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# Quantitation of entacapone glucuronide in rat plasma by on-line coupled restricted access media column and liquid chromatography–tandem mass spectrometry

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**Abstract**

A column-switching liquid chromatography–electrospray ionization-tandem mass spectrometric (LC–ESI-MS–MS) method was developed for the direct analysis of entacapone glucuronide in plasma. The plasma samples (5 μl) were injected onto a $C_{18}$-alkyl-diol silica (ADS) column and the matrix compounds were washed to waste with a mixture of 20 m*M* ammonium acetate solution at pH 4.0–acetonitrile (97:3). The retained analyte fraction containing (*E*)- and (*Z*)-isomers of glucuronides of entacapone and tolcapone glucuronide (internal standard) was backflushed to the analytical $C_{18}$ column, with a mixture of 20 m*M* ammonium acetate–acetonitrile (85:15) for the final separation at pH 7.0. The eluate was directed to the mass spectrometer after splitting (1:100). The mass spectrometer was operated in the negative ion mode and the deprotonated molecules $[M-H]^-$ were chosen as precursor ions for the analytes and internal standard. Collisionally induced dissociation of $[M-H]^-$ in MS–MS resulted in loss of the neutral glucuronide moiety and in the appearance of intensive negatively charged aglycones $[M-H-Glu]^-$, which were chosen as the product ions for single reaction monitoring. Quantitative studies showed a wide dynamic range (0.0025–100 μg/ml) with correlation coefficients better than 0.995. The method was repeatable within-day (relative standard deviation, RSD<7%) and between-day (RSD<14%) and the recovery (78–103%) was better than with the traditional, laborious pretreatment method. The use of tandem mass spectrometry permitted low limits of detection (1 ng/ml of entacapone glucuronide). The method was applied for the quantitation of (*E*)- and (*Z*)-isomers of entacapone glucuronide in plasma of rats used in absorption studies. 

*Keywords:* Restricted access media; Entacapone glucuronide

## 1. Introduction

Entacapone is a catechol-*O*-methyltransferase (COMT) inhibitor used as an adjunct in the drug treatment of patients with Parkinson's disease (PD)

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[1]. Levodopa, the most commonly used drug in PD, is strongly inactivated by COMT, and the addition of entacapone to the medication enhances the bioavailability [2]. It has been suggested that the poor oral bioavailability of entacapone would be improved in the presence of hydroxypropyl-β-cyclodextrin (HP-β-CD) [3]. Cyclodextrins (CDs), which are cyclic oligosaccharides, are capable of forming non-covalent inclusion complexes with drug molecules. Cyclodextrins can be used to increase the aqueous solubility, dissolution rate and bioavailability of drugs [4,5]. In a study of the human metabolism of entacapone, the main metabolites of entacapone in rat and human urine were determined to be 3-*O*-glucuronide conjugates of entacapone and its (*Z*)-isomer. No glucuronide conjugates were found in human or rat plasma, probably due to the insensitivity of the method [6].

There are several reports of the determination of other glucuronides in plasma, serum or blood. Glucuronide of mycophenolic acid and diastereomeric oxprenolol glucuronides were quantitated in plasma [7,8] and diastereomeric glucuronides of oxazepam in serum by high-performance liquid chromatography (HPLC) [9]. Conjugated metabolites of angiotensin II receptor antagonist, candesartan cilexetil, were characterized in rat plasma by liquid chromatography–tandem mass spectrometry (LC–MS–MS) [10]. Several LC–MS methods have been developed for the quantitation of morphine 3-β-D-glucuronide and morphine 6-β-D-glucuronide in serum, plasma or blood [11–16]. Some of these methods are appropriate for codeine glucuronide as well [13,16]. Recently, an LC–MS method was published for the determination of ethyl glucuronide, a minor metabolite of ethanol, in human serum [17]. All these studies require pretreatment of plasma samples before analysis. Plasma is one the most difficult biological matrices to analyze, as it includes interfering compounds at high levels. Proteins may interfere in the analysis, and also the protein binding of the analytes may distort quantitation. It is essential to remove proteins before conventional HPLC analysis in order to avoid clogging of the capillaries and the column.

