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Micellar electrokinetic capillary chromatography method for direct determination of glucuronides of entacapone and its (*Z*)-isomer in human urine

Päivi Lehtonen[a,*], Sanna Lehtinen[a], Liisa Mälkki-Laine[b], Tom Wikberg[c]

[a]*Department of Pharmacy, Division of Pharmaceutical Chemistry, P.O. Box 56, FIN-00014 University of Helsinki, Helsinki, Finland*
[b]*National Agency for Medicines, Pharmaceutical Department, P.O. Box 55, FIN-00301 Helsinki, Finland*
[c]*Orion Pharma, P.O. Box 65, FIN-02101 Espoo, Finland*

Abstract

This paper describes the validation of a micellar electrokinetic capillary chromatography method for the direct determination of the 3-*O*-glucuronides of entacapone and its (*Z*)-isomer, the main urinary metabolites of entacapone in humans. Entacapone is a novel drug which, as a potent inhibitor of catechol-*O*-methyltransferase (COMT), is used as an adjunct in the standard therapy of Parkinson's disease. The 3-*O*-glucuronide of another COMT inhibitor, nitecapone, was used as internal standard (I.S.). The validation experiments were performed by using spiked urine samples that were extracted with Sep-Pak C_{18} cartridges before analysis. Determinations were carried out in a buffer of pH 7.0 containing 25 m*M* of phosphate, 50 m*M* of borate and 20 m*M* of sodium dodecyl sulfate, and by applying 15 kV over a 67 cm (60 cm to the detector)×75 μm fused-silica capillary. UV detection was at 335 nm. The validity of the method was assessed by investigating the identity of the analytes, selectivity, limit of quantitation, linearity, within-day precision, extraction recovery, between-day precision and accuracy, electroosmotic flow stability and analyte stability. The method proved to be reproducible, sufficiently selective and accurate. Extraction recoveries of the analytes were >94%. The limit of quantitation (LOQ) was 2 μg/ml and the assay was linear in the range 2–150 μg/ml with correlation coefficients better than 0.999 for both glucuronides. The repeatability of the method, expressed as the ratio of corrected peak area of the analytes to that of I.S., gave RSD values of <5% even at the LOQ. Between-day precision (RSD) was <7.5% for both glucuronides at 7.5 μg/ml. Determination of the glucuronide concentrations in urine samples of 34 patients treated with entacapone either orally (200 mg) or intravenously (25 mg) showed the method to be suitable for monitoring the concentrations of the glucuronide of entacapone after both oral and intravenous administration and those of the glucuronide of its (*Z*)-isomer after oral administration. The limited long term stability of the system requires, however, frequent recalibration in applications involving long sample series.

Keywords: Pharmaceutical analysis; Validation; Glucuronides; Entacapone; COMT inhibitors

1. Introduction

Entacapone, [(*E*)-2-cyano-*N*,*N*-diethyl-3-(3,4-dihydroxy-5-nitrophenyl)propenamide], is a potent inhibitor of catechol-*O*-methyltransferase (COMT) [1]. It was recently approved in the European Union as an adjunct to standard preparations of levodopa/benserazide and levodopa/carbidopa for use in patients with Parkinson's disease and end-of-dose motor fluctuations, who cannot be stabilised on those combinations. It has been suggested that the benefi-

*Corresponding author. Tel.: +358-9-70859194; fax: +358-9-70859556; e-mail: paivi.k.lehtonen

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cial action of adding entacapone to this standard therapy is based on enhanced bioavailability of levodopa [2].

Before a new drug is introduced to the market, reliable methods must be available to monitor the concentrations of the parent drug and/or its metabolites in body fluids. The exact pharmacokinetic description of drug metabolism requires the determination not only of phase I metabolites but also the phase II conjugates of parent drugs [3]. Direct determination of glucuronides is usually not possible because glucuronide standards are difficult to obtain. Hydrolytic cleavage of glucuronide conjugates with acid [4] or with enzymes [4,5] is normally a prerequisite for their determination. We recently developed a micellar electrokinetic capillary chromatography (MECC) method for the separation of the 3-*O*-glucuronides of entacapone and its (*Z*)-isomer [6]. These glucuronides are the two main urinary metabolites of entacapone in humans [7]. The method development was based on urine samples spiked with 3-*O*-glucuronides of entacapone and its (*Z*)-isomer, and the 3-*O*-glucuronide of nitecapone was used as internal standard. The new method proved to be robust with respect to changes in running buffer pH, sodium dodecyl sulfate (SDS) concentration and applied voltage. Even small variations in running buffer ionic concentrations did not result in loss of baseline separation of the analytes [6].

Analytical methods used to determine drug concentrations in biological samples must be validated. Since there are no official guidelines referring to biological fluids, the extensive conference report published by Shah and co-workers [8] has generally been accepted as the authority for bioanalytical method validation criteria [9,10]. Analytical method validation has been defined as a procedure used to prove that the test method consistently does what it is expected to do, with adequate accuracy and precision [11]. Although capillary electrophoresis (CE) has been employed for clinical purposes, most of the publications thus far discuss possible applications and feasibility only. The number of fully validated assays published is still relatively small [12].

The aim of this study was to validate the developed MECC method for quantitative analysis of the glucuronides of entacapone and its (*Z*)-isomer in urine and to test its applicability for the analysis of urine samples collected from patients treated with entacapone. In this case, direct determination of the conjugates was possible because synthetic reference glucuronides were available for calibration. Validation of the method, during which a series of experiments were conducted, consisted of a before-study phase and a within-study phase. To establish the performance and validity of the method we investigated the following analytical parameters: identity of the analytes, selectivity, limit of quantitation, linearity, within-day precision, extraction recovery and between-day precision and accuracy. Electroosmotic flow (EOF) stability (precision of relative migration times) was investigated both within-day and between-days. Analyte stability during analysis was also tested. Structures of the analytes and internal standard are shown in Fig. 1.

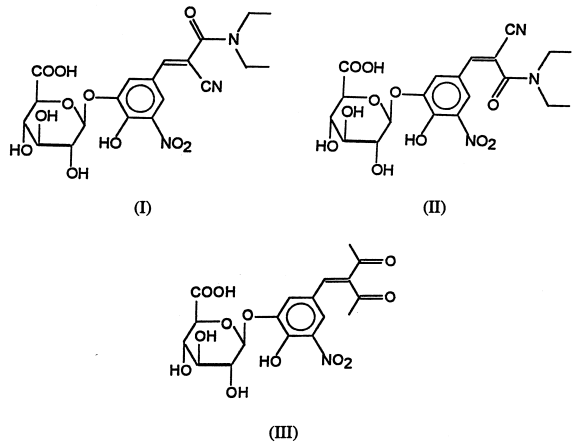

Fig. 1. Structures of the glucuronides of entacapone (I), its (*Z*)-isomer (II) and nitecapone (III) (internal standard).

