components. A small concentration of SDS (20 m*M*) was accordingly added to the running buffer to increase selectivity in the complex urine matrix. A further benefit is the ability of SDS to solubilize urinary proteins and prevent their adsorption on the capillary wall [29].

3.6. Effect of voltage

The effect of voltage on the separation of the glucuronides was studied in a phosphate (25 m*M*)–borate (50 m*M*)–SDS (20 m*M*) running buffer, pH 7.0. Baseline separation of the glucuronides was achieved with all voltages tested (7, 10, 13, 15, 20 and 25 kV). Resolution of the analyte glucuronides was best at 13 kV to 20 kV, where the R_s value was approximately 1.2. The migration times of the analytes were about 8.5 min with the highest and about 33.5 min with the lowest applied voltage.

On the basis of these experiments, a phosphate (25 m*M*)–borate (50 m*M*) buffer (pH 7.0) containing 20 m*M* of SDS was chosen as running buffer for the separation of the glucuronides of entacapone and its (*Z*)-isomer in urine. The maximum operating voltage established from the Ohm's law plot (the plot of current versus voltage) [13] was 15 kV at 25°C in a 75 μm I.D., 60/67 cm capillary, resulting in typical current of 38–42 μA. Fig. 4. shows the separation of the glucuronides achieved under these conditions. The glucuronide of entacapone migrated slightly faster than the glucuronide of the (*Z*)-isomer. The glucuronide of nitecapone, which is of lower molecular mass, moved electrophoretically faster towards the anode and was detected last. The total run time of the method was about 17 min.