Restricted access media (RAM) are a class of column packing materials that were developed to allow direct injections of untreated plasma and other biological fluids. Boos and Rudolphi [18] have published a comprehensive review of these materials. Although RAM materials are prepared by a variety of methods, the principle of their operation is the same. Large molecules such as proteins only reach the hydrophilic, non-absorptive layers on the outer surface of silica particles and the access to the inner bonded phase is prevented. Small analyte molecules, in turn, penetrate the outer surface, reach the adsorption sites and are retained. RAM columns have great potential as pre-columns, used in on-line analysis with column-switching or hyphenated techniques [19]. In drug analysis as elsewhere, an increasing number of applications now rely on RAM materials [20–32], but there are no applications for glucuronides.

The aim of our study was to develop a direct and sensitive method for the quantitation of entacapone glucuronide in plasma and to test it in the analysis of rat plasma samples collected after administered entacapone dose with and without HP-β-CD. Because of the lack of commercially available standard glucuronides, glucuronides are often analyzed indirectly after acidic or enzymatic hydrolysation. Indirect methods are slow, however, and not always reliable enough. In this work enzymatically synthesized standard glucuronides made direct analysis possible. As the amount of entacapone glucuronide in rat plasma could be assumed to be minimal, the analytical technique selected was LC–MS–MS. To confirm the validity of the method we studied the following parameters: identity of the analytes, selectivity, limit of detection (LOD), limit of quantitation (LOQ), linearity, in-day and between-day precision and recovery of the extraction.

## 2. Experimental

### *2.1. Materials*

3-*O*-β-D-Glucuronides of (*E*)-entacapone (EEG) (purity over 99%), (*Z*)-entacapone (EZG) and tolcapone (TG, internal standard, I.S.) (Fig. 1) were enzymatically synthesized in our laboratory [33]. HPLC-grade acetonitrile and acetic acid were obtained from Rathburn (Walkerburn, UK). Ammonium acetate, ammonium hydroxide and perchloric

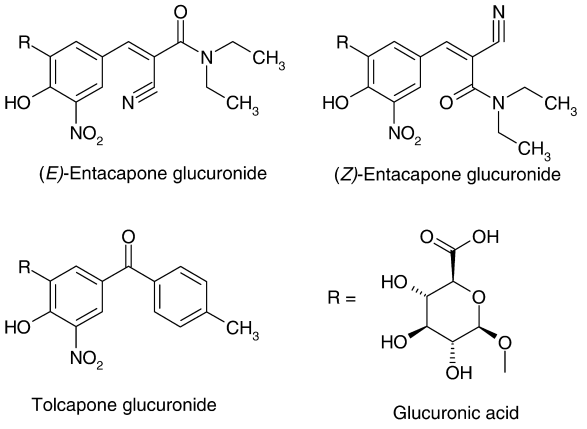


Fig. 1. Structures of the studied compounds.

acid (70–72%) of analytical-reagent grade were from Merck (Darmstadt, Germany). Entacapone (purity over 98%) was obtained from Orion Pharma (Espoo, Finland) and hydroxypropyl-β-cyclodextrin (Encapsin) from Janssen Biotech (Belgium). Water was purified in a Milli-Q water purification system (Millipore, Molsheim, France). Blank human plasma was obtained from a blood bank (SPR, Helsinki, Finland) and blank rat plasma was from Wistar rats (National Laboratory Animal Centre of the University of Kuopio, Finland).

### 2.2. Column-switching set-up

A schematic drawing of the column-switching system is given in Fig. 2. The pre-column LiChrospher RP-18 ADS (25×4 mm, 25 μm) (Merck) was connected via a six-port switching valve (Rheodyne, Cotati, CA, USA) to an analytical column, Waters Symmetry $C_{18}$ (150×3.9 mm, 5 μm) (Waters, Milford, MA, USA). The samples (5–100 μl), in water or in plasma, were loaded onto the pre-column at valve position A. Loading eluent (20 m*M* ammonium acetate, pH 4–acetonitrile, 97:3, v/v) was delivered by a HP 1100 series HPLC system (Hewlett-Packard, Waldbronn, Germany) at a flow-rate of 0.8 ml/min. After the valve had been switched to position B, the analytes were backflushed to the analytical column with a separation eluent (20 m*M* ammonium acetate, pH 7–acetonitrile, 85:15, v/v) at a flow-rate of 1.3 ml/min delivered by a Waters 510 HPLC pump. The valve was switched back to its original position after complete transfer of the analytes. The pre-column was washed during the separation step for 6 min with eluent containing 15% of acetonitrile and re-equilibrated with the loading buffer for 5 min. A Hewlett-Packard UV detector was used for the detection of the analytes (365 nm) and matrix compounds (280 nm) during method development and recovery testing. In LC–MS–MS analysis the eluate flow from the column (1.3 ml/min) was split 1:100 with a splitter (Acurate, LC Packings, San Francisco, CA, USA), and 13 μl/min (measured after splitting) was delivered to the electrospray source of the mass spectrometer.