2. Experimental

2.1. Materials

The 3-*O*-glucuronides of entacapone [(*E*)-g.], its (*Z*)-isomer [(*Z*)-g.] and nitecapone were synthesised at the Division of Pharmaceutical Chemistry, Department of Pharmacy, University of Helsinki, Finland [13]. All organic solvents and other chemicals were

of analytical or chromatographic grade. Acetone and boric acid (H_3BO_3) were purchased from Riedel-de Haën (Seelze, Germany). Methanol was of HPLC grade and was obtained from Rathburn (Walkerburn, UK), SDS was supplied by Serva Feinbiochemica (Heidelberg, Germany), and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$), hydrochloric acid (Titrisol 0,1 mol/l), sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$, extra pure), sodium hydroxide (NaOH) and disodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) were obtained from Merck (Darmstadt, Germany). D-Saccharic acid-1,4-lactone was purchased from Sigma (St. Louis, MO, USA) and β-glucuronidase (from *Escherichia coli* K 12, catalogue No. 127 051) was from Boehringer Mannheim (Germany). Water was purified in a Milli-Q plus water purification system (Millipore, Molsheim, France). Blank urine was obtained from a healthy volunteer and urine samples were obtained from two clinical patient studies. Urine fractions collected 0–12 h after an oral (200 mg) or an intravenous (25 mg) dose of entacapone were used for quantitative analysis. The urine samples were stored at −70°C until analysed.

2.2. Preparation of urine samples

All urine samples used in the method validation experiments were prepared in the same way except for the samples used in the identification (hydrolysis) and limit of quantitation experiments (see Sections 2.5 and 2.6). Before sample preparation a CE run of the blank urine was performed to confirm that no interfering compounds were present in the migration region of the analytes and internal standard. Isomerisation of the analytes was avoided by protecting all samples from light during handling.

A standard stock solution (1500 μg/ml) of the glucuronides of entacapone and its (*Z*)-isomer was prepared in water. The stock solution was further diluted with water to get standards with concentrations of 20 to 1000 μg/ml. Aliquots of 100 μl of these glucuronide standard solutions were pipetted into 2 ml cryo-vials and stored at −70°C until used. A solution containing 300 μg/ml of nitecapone glucuronide (internal standard) was prepared in water and stored at −20°C.

Before the calibration samples were prepared, both the blank urine and the standards were allowed to thaw. The calibration samples were prepared daily by mixing 1 ml of blank urine, 100 μl of glucuronide standard, 50 μl of internal standard and 450 μl of water. The samples were acidified (to pH 1–2) with 160 μl of 1 *M* hydrochloric acid solution before being extracted.

Urine samples of patients were allowed to thaw and mixed well and then prepared for analysis in the same way as the calibration samples: 1 ml of urine, 50 μl of internal standard and 550 μl of water were mixed and the solution was acidified and extracted.

Pre-dose urine samples were prepared in the same way as urine samples collected after dosing; 1 ml of urine was mixed with 600 μl of water and samples were acidified before extraction.

To determine the recoveries of the solid-phase extraction used in sample preparation, six samples were prepared by mixing a 100 μl aliquot of glucuronide standard, 50 μl of internal standard and 850 μl of water. Two samples each of three concentrations (2, 50 and 150 μg/ml) were prepared and assayed without the extraction step.

To determine between-day precision and accuracy, a batch of 20 homogeneous quality control (QC) samples were prepared by spiking 1 ml of blank urine with 100 μl of glucuronide standard solution. These urine samples contained either 7.5 or 75 μg/ml (ten samples of each concentration) of the glucuronides of entacapone and its (*Z*)-isomer. In connection with each calibration, two samples of each concentration were allowed to thaw and mixed with 50 μl of internal standard and 450 μl of water. The samples were acidified and extracted in the same way as the calibration samples and the samples to be analysed.

2.3. Sample pretreatment (solid-phase extraction)

All urine samples were pretreated in the same way by using Sep-Pak Vac C_{18} cartridges 1 ml (sorbent amount 100 mg) (Waters, Milford, MA, USA). In the limit of quantitation and identification experiments, sample pretreatment was performed in a slightly different way (for details see Sections 2.5 and 2.6). The solid-phase extractions (SPEs) were carried out with a Baker-10 SPE System (J.T. Baker, Phillipsburg, NJ, USA) vacuum manifold. The cartridges

were conditioned with 1 ml of methanol and 1.5 ml of 0.05 *M* hydrochloric acid solution that contained 2% (v/v) of methanol. The vacuum was turned off as soon as the applied solutions reached the sorbent bed to prevent the sorbent from drying. The acidified urine samples were slowly (~1 ml/min) loaded onto the cartridges, after which the cartridges were washed successively with 1 ml of 0.002 *M* hydrochloric acid solution and 1 ml of methanol–water (5:95, v/v) solution. The glucuronides were manually eluted into glass vials with 1.25 ml of (55:45, v/v) methanol–water solution. Extracts were concentrated under a stream of nitrogen and residues were dissolved in 1 ml of water. Samples were stored at −70°C until analysed. All samples were filtered (Millipore HV, 0.45 μm, Nihon Millipore, Yonezawa, Japan) and sonicated before injection.

2.4. CE instrumentation and conditions

All validation experiments and quantitative determinations were performed with use of a P/ACE 2200 CE instrument (Beckman, Fullerton, CA, USA) controlled with System Gold software. Temperature was controlled through liquid cooling. A UV absorbance detector with cut-off filter (335 nm) was employed. 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 60 cm. Throughout the work a running buffer solution containing 25 m*M* phosphate, 50 m*M* borate and 20 m*M* SDS, pH 7.0 was employed. The running buffer solution was prepared and adjusted to pH 7.0 (PHM83 Autocal pH meter, Radiometer, Copenhagen, Denmark) by mixing a buffer (pH ~4.7) containing 25 m*M* sodium dihydrogen phosphate, 50 m*M* boric acid and 20 m*M* SDS with a buffer (pH ~8.3) containing 25 m*M* sodium dihydrogen phosphate, 12.5 m*M* disodium tetraborate and 20 m*M* of SDS. A constant voltage of 15 kV (current ~40±1.5 μA) was applied and the temperature was set at 25°C.

All samples were injected at the anode end by pressure injection, 0.5 p.s.i. (~3450 Pa), for 8 s. Acetone (3%, v/v, aqueous solution) was used as a neutral marker to monitor the EOF and was injected for 4 s. The electroosmotic breakthrough time (t_0) (detected as a negative peak) was used as the reference time when relative migration times were determined.

New capillaries were conditioned before first 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 always 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 rinsing. The running buffer vial (the inlet) was changed every seventh run to prevent ion depletion from the buffer vial. Before each run the capillary was purged for 2 min with the running buffer. The quantitative determinations required more efficient rinsing procedures (water+0.1 *M* NaOH+water+running buffer, ~2 min each) that were performed every second or third run. 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.