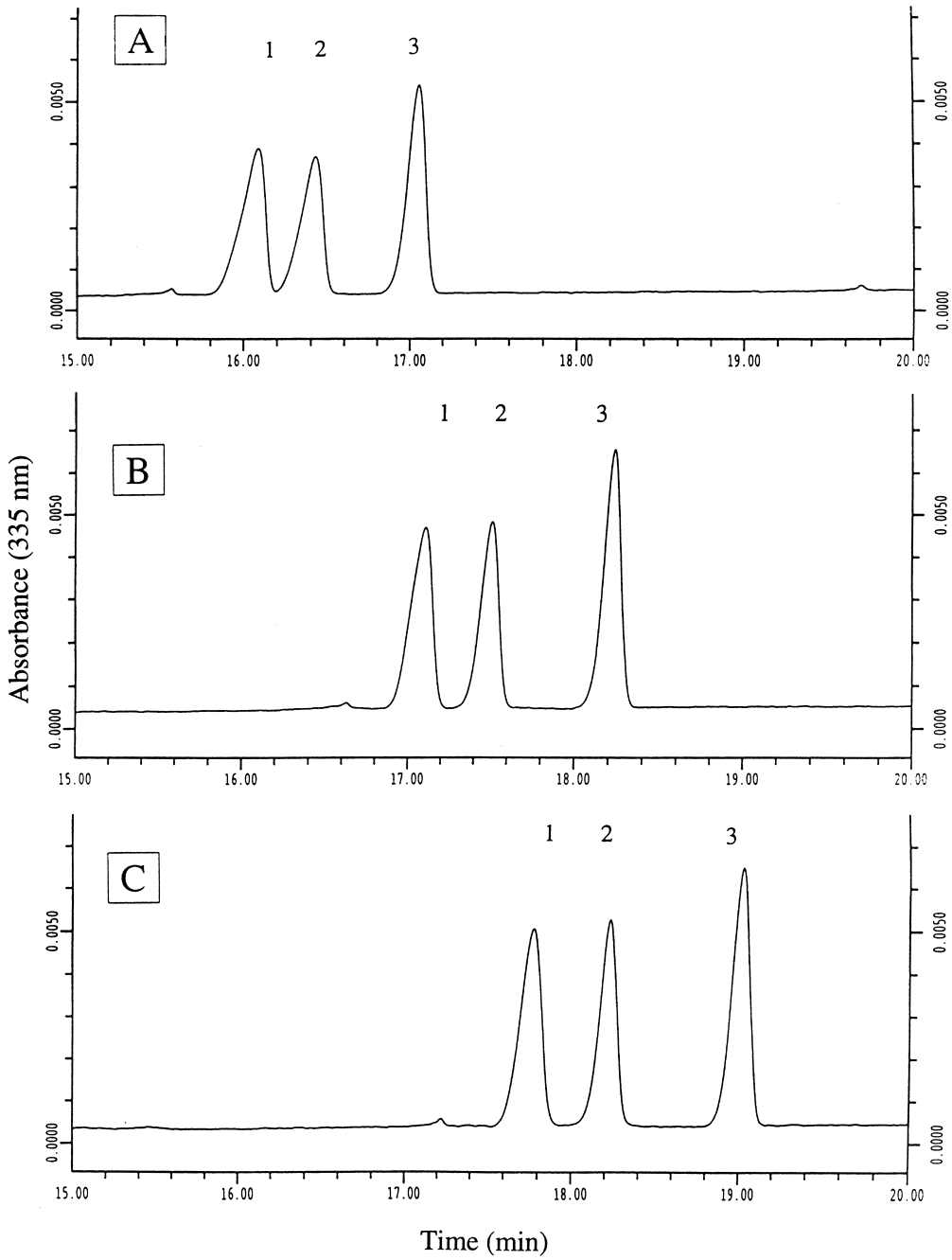

Fig. 3. Effect of running buffer ionic concentration on separation and migration times of the 3-*O*-glucuronides of entacapone (1), its (*Z*)-isomer (2) and nitecapone (3). Capillary: fused-silica 60/67 cm, 75 μm I.D. Running buffer : **A** phosphate (15 m*M*)–borate (36 m*M*)–SDS (20 m*M*), pH 7.0; **B** phosphate (20 m*M*)–borate (40 m*M*)–SDS (20 m*M*), pH 7.0; **C** phosphate (25 m*M*)–borate (50 m*M*)–SDS (20 m*M*), pH 7.0. Voltage and temperature: 13 kV, 25°C ; Sample injection with pressure (0.5 p.s.i.) for 8 s.