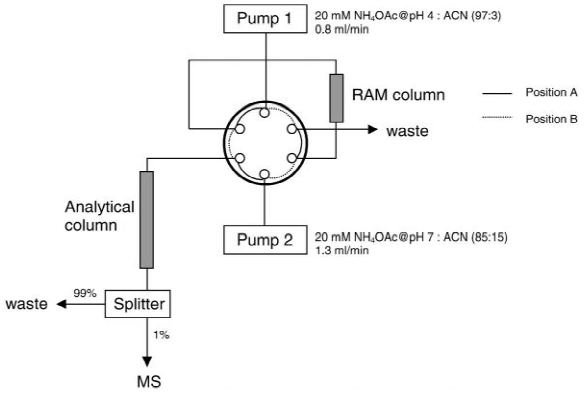


Fig. 2. Schematic drawing of the column-switching system.

### 2.3. Mass spectrometry

A Sciex API300 triple quadrupole mass spectrometer (Sciex, Concord, Canada) equipped with an electrospray ion source was used. The instrument was operated in the negative ion mode. Selected reaction monitoring (SRM) was used to monitor the loss of the glucuronide moiety. The monitored reaction was $m/z$ 480→304 for EEG and EZG, and $m/z$ 448→272 for TG. Nitrogen produced with a Whatman 75-720 (Whatman, Haverhill, MA, USA) nitrogen generator was used as curtain and collision gas, and high-purity air (99.998%) was used as nebulizing gas. The ion source and the collision conditions were tuned to produce maximum intensity of the product ions monitored. This was done manually by a direct infusion of EZG solution delivered by a micro syringe pump (Harvard Apparatus, Quebec, Canada).

### 2.4. Preparation of standards and plasma samples

All stock and working solutions of glucuronides were prepared in water and stored in a freezer (−20°C). Spiked plasma samples were prepared to cover the ranges 2.5–50 ng/ml, 50–300 ng/ml and 2–100 μg/ml by adding appropriate amounts of working solution to micro test-tubes. The amounts of internal standard (TG) were 50 ng/ml, 200 ng/ml and 20 μg/ml, respectively. Water was evaporated in a freeze–drier and the residues were dissolved in blank plasma (0.5 ml) by vortex mixing. Internal standard (TG) was added similarly to rat plasma samples. All the samples were centrifuged (4000 *g*, 10 min) and filtered (Millipore HV, 0.45 μm; Nihon Millipore, Yonezawa, Japan) before injection. Injection volumes were 5 or 100 μl.

Two parallel series of spiked plasma samples were prepared for recovery testing. The samples in one series were injected without pretreatment to the RAM column and analyzed by HPLC. The samples in the other series were pre-treated manually before analysis. In this procedure, 200 μl of 4 *M* perchloric acid was added to samples (0.5 ml) and the samples were centrifuged for 10 min at 4000 *g*. The resulting supernatants were purified by solid-phase extraction (SPE) with Sep-Pak $C_{18}$ cartridges (Waters) that had been conditioned with 2 ml of methanol and 2 ml of 50 m*M* hydrochloric acid in 2% methanol solution. After loading to the cartridges the supernatants were washed with 2 ml of 5 m*M* hydrochloric acid and 2 ml of water. The analytes were eluted with 1.5 ml of methanol and evaporated to dryness under nitrogen flow. The residues were dissolved in 0.5 ml of water, filtered and analyzed by HPLC without a RAM column. The recoveries for both pretreatment procedures were calculated by comparing the obtained results with the results obtained in direct analysis of standards prepared in water.

The matrix effect was studied to ensure that the MS–MS response was the same for samples originating from different rats. Plasma samples of five rats were spiked with EEG, EZG and I.S. (20 μg/ml) and analyzed by LC–electrospray ionization (ESI) MS–MS. The individual peak areas of both analyte and those of I.S. were examined and the precision of the results was calculated. Also the precision of the peak area ratios (analyte/I.S.) was calculated.