2.5. Identification of the glucuronides

For identification of the glucuronides, spiked, authentic and pre-dose urine samples were subjected to sample pretreatment, concentration and enzymatic hydrolysis. To an aliquot (5 ml) of blank urine spiked with 400 μl of aqueous glucuronide solution that contained 1500 μg/ml of the glucuronides of entacapone and its (*Z*)-isomer, water (2 ml) was added and the solution was acidified to pH 1–2 with 700 μl of 1 *M* hydrochloric acid solution. The sample was passed through a Sep-Pak Light C_{18} cartridge (Waters) conditioned with 2 ml of methanol and 1.5 ml of 0.05 *M* hydrochloric acid solution that contained 2% (v/v) of methanol. The cartridge was washed successively with 4 ml of 0.002 *M* hydrochloric acid solution and 4 ml of methanol–water (5:95, v/v) solution. The glucuronides were then eluted from the cartridge with 3.5 ml of methanol–water (55:45, v/v) solution. The extract was concentrated under a stream of nitrogen and the residue was dissolved in 2 ml of water (the concentration of the glucuronides was ca. 300 μg/ml).

An aliquot (5 ml) of an authentic urine sample (collected from one subject 0–12 h after an oral dose of 200 mg of entacapone) was diluted with 2.4 ml of water and acidified to pH 1–2 with 700 μl of 1 *M* hydrochloric acid solution. The sample was then extracted and treated in the same way as the spiked urine sample.

An aliquot (5 ml) of pre-dose urine of the same subject as above was treated similarly to the authentic urine sample and used as control urine.

Enzymatic hydrolysis of the glucuronides was carried out by treating the above aqueous extracts with β-glucuronidase (from *Eschericia coli*). Aliquots (200 μl) of aqueous extract were pipetted into four Eppendorf vials and 100 μl of water was added to each. In all hydrolysis experiments the buffer was 12.5 m*M* phosphate (Na_2HPO_4/NaH_2PO_4), pH 6.85. To the first vial, 660 μl of buffer and 0.08 units of β-glucuronidase (in 40 μl of buffer) were added with mixing. To the second vial, 100 μl of buffer, 5.6 m*M* D-saccharic acid-1,4-lactone (in 560 μl of buffer) and 0.08 units of β-glucuronidase (in 40 μl of buffer) were added with mixing after each addition. Both samples were incubated at 37°C for 3 h. The aqueous extracts of the two control vials were mixed with 700 μl of the buffer, whereafter one was frozen immediately (−70°C) and the other was incubated at 37°C for 3 h. During the incubation, aliquots of 300 μl were taken from the β-glucuronidase-treated and control samples at 20 min and 1 h. All samples were immediately frozen and stored at −70°C until analysed.

Before CE analysis the hydrolysed samples were allowed to thaw at room temperature. The hydrolysates were first filtered (Millipore HV, 0.45 μm, Nihon Millipore), then pipetted into the sample reservoir of a Microcon-50 Ultrafiltration Devices (Amicon, Beverly, MA, USA) and centrifuged (5 min, 10 300 *g*) to remove proteins larger than M_r 50 000 present in the β-glucuronidase preparation. The samples were sonicated (5 min) before injection. Aliquots of control samples were treated in the same way.

2.6. Selectivity and limit of quantitation

Blank urine and pre-dose urine samples of six subjects, obtained from a clinical study, were used to evaluate method selectivity. Sample pretreatment was slightly modified from that described in Section 2.3. Aliquots (1 ml) of urine were diluted with 1 ml of water and acidified to pH 1–2 with 200 μl of 1 *M* hydrochloric acid solution. Elution was performed with 1 ml of methanol–water (55:45, v/v) solution. Extracts were concentrated as described in Section 2.3, and the residue of the blank urine sample was redissolved in 1 ml of aqueous glucuronide solution, while the residues of the pre-dose urine samples were redissolved in 1 ml of water. The concentration of the glucuronides of entacapone and its (*Z*)-isomer in this spiked urine sample was 2 μg/ml and that of nitecapone glucuronide 10 μg/ml. Another blank urine sample was prepared as above and spiked with the analytes at concentration 1 μg/ml. To determine the LOQ the spiked urine samples were injected twice, before and after the six pre-dose urine samples. Both the spiked and pre-dose urine samples were assayed and the mean peak heights of the analytes were compared with the average noise level of the six blanks.

2.7. Linear range, within-day precision and recovery

The linear range, within-day precision of the method and recovery were determined in a single set of experiments. Linearity of the method for the glucuronides was assessed over the range 2–150 μg/ml by using calibration samples of seven different concentrations, each containing 15 μg/ml of internal standard. The curves were constructed by plotting corrected peak area (peak area divided by migration time) ratios of the analytes to internal standard as a function of the concentration of the analytes (seven data points). At the concentrations 2, 50 and 150 μg/ml, the means of six calibration samples were used for calibration, while the standard deviations served to estimate the repeatability of the method. At the remaining four concentrations only one calibration sample was prepared and it was injected once. The within-day precision of the method was evaluated as relative standard deviations of both the corrected peak areas and corrected peak area ratios of the analytes to internal standard.

Recoveries of the solid-phase extraction were measured at the concentration levels 2, 50 and 150

μg/ml. Two aqueous solutions containing 2, 50 or 150 μg/ml of the analytes were injected in duplicate and the recoveries were calculated by comparing the means of the corrected peak areas of the six spiked and extracted urine samples with those of these solutions.

The repeatability of injection was tested separately with six consecutive injections of aqueous solutions containing 2, 50 or 150 μg/ml of the glucuronides of entacapone and its (*Z*)-isomer and 15 μg/ml of internal standard.

The within-day precision (RSD) of relative migration times (for details see Section 2.4) was determined from the migration times of the calibration runs.

2.8. Between-day precision and accuracy

The between-day precision and accuracy of the method was evaluated with spiked (7.5 and 75 μg/ml) homogeneous urine samples (QC samples). One or two samples of each concentration were analysed and quantified in connection with five separate calibrations.

2.9. Analyte stability

Blank urine spiked with the glucuronides of entacapone, its (*Z*)-isomer (25 μg/ml each) and nitecapone (15 μg/ml) was used to test the stability of the analytes. Sample preparation, pretreatment and storage were as described for calibration samples (see Sections 2.2 and 2.3). The spiked sample, and an aqueous reference sample of similar composition, were analysed in duplicate. The test sample was allowed to stand (in microvials) in the autosampler for 4.5–5 h, whereafter it was re-analysed together with a freshly prepared reference sample. The peak areas of the test sample (corrected peak area ratio of the analytes to internal standard) were compared with those of the reference samples.