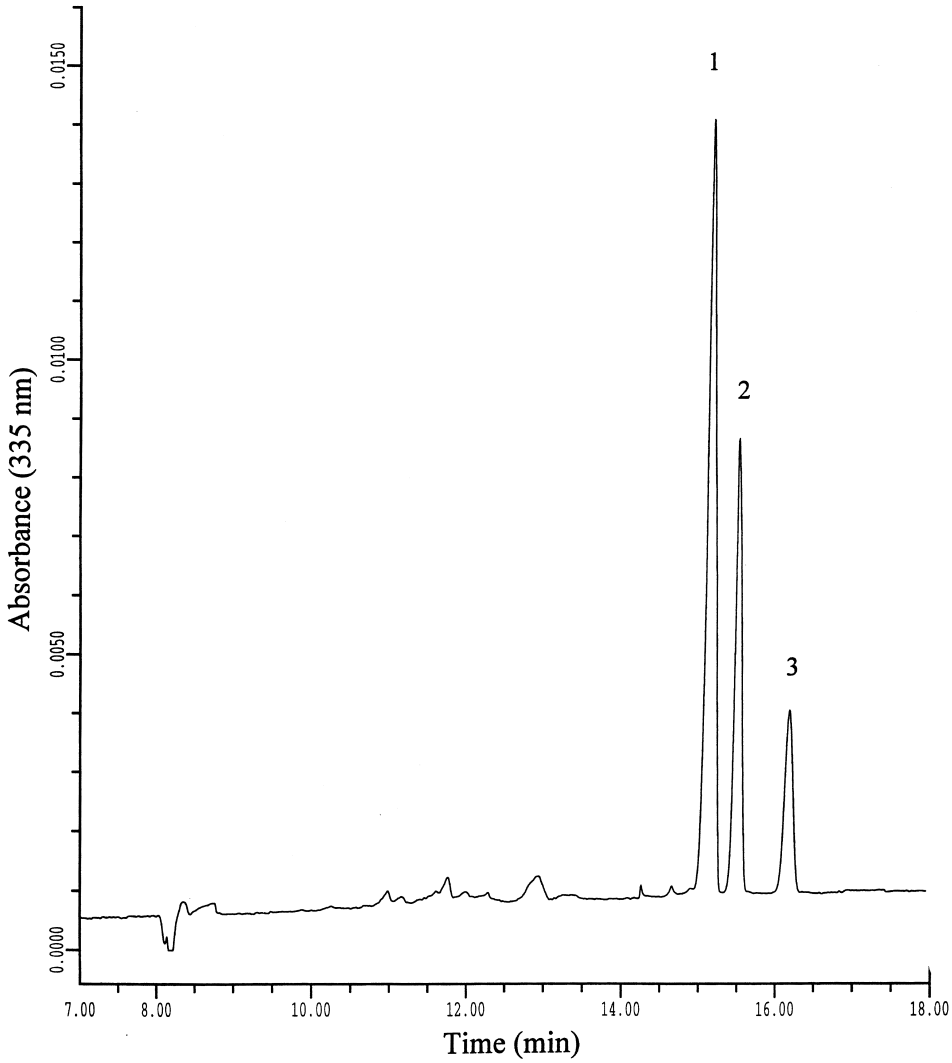

Fig. 4. Separation of the glucuronides of entacapone (1) and its (*Z*)-isomer (2) in a patient urine sample by MECC. Urine was collected 0–9.3 h after administration of four oral 200 mg doses of entacapone. The sample is diluted six-fold with water and injected with pressure (0.5 p.s.i.) for 5 s. The concentration of nitecapone glucuronide (internal standard) (3) is 25 μg/ml. Capillary: fused-silica 60/67 cm, 75 μm I.D. Running buffer: phosphate (25 m*M*)–borate (50 m*M*)–SDS (20 m*M*), pH 7.0. Voltage and temperature: 15 kV, 25°C.

3.7. Applicability of the method

The method did not allow resolution of the unconjugated entacapone from its (*Z*)-isomer. Neither could the trace amounts of entacapone or its phase I metabolite be detected in urine samples of patients since these compounds co-migrated with some of the urine components at 11–12 min. In contrast, the glucuronides were well separated from each other and from the matrix components, most of which migrated before the doubly charged late migrating glucuronides.

The suitability of the method for quantitative determination of the glucuronides of entacapone and its (*Z*)-isomer was also evaluated. Sensitivity of the instrument was tested with aqueous glucuronide solutions. Detection limits (LOD) were determined with diluted glucuronide standard solutions using the

capillary and injection conditions (0.5 p.s.i. for 8 s.) described in Experimental. For both glucuronides, the concentration that gave a signal approximately three times the baseline noise was 0.25 μg/ml ($5.2 \cdot 10^{-7}$ *M*). The linear detection range for both analytes was 2–100 μg/ml with correlation coefficients better than 0.999. The repeatability of injection was tested with six consecutive injections of the same aqueous sample, which contained 2 μg/ml or 75 μg/ml each of the glucuronides of entacapone and its (*Z*)-isomer. The RSD values ($n=6$) on peak-area (corrected peak-area ratio of the analytes to that of internal standard) were <1.5% for the lower concentration and <0.61% for the higher concentration for both glucuronides. Separation of the glucuronides was possible even with concentrations of 150 μg/ml. Good within-day precision of the relative migration times was achieved (RSD 0.4%, $n=19$) with aqueous solutions. Both current (39.5±1 μA) and temperature (25±0.1°C) were constant from run to run indicating stable separation conditions.

4. Conclusions

A MECC method requiring only filtration for sample clean-up was developed for the separation of the glucuronides of entacapone and its (*Z*)-isomer in urine. A phosphate–borate buffer that contained SDS micelles provided a suitable electrophoresis medium for the separation. Baseline resolution of the glucuronides was achieved over a wide pH range for all ionic concentrations of the buffering species investigated. The running buffer solution with highest ionic concentration gave the best resolution of the analytes, and without excessive current generation. The method is now being validated for determination of the glucuronides of entacapone and its (*Z*)-isomer in urine samples of patients treated with entacapone.

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