### 2.5. Entacapone treatment of rats and analysis of rat plasma samples

The method was applied to the quantitation of entacapone glucuronide and its (*Z*)-isomer in rat plasma samples collected from 16 rats. Male Han/Wistar rats (National Laboratory Animal Centre of the University of Kuopio) were housed in stainless steel cages and kept on a 12-h light–12-h dark cycle (lights on at 07:00) at an ambient temperature of 22±1°C. The relative air humidity was 50±10%. Pelleted food (Lactamin R36; Lactamin, Södertälje, Sweden) was removed 24 h before the experiment, but water was available at all times. Rats were 8 to 10 weeks old and weighed 260–330 g.

Entacapone was administered orally by gavage in a volume 0.5 ml 100 $g^{-1}$ water suspension (pH 5.0), $n=8$, or as an HP-β-CD solution (entacapone in 18%, w/v, HP-β-CD), $n=8$ (see Table 1 for details). Entacapone content of the preparations was 12 mg/ml. Animals were decapitated 60 and 120 min after drug administration and blood samples were collected into glass tubes containing EDTA to prevent coagulation. Plasma was separated by centrifugation at +4°C at 1500 *g* for 10 min. The plasma samples were transferred to plastic tubes and stored at −80°C until analyzed.

Table 1
Treatment of rats and sample collection

| Rat number | Weight (g) | Drug[a] | Dose (ml) | Time (min) |
|---|---|---|---|---|
| 1 | 250 | E | 1.25 | 60 |
| 2 | 260 | E | 1.3 | 60 |
| 3 | 270 | E | 1.35 | 60 |
| 4 | 260 | E | 1.3 | 60 |
| 5 | 300 | E | 1.5 | 120 |
| 6 | 290 | E | 1.45 | 120 |
| 7 | 280 | E | 1.4 | 120 |
| 8 | 300 | E | 1.5 | 120 |
| 9 | 260 | E+CD | 1.3 | 60 |
| 10 | 260 | E+CD | 1.3 | 60 |
| 11 | 270 | E+CD | 1.35 | 60 |
| 12 | 270 | E+CD | 1.35 | 60 |
| 13 | 280 | E+CD | 1.4 | 120 |
| 14 | 330 | E+CD | 1.65 | 120 |
| 15 | 330 | E+CD | 1.65 | 120 |
| 16 | 310 | E+CD | 1.55 | 120 |

[a] E=Entacapone suspension (pH 5.0), CD=entacapone-hydroxypropyl-β-cyclodextrin solution (pH 5.0), dose 60 mg/kg.

All procedures with animals were reviewed and approved by the Animal Ethics Committee of the University of Kuopio. The analyses were performed on 3 separate days and calibration graphs with six data points were constructed on each day.

## 3. Results and discussion

The objective in including the column-switching system was to enable the analysis of entacapone glucuronide in rat plasma without laborious sample preparation. The most important parameters to be optimized in the system are the breakthrough time ($t_B$) for the analytes on the RAM column, the time for wash-out of sample matrix ($t_W$), and the transfer time ($t_T$) of the analyte fraction from the pre-column to the analytical column.

### *3.1. Optimization of column-switching conditions and chromatography*

All matrix compounds should be washed to the waste from the RAM column during the first column-switching time, which determines the end point of the fractionating step. If $t_B$ is greater than 10 min, the switch can be made at $t_W$ +5 min [34]. The time needed for wash-out of sample matrix ($t_W$) was observed visually, as the time needed for the response to return back to the baseline level after plasma injection. The breakthrough of the analytes (5 μg/ml in water) was tested using 20 m*M* ammonium acetate solution at pH 7 with various amounts of acetonitrile (ACN, 0–5%) as loading eluent. The flow-rate was 0.8 ml/min. pH of the loading eluent should be kept close to physiological pH (7.4) in order to avoid precipitation of the proteins and to enhance the recovery of the analytes [20–27]. Lower pH has also been used successfully [28,29,31]. In this study, neutral mobile phase led to early breakthrough of the acidic analytes. Accordingly, 20 m*M* ammonium acetate buffer at pH 4.0 was chosen for the analysis. The addition of organic solvent to the loading eluent is advisable in order to solubilize lipid materials and thus prolong the column life-time, but the amount should be kept to a minimum to avoid protein precipitation [34]. In this work, 3% of ACN was added to the loading eluent. With this composition (20 m*M* ammonium acetate, pH 4.0–ACN, 97:3, v/v) the matrix was washed to the waste in 3 min, while $t_B$ was 15–16 min, providing a sufficiently wide window for switching. The first switching time was set to 8 min as proposed by Majors et al. [34].