2.1. Analysis of patient urine samples

The MECC method was applied to determine the concentrations of the glucuronides of entacapone and its (*Z*)-isomer in samples collected from 34 patients treated with entacapone. The analyses were performed on five days, with 10 to 18 samples analysed per day. Each analysis sequence included calibration samples of six concentrations and three to four spiked QC samples. Linear regression equations calculated from corrected peak area ratios of the analytes to internal standard versus analyte concentration were used for calibration. Separate calibration curves, each consisting of at least four data points, were calculated for the low (2–75 μg/ml) and high (50–150 μg/ml) concentration ranges.

3. Results and discussion

The validation criteria generally employed in assessing a CE method are similar to those used in evaluating the performance of HPLC methods [14]. The criteria evaluated in this study included identity of the analytes, selectivity, limit of quantitation, linearity, within-day precision (repeatability), extraction recovery, between-day precision (reproducibility) and accuracy and analyte stability during and after sample pretreatment. Migration time reproducibility (EOF stability) is an additional CE-specific aspect [15] that was evaluated.

3.1. Identity

The first approach to identifying the analytes in an authentic urine sample as 3-*O*-glucuronides of entacapone and its (*Z*)-isomer was based on the similarity of migration times with those of synthetic reference glucuronides. To confirm the identity of the compounds, we hydrolysed enzymatically, a blank urine sample spiked with the synthetic reference glucuronides, an authentic urine sample and a pre-dose urine sample. The electropherograms are presented in Fig. 2. Panel A1 clearly shows a rapid decay of the synthetic reference glucuronides and a concomitant appearance of a new compound with a 12 min migration time when the spiked urine sample was incubated 20 min at 37°C with β-glucuronidase. The new peak represents both entacapone and its (*Z*)-isomer, which co-migrated with a migration time of ~12 min. Similar changes can be seen in panel B1 in the electropherogram of the authentic urine sample incubated for 20 min with β-glucuronidase. The results indicate that the peaks with similar migration

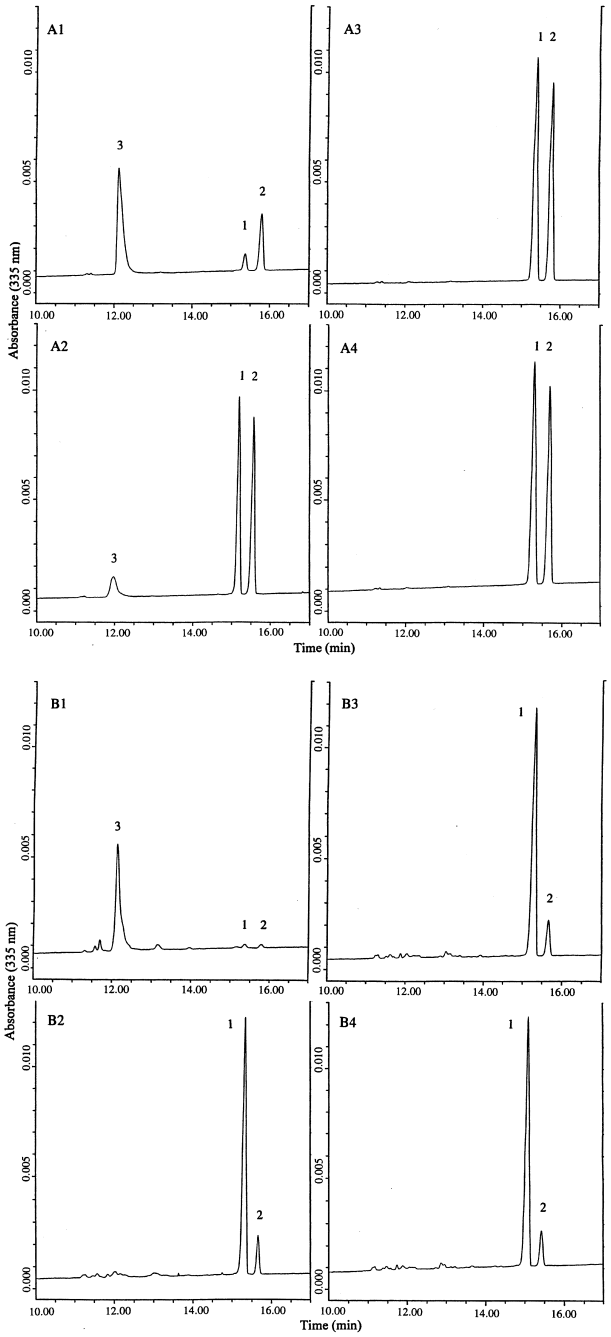

Fig. 2. Identification of the analytes in hydrolysis experiments. Blank urine spiked with the glucuronides of entacapone and its (*Z*)-isomer (A) and an authentic urine sample (collected 0–12 h after an oral dose of 200 mg of entacapone) (B) were incubated at 37°C for 20 min with β-glucuronidase in the absence (panels A1 and B1) and presence (panels A2 and B2) of D-saccharic acid-1,4-lactone, a specific inhibitor of β-glucuronidase. In control samples the enzyme solution was replaced with 12.5 m*M* phosphate buffer, pH 6.85. Panels A3 and B3 show the electropherograms of control samples incubated in the same way, and panels A4 and B4 the electropherograms of immediately frozen (−70°C) controls. Conditions as in Section 2. Peaks: glucuronide of entacapone (1), glucuronide of (*Z*)-isomer of entacapone (2) and entacapone in co-migration with its (*Z*)-isomer (3).

times are identical. No corresponding peaks were seen in the pre-dose urine sample (data not shown). Control samples without the enzyme were incubated in the same way to confirm that the hydrolysis was catalysed by the enzyme. Since some β-glucuronidase preparations contain sulfatase and phosphatase activity [5], the hydrolysis was also carried out in the presence of the specific enzyme inhibitor D-saccharic acid-1,4-lactone to demonstrate that the hydrolysis was β-glucuronidase-dependent. As shown in panels A2 and B2, the hydrolysis was almost completely inhibited at 20 min (and also at 3 h, data not shown), whereas in the absence of the inhibitor all glucuronide had disappeared after 3 h incubation with the enzyme (data not shown).

3.2. Selectivity and limit of quantitation

The ultimate goal of the selectivity experiments described in Section 2.6 was to evaluate the ability of the method to determine only the glucuronides of entacapone and its (*Z*)-isomer. It has been recommended that the selectivity of a method should be tested using six sources of the matrix [8]. Investigation of the electropherograms of six pre-dose urine samples revealed that the region after 15 min, where the analytes and the internal standard migrate, was relatively free from interferences and that most of the matrix components migrated faster than the doubly charged slowly migrating glucuronides (the pK_{a_2} values of the three glucuronides are about 4.1–4.6 [16]). Typical electropherograms of two pre-dose urine samples and a spiked urine sample are shown in Fig. 3. As mentioned, the parent drug entacapone co-migrated with its (*Z*)-isomer at 12 min and did not interfere with the determination of the glucuronides (Fig. 2).