The eluent in the transfer and separation steps was 20 m*M* ammonium acetate at pH 7–acetonitrile (85:15, v/v). We earlier found this composition to be suitable for the separation of EEG and EZG with the column used and also to be compatible with ESI-MS [35]. After 8 min loading time the valve was switched to position B and the adsorbed analytes were backflushed to the analytical column for the separation. The transfer of the analyte fraction was complete in 1.2 min and the valve was switched back to position A at 10.2 min as proposed by Majors et al. [34]. The parent compound, entacapone, was retained on the RAM column. Typical product ion chromatograms of blank plasma, spiked standard and authentic rat plasma samples are presented in Fig. 3. EZG and EEG as well as the internal standard (TG) were fully separated and the retention factors in the analytical co1umn for the compounds were 1.63, 2.55 and 6.60, respectively. The total analysis time was 22 min. The system was kept as simple as possible by not including gradient elution.

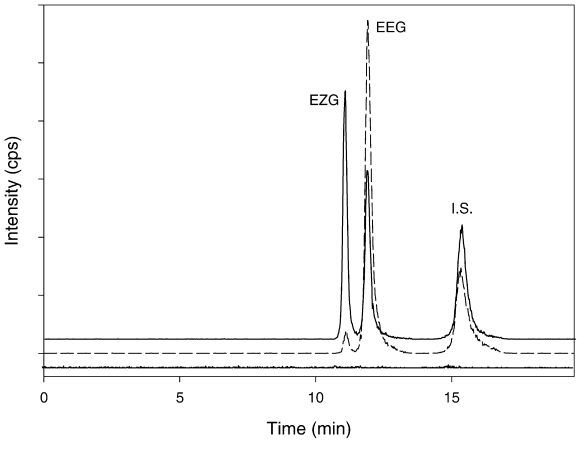


Fig. 3. Chromatograms obtained in the LC–ESI-MS–MS analysis of blank plasma, plasma spiked with EEG, EZG and I.S. (20 μg/ml) and authentic rat plasma sample (dashed line).

### 3.2. Optimization of mass spectrometry

Negative ion mode MS–MS conditions were similar to those optimized for the compounds earlier [36]. The only product ion in the negative ion mode ESI-MS–MS spectra of the compounds is deprotonated aglycone $[M-H-Glu]^-$, formed by the loss of the glucuronide moiety after cleavage of the glycoside bond. Negatively charged ether oxygen is retained on the aglycone part and the resulting product is extremely stable due to the resonance stabilization. No other ions appear in the negative product ion spectra when collision energies are low and these product ions were chosen for the SRM experiments. Monitored reactions were $m/z$ 480→304 for EEG and EZG and $m/z$ 448→272 for TG. The maximum intensity of the product ion chosen for SRM was obtained at collision energy of 30 eV.

### 3.3. Specificity and the matrix effect

Specificity of the method was evaluated by the analysis of blank plasma, standard solution of EEG, EZG and TG in water (20 ng/ml), and plasma spiked with EEG, EZG and TG (20 ng/ml). No peaks were present in the ion chromatogram of the blank plasma, and the ion chromatogram of plasma spiked with the analytes was similar to that of the standard sample (data not shown). In MS–MS both EEG and EZG produced only a negatively charged aglycone ion ($m/z$ 304), formed by the loss of the glucuronide moiety (176 u) when low collision energy (30 eV) was used. Further energy (45 eV) produced an additional ion at $m/z$ 66, which also was seen in the spectra of the standards. Specificity of the method and the absence of cross-talk between MS–MS channels was proved by injecting EEG (100 μg/ml) to the system and monitoring the response at the channel of I.S. at the LOQ setting of the $y$-axis (Fig. 4). Internal standard (20 μg/ml) was also injected to the system and the lack of response in the channel of the analytes was confirmed. No disturbing peaks in either of the channels were noticed after injection of blank plasma.

Matrix effect can affect on the reproducibility of the assay [37,38]. The matrix effect is caused by high concentrations of coeluting matrix components leading to ion supression. This can be seen as unacceptably high RSD values of the peak area of the analyte or the internal standard [37]. In our study the precision of the absolute peak areas for both analytes (20 μg/ml) and I.S. (20 μg/ml) spiked into five different batch of plasma was 7.3% for EZG and EEG and 8.5% for TG, respectively. The precision of the peak area ratios (analyte/I.S.) was even better being 6.9% for EZG and 5.7% for EEG indicating that I.S. improved the precision of the method and that the extraction efficiency of both analytes and I.S. is similar despite the source of the matrix. The data proves the lack of the matrix effect and thus no need for further examination of the matrix effect exist.