Limit of quantitation (LOQ) is defined as the smallest quantity of analyte that can be determined with acceptable accuracy and precision [11]. Since our original idea was to develop a method to determine the glucuronides of entacapone and its (*Z*)-isomer in human urine with direct sample injection, with no other pretreatment than dilution, filtration and sonication, we first investigated the feasibility of direct urine injection. Unfortunately the LOQ achieved, 5 μg/ml was too high for our purposes.

An improvement in sensitivity was achieved when a solid-phase extraction procedure was included in the method. The LOQ for the glucuronides of entacapone and its (*Z*)-isomer in pretreated urine samples was set at 2 μg/ml. At this concentration, the signal-to-noise ratio (S/N) for the glucuronide of entacapone was 14 and that for its (*Z*)-isomer 12.

3.3. Linear range

If peak areas are used in CE quantitation, calibration curves (with UV detector) are usually linear over a wide concentration range [15,17]. Peak heights are less reliable in CE quantitations since height does not increase linearly at high concentrations [18]. The concentration ranges tend to be wide in bioanalysis [19]. It has been suggested that in the analysis of drugs in body fluids such as bovine plasma, human serum and urine, the calibration graphs show good linear correlations when assessed over a concentration range of not more than about two orders of magnitude [12]. For our analytes, a good linear relationship between response and concentration was obtained over the range 2–150 μg/ml. The equation was $y=0.068x+0.105$ ($r^2=0.998$) for the glucuronide of entacapone and $y=0.054x+0.092$ ($r^2=0.998$) for its (*Z*)-isomer.

Although these results of before study validation seemed good, considering that the samples were extracted before the CE separation step, there was a slight deviation from linearity at the highest calibration point (150 μg/ml). Probably because of saturation of the detector, the same deviation from linearity was seen in the calibration curves constructed for the between-day validation experiments. Evidently the range selected for validation was somewhat too wide, which emphasizes the importance of determining not only the 'lower' but also the 'upper' LOQ. An asymmetric peak shape was further evidence of capillary overloading (Fig. 4).

3.4. Within-day precision

The within-day precision (repeatability) of the method was studied at three concentrations representing the entire range of calibration. The results are given in Table 1. For the analytes, the RSD values of corrected peak areas were good (<3.2%) for the

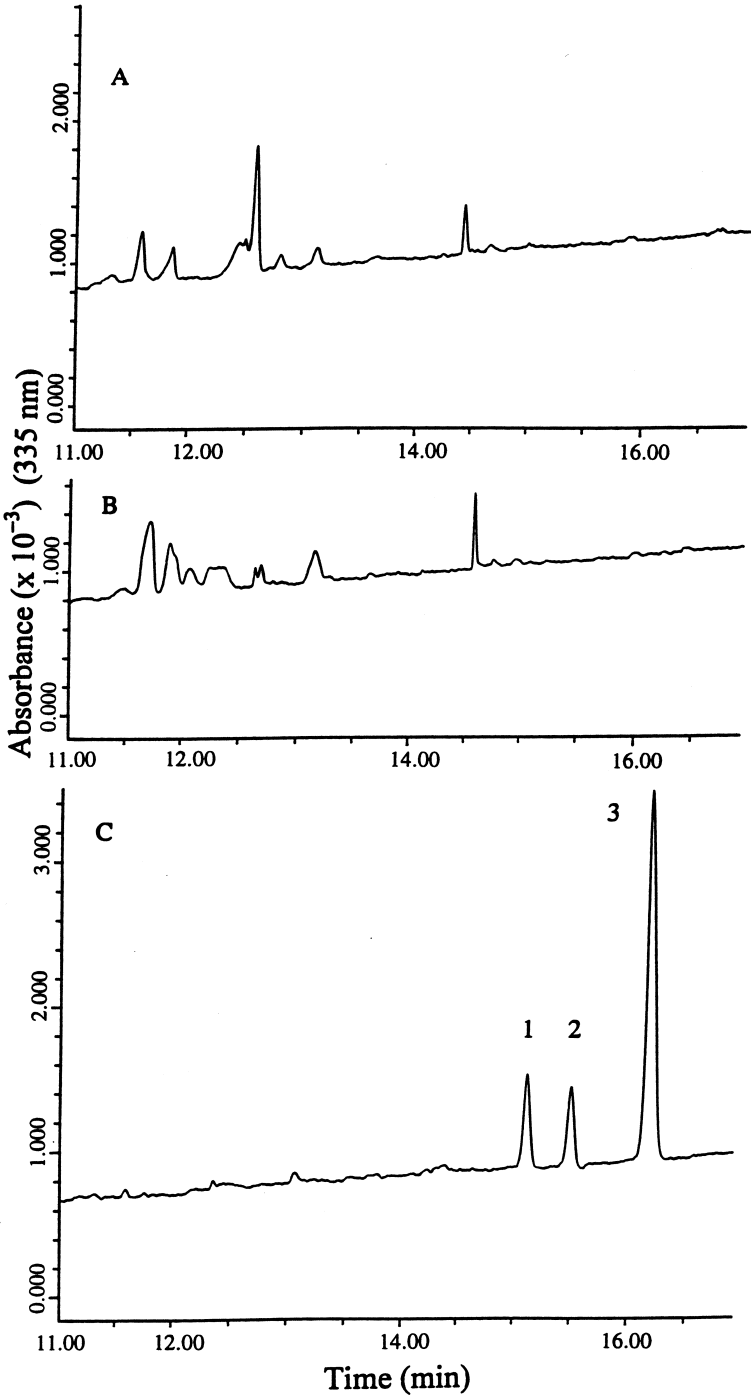

Fig. 3. Selectivity of the method. Electropherograms of two pre-dose urine samples of patients are shown in panels A and B and an electropherogram of blank urine spiked with the glucuronides of entacapone (1) (2 μg/ml), its (Z)-isomer (2) (2 μg/ml) and nitecapone, I.S. (3) (10 μg/ml) is shown in panel C. Conditions as in Section 2.

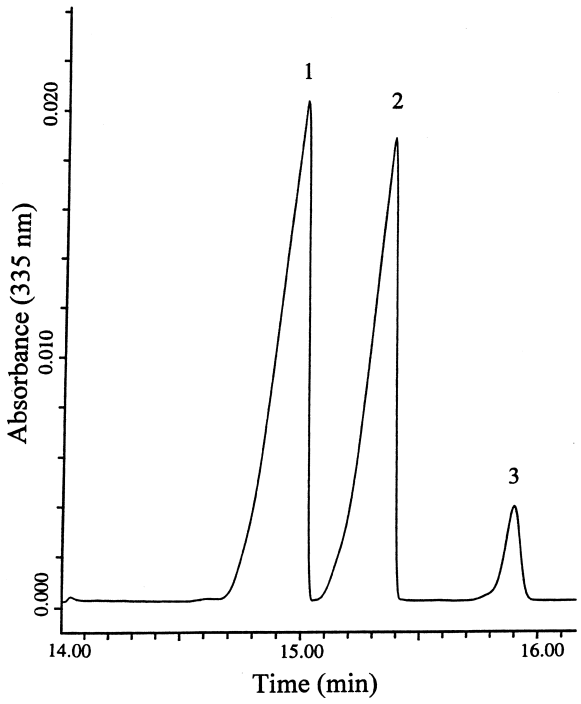

Fig. 4. Electropherogram of a calibration sample 150 μg/ml. The peaks are baseline-separated, although they are asymmetric. The figure clearly illustrates that CE quantitation based on peak heights would lead to non-linear response at high concentrations. Conditions as in Section 2. Peaks as in Fig. 3. The concentration of I.S. is 15 μg/ml.

lowest concentration of 2 μg/ml and excellent (<1.5%) for the medium concentration of 50 μg/ml. The poorer precision (<6.3%) at the highest concentration of 150 μg/ml is probably related to capillary overloading effects. The RSD values for the internal standard were slightly larger than those of the analytes and this is reflected in the results (corrected peak area ratio of the analytes to that of I.S.) as poorer precision except at the highest concentration.

The results of the injection repeatability test are also shown in Table 1. An RSD value of about 1.5% was obtained for corrected peak areas at the lowest concentration level [for (*E*)-g.], about 0.5% at the medium and about 1% at the highest concentration level. The results were very good [except for the value 3.58% for (*Z*)-g.] since the objective is to reach intermediate precisions corresponding to RSD values of below 1% [15]. Again a slight worsening of RSD values, except for the concentration 150 μg/ml, was noticed with the use of internal standard. Generally the use of internal standard is recommended in CE analysis, since it compensates for fluctuations in injection volume and usually improves the quantitative precision [15,20–22]. Sometimes, however, no improvement in the results [23] or even less reproducible results have been achieved with use of internal standards [24]. In our study,

Table 1
Validation parameters for the glucuronides of entacapone and its (*Z*)-isomer; repeatability and recovery

Parameter	Concentration (μg/ml)	*n*	(*E*)-g.[a]	(*Z*)-g.[b]	N.g.[c]	(*E*)-g./N.g.	(*Z*)-g./N.g.
Repeatability of	2	6	3.16	2.32	3.28	3.66	4.87
the method	50	6	1.48	1.54	4.21	3.25	2.92
(RSD%)	150	6	6.20	6.28	7.26	3.09	3.04
Repeatability of	2	6	1.55	3.58	1.46	2.44	2.82
injection	50	6	0.47	0.56	0.85	0.96	0.73
(RSD%)	150	6	1.10	1.02	1.00	0.53	0.60
Recovery of	2	6	94.3	96.9	90.7		
extraction	50	6	100.5	101.3	94.7		
(%)	150	6	97.9	98.8	91.0		

[a] (*E*)-g.=glucuronide of entacapone.
[b] (*Z*)-g.=glucuronide of (*Z*)-isomer of entacapone.
[c] N.g.=glucuronide of nitecapone.

incorporation of an internal standard was necessary to compensate not only for variability in injection but for errors arising during sample preparation and pretreatment. Although the peak shape of nitecapone glucuronide was good, a larger amount of internal standard could have minimised integration errors and most probably improved precision [14,15]. In any event, the RSD values obtained were well below the 15% proposed as the upper limit of acceptable precision in bioanalytical methods [8].

The stability of EOF is an important criterion for CE validation [15]. The within-day precision of the relative migration times of the glucuronides of entacapone and its (*Z*)-isomer was <0.75%, which indicates that the method conditions gave a stable and repeatable separation. Repeatability of relative migration times is shown in Table 3.

Table 3
Repeatability (within-day precision) and reproducibility (between-day precision) of relative migration times

	(*E*)-g.[a]	(*Z*)-g.[b]	*n*[c]	Days
Within-day precision				
Mean	1.833	1.877	22	1
SD	0.0132	0.0138		
RSD (%)	0.72	0.74		
Between-day precision				
Mean	1.874	1.922	17	5
SD	0.026	0.028		
RSD (%)	1.38	1.46		

[a] (*E*)-g.=glucuronide of entacapone.
[b] (*Z*)-g.=glucuronide of (*Z*)-isomer of entacapone.
[c] *n*=the number of runs used to calculate relative migration times.

3.5. Recovery

Extraction recoveries of the analytes were >94% at the three concentrations tested (Table 1). The recoveries of nitecapone glucuronide were always slightly lower. The somewhat lower recovery may explain why RSD values for repeatability were higher for nitecapone glucuronide than for the analyte glucuronides.

3.6. Between-day precision and accuracy

The between-day precision (reproducibility) and accuracy were estimated by determining the glucuronide concentrations of homogeneous QC samples on five separate days when urine samples of patients were analysed. As can be seen in Table 2, the results are surprisingly similar for the two analytes: between-day precision (RSD) was <7.5% for the lower and <5.5% for the higher concentration. The determinations were carried out by two analysts using three capillaries from the same batch. The results indicate that the method can be satisfactorily repeated by different analysts and with different capillaries.

Data for the reproducibility of relative migration times between-days is shown in Table 3. Note that the total number of runs is greater than *n* (ca. 27 runs/day) since each patient sample run was preceeded by a blank run and some runs were abandoned due to low internal standard concentration or current irregularities caused by air bubbles in the sample or buffer. The results show a greater variability in relative migration times compared with the within-day data. In part this is explained by the vast individual differences in endogenous matrix components and salt concentrations of the patient samples.

Table 2
Between-day precision and accuracy for homogeneous spiked urine samples (*n*=9)

Nominal concentration (μg/ml)	Determined mean concentration (μg/ml)		Inaccuracy		Variation SD (μg/ml)		Days
	(*E*)-g.[a]	(*Z*)-g.[b]	(*E*)-g.	(*Z*)-g.	(*E*)-g.	(*Z*)-g.	
7.5	7.68	7.69	+2.4%	+2.5%	0.56	0.57	5
75	79.21	79.11	+5.6%	+5.5%	4.38	4.36	5

[a] (*E*)-g.=glucuronide of entacapone.
[b] (*Z*)-g.=glucuronide of (*Z*)-isomer of entacapone.