### 3.4. Linearity and precision

The linearity of the method was studied between 0.0025 and 100 μg/ml (Table 2). Three calibration graphs for EEG and EZG were constructed using spiked plasma samples of at least five different concentrations in each series to cover the range, and by plotting the peak-area ratios of compounds to the internal standard as a function of concentration. The lowest standard curve was 2.5–50 ng/ml, the next 50–300 ng/ml and the highest 2–100 μg/ml. The amounts of internal standard were 50 ng/ml, 200 ng/ml and 20 μg/ml, respectively. Although all the curves were linear, with correlation coefficients better than 0.995, only the highest range was further validated and used for the quantitation of the samples, since samples were found to contain EEG in high concentration. The linearity was very good with correlation coefficient 0.999, slope 1.817 and intercept 0.0017. The within-day precision of the method was evaluated by injecting five plasma samples from different rats spiked with EZG and EEG (20 μg/ml). RSDs of the peak area ratios were found to be 6.9% and 5.7% for EZG and EEG, respectively. RSDs of repetitive injections ($n=7$) of one sample were 1.9% and 1.3% for EZG and EEG, respectively, demonstrating the reliability of the instrument. The between-day precision was estimated from the results obtained in the analysis of the one sample on 7 different days and determined to be 7.7% for EZG and 14% for EEG. All values are acceptable for biological samples.

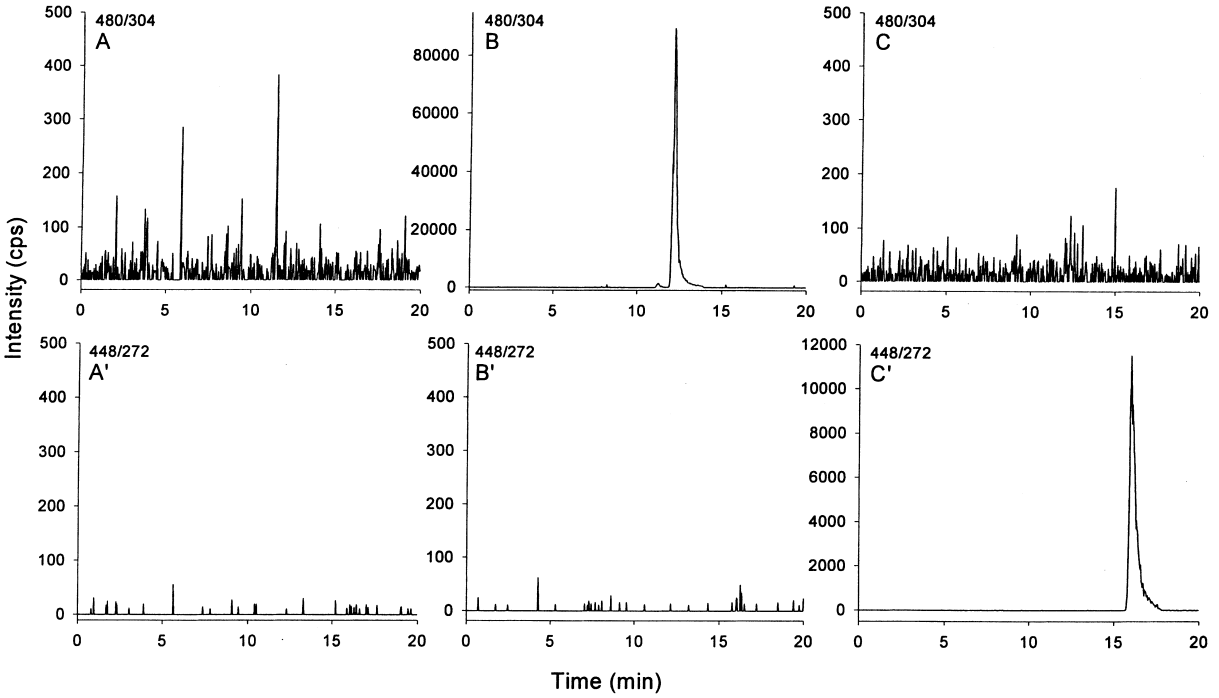


Fig. 4. Representative LC–ESI-MS–MS chromatograms of plasma (5 μl) obtained by multiple reaction monitoring at $m/z$ 480→304 (channel ''a'') for entacapone glucuronide and $m/z$ 448 →272 (channel ''b'') for internal standard; A, A′, blank plasma monitored at channels ''a'' and ''b'', respectively; B, B′, plasma spiked with 100 μg/ml EEG and monitored at channels ''a'' and ''b'', respectively; C, C′, plasma spiked with 20 μg/ml of I.S. and monitored at channels ''a'' and ''b'', respectively.