3.7. Analyte stability

No essential changes were observed in the two electropherograms of the test sample after storage in the autosampler for 4.5–5 h. The difference (%) between the corrected peak area ratios (analyte to I.S.) of the test sample and the reference sample at time 0 was 2.8 for (*E*)-g. and 2.6 for (*Z*)-g. and is due to sample pretreatment (the recovery of I.S. is lower than that of the analytes). The corresponding values after storage were 4.3 for (*E*)-g. and 3.7 for (*Z*)-g. The somewhat greater difference was probably due to slight concentration of the test sample. The corrected peak area ratio, (*E*)-g./(*Z*)-g., for the test sample was 1.34 and 1.33 before and after storage, and consistent with that of the two reference samples (1.34 and 1.32). Further information on analyte stability was obtained from the hydrolysis experiments. No significant changes were observed in the corrected peak areas of the analytes when the spiked control sample, incubated in phosphate buffer for 3 h (see Section 2.5), was compared with the immediately frozen control sample. Both of these experiments suggest that the glucuronides of entacapone and its (*Z*)-isomer are relatively stable; neither isomerisation nor non-specific hydrolysis occurred in the experimental conditions. The results are in agreement with an earlier report concerning the stability of ether glucuronides in urine [25]. Freeze/thaw stability of the analytes was not tested. However, stability of the parent drug entacapone has been investigated in frozen samples and a decrease of 5–10% in the concentrations was found when spiked urine samples were stored at −20°C for 17 weeks [26].

3.8. Analysis of patient urine samples

Urine samples collected from patients after an oral (200 mg) or intravenous (25 mg) dose of entacapone were analysed. Glucuronide concentrations were determined in 17 samples from each patient group. Because the concentration range required was relatively wide, separate calibrations were used for low and high concentrations. The calibration curves were constructed by using four or five data points (2–75 or 50–150 μg/ml, except for the calibrations III and V where the highest data points were 75 and 100 μg/ml, respectively). The results of the calibrations are shown in Table 4. Except for one calibration, correlation coefficients better than 0.999 were obtained in the range 2–75 μg/ml. The correlation coefficients in the range 50–150 μg/ml were not so good. The differences between nominal concentrations and the mean determined concentrations of the calibration samples are shown in Table 5. The differences were largest at the LOQ where the deviation from the nominal value was ±16% for the glucuronide of entacapone and ±15% for its (*Z*)-isomer (two outliers accounted for the high values). At the other calibration points the difference between nominal and mean determined concentrations was considerably smaller, <4%, except at the calibration point 100 μg/ml where the difference was about 7%.

Table 4
Calibration data of the glucuronides of entacapone and its (*Z*)-isomer collected during five analysis days

	2–75 μg/ml			50–150 μg/ml[a]		
	Slope	Intercept	Correlation coefficient	Slope	Intercept	Correlation coefficient
Glucuronide of entacapone						
Mean	0.0718	−0.00178	0.99942	0.0628	0.6467	0.9915
SD	0.0036	0.040	0.0008	0.0060	0.4872	0.0059
Glucuronide of (Z)-isomer of entacapone						
Mean	0.0586	0.0007	0.99943	0.0510	0.5406	0.9923
SD	0.0028	0.0325	0.0009	0.0053	0.4256	0.0055

[a] The data are based on four calibrations only (three calibrations with the highest data point 150 μg/ml; one calibration with the highest data point 100 μg/ml).

Table 5
Systematic error (%) at seven calibration points for the glucuronides of entacapone and its (*Z*)-isomer during five analysis days

	Concentration (μg/ml)						
	2	7.5	25[a]	50	75	100[a]	150[a]
Glucuronide of entacapone							
Mean	16	1	1.92	2.18	2.96	7.12	2.26
SD	16.14	1.97	2.19	2.57	2.2	3.00	1.94
Glucuronide of (Z)-isomer of entacapone							
Mean	15	3.99	2.39	1.76	2.95	6.66	2.08
SD	13.75	1.99	2.53	2.6	2.29	2.73	1.81

[a] The data are based on three calibrations only.

From the results obtained with QC samples, it is possible to evaluate indirectly the appropriateness of daily calibration lines [19].

The glucuronide concentrations determined in the urine samples of patients varied from 2 to 150 μg/ml. After an oral administration of 200 mg of entacapone, the average concentration of the glucuronide of entacapone was >30 μg/ml, and it exceeded the calibrated range in two samples (such samples may be diluted with blank urine and reanalysed). After i.v. administration of 25 mg, concentrations were normally about 10 μg/ml, and in one sample the concentration was under 2 μg/ml. After oral administration, the concentration of the glucuronide of the (*Z*)-isomer was 5–25 μg/ml. Except for one sample the concentration of the glucuronide of the (*Z*)-isomer of entacapone after i.v. administration was under LOQ. In spite of the low concentration the peak could easily be detected in all samples. Fig. 5 shows the typical electropherograms of two patient samples, while Fig. 6 presents the relative amounts of the dose excreted in the 0–12 h urine fraction as glucuronides of entacapone and its (*Z*)-isomer after a single 200 mg oral dose of entacapone.

The experiments showed the mode of administration to have a clear effect on the proportions of the glucuronides of entacapone and its (*Z*)-isomer in urine. As the concentration of the (*Z*)-isomer glucuronide after i.v. administration was generally below the LOQ, we used corrected peak areas to evaluate the relative proportions of the glucuronides in urine. The ratio of the corrected peak area of the (*Z*)-isomer glucuronide to the summed area for both peaks was calculated. The values of the ratios for oral and i.v. administration were 0.25 ± 0.046 and 0.098 ± 0.03, respectively ($n=10$). The data strongly suggest that entacapone, when administered orally, is metabolised in notable amount to its (*Z*)-isomer during first-pass metabolism in the intestine and liver. Comparison of corrected peak area ratios may thus be used to estimate the impact of the mode of administration on the metabolism of drugs, even though amounts are too low for determination of the actual concentrations.

Steady EOF is crucial for reliable CE separations. However, the EOF may be strongly influenced by adsorbed analytes or matrix constituents [27]. Although the validation experiments showed the method to be robust and suitable for the intended use, problems were encountered in the analytical phase of the study. As revealed in the sudden drifts in migration times, probably the main source of the problems was the tendency of endogenous matrix components to adhere to the capillary wall. When this occurred, a more effective rinsing procedure was carried out to restore the correct operating conditions. In spite of effective rinsing the performance of the capillary was lost during the third day of analysis and only minor peaks were observed in the electropherogram of the calibration sample 150 μg/ml. The reliability of the results was ensured by replacing the capillary three times over the five-day period of analysis. Clearly, rinsing procedures are an important part of CE validation [15], at least in bioanalytical applications. Clearly, too, methods intended for clinical use must be extremely robust to meet the special demands of patient samples in