### *3.5. Recovery*

Recovery of the RAM-LC–MS method was determined at three concentration levels: 2, 20 and 100 μg/ml (Table 3). The recoveries were calculated by comparing the peak areas of spiked plasma samples obtained in the analysis after pretreatment, as described in the Experimental section, with those of standards prepared in water. The SPE method applied was earlier successfully used in the analysis of the same compounds in urine samples [35,36,39]. However, the low recoveries obtained here (10–61%) showed SPE to be inappropriate for plasma samples. The recoveries obtained with the RAM column set-up were significantly better, being 78–95% and 97–101% for EEG and EZG, respectively. The reason for the lower recovery of EEG, at all concentration levels, was studied by preparing water

Table 2
Regression data from the linearity study of EEG and EZG

| Compound | Concentration (μg/ml) | *n* | Correlation coefficient | Slope | Intercept |
|---|---|---|---|---|---|
| (*E*)-Entacapone glucuronide | 0.0025–0.05 | 7 | 0.9959 | 0.0166 | 0.0394 |
| | 0.1–0.3 | 5 | 0.9954 | 0.0481 | 0.00027 |
| | 2–100 | 6 | 0.9992 | 1.8175 | 0.0017 |
| (*Z*)-Entacapone glucuronide | 0.0025–0.05 | 7 | 0.9980 | 0.0276 | 0.0248 |
| | 0.1–0.3 | 5 | 0.9953 | 0.0732 | −0.00017 |
| | 2–100 | 6 | 0.9984 | 2.0674 | −0.01408 |

Table 3
Results of the recovery studies ($n=6$)

| Concentration (μg/ml) | (*E*)-Entacapone glucuronide (%) | | (*Z*)-Entacapone glucuronide (%) | |
|---|---|---|---|---|
| | SPE | RAM | SPE | RAM |
| 2 | 10.0 | 94.7 | 16.4 | 100.8 |
| 20 | 29.4 | 77.9 | 40.4 | 96.7 |
| 100 | 51.4 | 88.6 | 61.0 | 102.6 |

and plasma samples spiked with equal amounts of EEG and EZG. Analysis of the sample prepared in water gave 52% of EEG and 48% of EZG, but the spiked plasma sample gave 42.3% of EEG and 57.7% of EZG. The probable reason for the lower value of EEG is that the structure of the (*E*)-isomer is more open and has a larger surface to bind with the proteins in the plasma. Being tightly bound to the proteins, some of the EEG is eluted with them, through the RAM to the waste. The difference in three-dimensional structure of the compounds also explains the elution order of the compounds in reversed-phase column; the (*Z*)-isomer with more closed structure elutes first.

### *3.6. Limit of detection and limit of quantitation*

High sensitivity is essential for methods to be used in bioanalysis. Analytes may be present in extremely small amounts, as at the very beginning or end of the series in pharmacokinetic studies. Larger sample volumes can be injected to improve the sensitivity of the method, and this approach is commonly used with RAM columns [20,22–26,29,31]. RAM columns typically tolerate over 1 ml of plasma without loss of extraction efficiency or pressure rise. The injection volume in this work was limited to 100 μl, however, owing to the autosampler available, and the LOD ($S/N=3$) was 1 ng/ml for the (*E*)- and (*Z*)-entacapone glucuronides. After splitting (1:100), the amount of compound introduced to the mass spectrometer was 1 pg (2.1 fmol). The LOQ was determined by injections of plasma spiked with EEG and EZG in decreasing concentrations. The injection volume was 5 μl, the same as in the analysis of the rat plasma samples. The reproducibility of the injections ($n=6$) was calculated for each concentration. The LOQ for EEG and EZG was 0.08 μg/ml with RSDs below 7 and 9%, respectively (Fig. 5).

### *3.7. Analysis of rat plasma samples*

The method was applied to the analysis of plasma samples collected from 16 rats. The concentration of EEG in the samples was relatively high and thus only the highest calibration (2–100 μg/ml) was needed. The average EEG concentrations determined in the plasma samples collected from rats treated with entacapone suspension (pH 5.0) and with entacapone and HP-β-CD solution (pH 5.0) were 26.84 and 37.32 μg/ml 1 h after treatment and 33.83 and 38.54 μg/ml 2 h after treatment, respectively. As can be seen from the results, HP-β-CD appears to improve the absorption of entacapone. The rats treated with HP-β-CD as well as entacapone exhibited noticeably higher concentration of EEG in plasma after 1 h than did rats treated with entacapone alone after 2 h. Entacapone and its (*Z*)-isomer isomerize to each other in plasma fairly slowly in vitro [6]. Equilibrium concentrations were reached in 72 h, at which point the incubations contained 68% of entacapone and 32% of its (*Z*)-isomer. In the rat

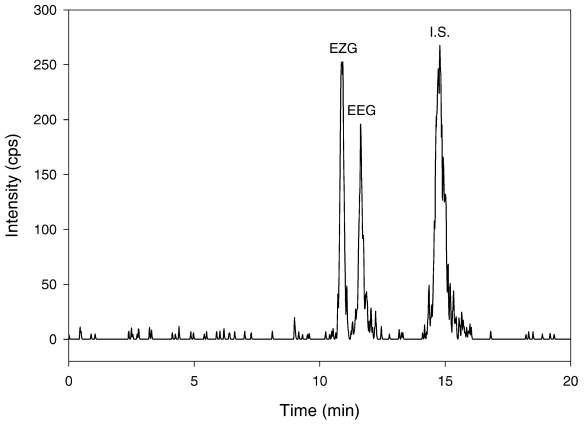


Fig. 5. Representative chromatogram obtained at the limit of quantitation (0.08 μg/ml).

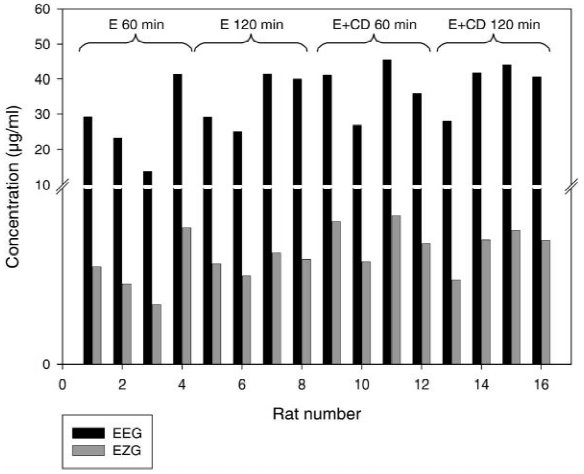


Fig. 6. Results obtained in the analysis of rat plasma samples. Black barrels: EEG=(*E*)-entacapone glucuronide, grey barrels: EZG=(*Z*)-entacapone glucuronide. Concentration of EZG is multiplied by 10. E=Entacapone, CD=encapsin. Please note the cut on the *y*-axis.

plasma samples the concentrations of EZG were clustered around the lowest calibration point, and some of them were even under the range, so that the results are only approximate. The average concentration of EZG varied between 1.59 and 2.19 μg/ml, that is, between 5.1 and 5.9% of EEG. However, reliable simultaneous analysis of the (*Z*)-isomer would demand another internal standard for the method and a lower calibration range. The results of the analysis of individual samples are presented in Fig. 6. The wide variation in the results is due to the small number of animals and individual metabolism profiles.

## 4. Conclusions

The hyphenation of RAM column clean-up, HPLC separation and MS–MS detection provides fast and automated analysis of complex protein-containing samples with minimal sample pretreatment. In this study, a column-switching LC–ESI-MS–MS method was developed for direct quantitative determination of entacapone glucuronide in plasma. The total analysis time was just 22 min, as samples required only centrifuging and filtering before analysis. The validity of the method for quantitative analysis was established. The within-day repeatability was below 6% and the between-day repeatability below 14% for entacapone glucuronide, which is acceptable for the intended application of the method. The method was linear ($r$=0.9954–0.9992) in a wide concentration range and, with a detection limit of 2.1 fmol, extremely sensitive. The applicability of the method was confirmed in the analysis of 16 rat plasma samples. Entacapone was excreted in plasma as glucuronide conjugates and both (*E*)- and (*Z*)-isomers were characterized and quantitated.

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