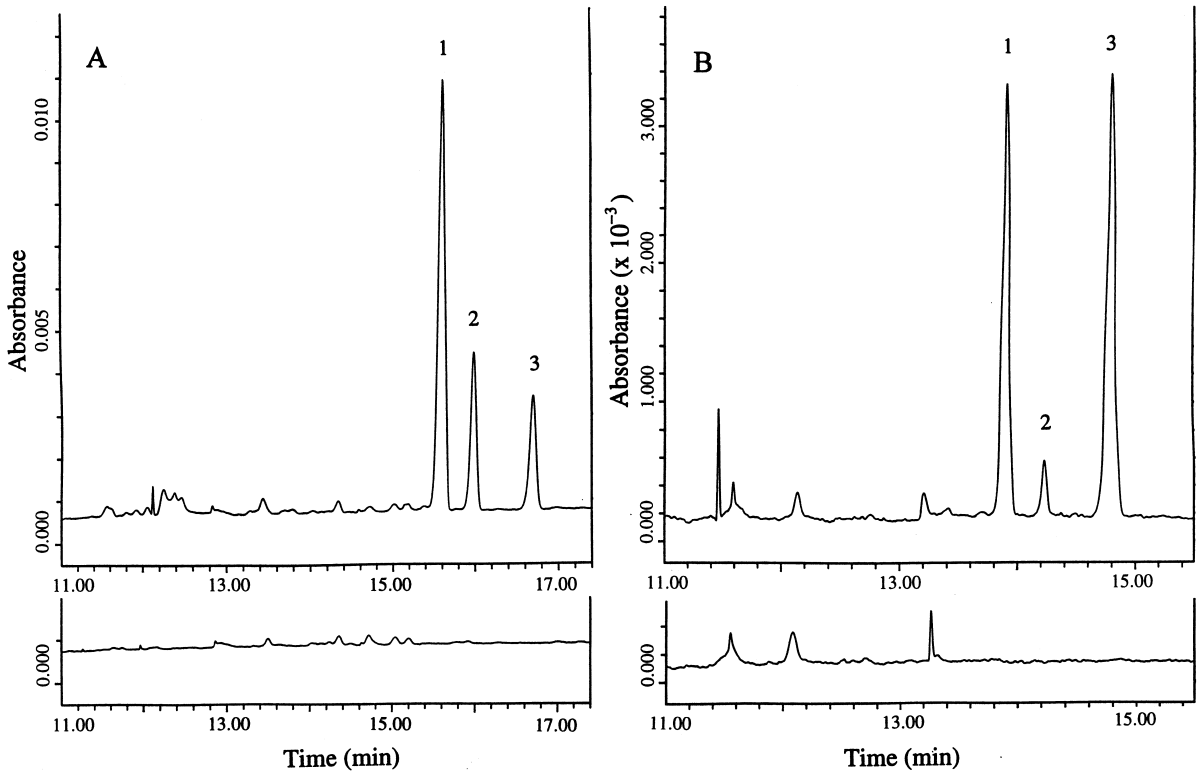

Fig. 5. Typical electropherograms of two patient samples before (lower panel) and after (upper panel) administration of entacapone. Urine was collected 0–12 h after a single 200 mg oral dose A and after a single 25 mg i.v. dose B of entacapone. The relative concentration of the (*Z*)-isomer glucuronide is higher after oral administration than after i.v. administration. The determined concentrations were in A 50.71 μg/ml (1) and 20.63 μg/ml (2) and in B 10.93 μg/ml (1) and <2 μg/ml (2). Conditions as in Section 2. Peaks as in Fig. 3. The concentration of I.S. (3) is 15 μg/ml in both samples.

which the amounts of endogenous compounds (proteins) may be many-fold those in urine samples obtained from healthy persons. (Part of the urine samples used in this study were collected from patients that suffered from impaired renal function.) The relative long term instability of this method will require frequent recalibration. Possible ways to overcome matrix effects and to improve long term stability would be to add more SDS to the running buffer solution to facilitate protein solubilisation [12], and to raise the running buffer pH. Running buffer solutions (pH 8.4–9.3) containing 65–75 m*M* of SDS are widely employed in urine analysis [4,5,23,28,29]. However, the presented validation and quantitations show that polar glucuronides can be reliably determined by CE.

4. Conclusions

CE provides a relatively new and efficient technique for modern bioanalytical research. In this study, the MECC method we recently developed for the separation of the 3-*O*-glucuronides of entacapone and its stereoisomer was validated for quantitative analysis. Although MECC assays generally require no or only minimal sample pretreatment, a solid-phase extraction procedure was included in the method to gain sensitivity. The validity of the method was established by investigating several analytical parameters for which reproducibility was good enough for the intended application of the method. The within-day repeatability with internal standardisation (corrected peak area ratios were

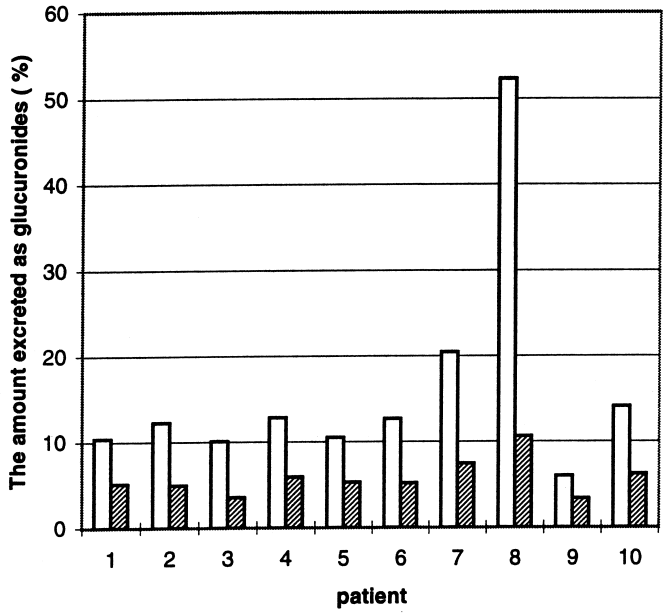

Fig. 6. Relative amounts of the dose excreted in the 0–12 h urine fraction as glucuronides of entacapone and its (*Z*)-isomer after a single 200 mg oral dose of entacapone. Open barrels: glucuronide of entacapone; striped barrels: glucuronide of (*Z*)-isomer of entacapone.

employed as the basis for data evaluation) was <5% and somewhat better without the use of internal standard (except for the highest concentration). The between-day reproducibility was <7.5% at 7.5 μg/ml. For both analytes, LOQ was 2 μg/ml. Experiments in which the validated method was applied for the analysis of urine samples collected from patients after entacapone treatment demonstrated that the method is suitable for monitoring the concentrations of the glucuronide of entacapone after both single oral (200 mg) and i.v. (25 mg) administration, and the concentrations of the (*Z*)-isomer after oral administration. After i.v. administration the concentration of the glucuronide of the (*Z*)-isomer was generally too low to be quantitated with this method. The major drawback of the method is the limited long term stability that is possibly related to adsorption of matrix components of urine on the capillary wall. These problems must be overcome before the method (without frequent recalibration) can be adopted for routine use in bioanalytical applications where long, automated analysis sequences are needed. To obtain better long term stability of the system will require further investigation of the effect of modifying the running buffer solution (increasing buffer pH and the amount of SDS or adding an organic solvent).

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