ELSEVIER

Journal of Chromatography A, 794 (1998) 75–83

JOURNAL OF CHROMATOGRAPHY A

Analysis of catechol-type glucuronides in urine samples by liquid chromatography–electrospray ionization-tandem mass spectrometry

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Abstract

A direct and fast liquid chromatographic–electrospray ionization-tandem mass spectrometric (LC–ESI-MS–MS) method is described for the determination of 3-O-glucuronides of *E*- and *Z*-entacapone, nitecapone and tolcapone and 1-O- and 2-O-glucuronides of 4-nitrocatechol in urine. *p*-Nitrophenyl β-D-glucuronide was used as internal standard. Spiked urine samples were prepared by solid-phase extraction and analysed by isocratic LC–ESI-MS–MS in negative ion mode. The ESI mass spectra showed an abundant deprotonated molecule $[M-H]^-$, which was chosen as precursor ion. Collisionally induced dissociation of $[M-H]^-$ in MS–MS resulted in the loss of neutral glucuronide moiety and in the appearance of an intense negatively charged drug molecule, which was chosen as the product ion to be monitored in the LC–MS–MS analysis. The new method showed good linearity ($r^2>0.997$) and repeatability of the method (relative standard deviation <2.56%). The limits of detection were determined to be 0.1–0.2 μg/ml when 5 μl of the spiked urine was used for the analysis (5–10 pg of glucuronide introduced to ESI-MS–MS).

Keywords: Detection, LC; Mass spectrometry; Glucuronides

1. Introduction

Catechol-O-methyltransferase (COMT) is an important enzyme, found in nearly all human tissue. COMT has an important role in inactivating catecholamines of physiological origin (e.g. noradrenaline, adrenaline and dopamine) as well as of environmental origin (e.g. in food and medicines). Levodopa, the most widely used drug for Parkinson's disease, is one of the xenobiotics metabolized by COMT. The effects of levodopa can be enhanced by inhibiting COMT. Nitrocatechol derivatives are potent inhibitors of COMT and some of them, such as entacapone, may be beneficial when used together with levodopa and a dopa decarboxylase inhibitor in the medical treatment of patients with Parkinson's disease [1].

Biotransformation reactions of xenobiotica are generally divided into two classes. In phase I reactions, polar functional groups are generated in the molecule. Typical phase I reactions are oxidation, reduction and hydrolysation. In phase II reactions, which are typical for nitrocatechol derivatives, the drug and/or its phase I metabolites are conjugated with an endogenous species such as glucuronic acid, sulfate or a small peptide. These conjugated products are polar and water soluble, and thus readily excreted in bile or urine. Glucuronidation is generally consid-

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0021-9673/98/$19.00
PII S0021-9673(97)00885-6

ered to be a major conjugation mechanism for xenobiotics in mammals. For the compounds studied here the site of the glucuronidation is the OH group in *meta* or *para* position relative to the nitro group (Fig. 1) [2]. Nitrocatechol derivatives can also undergo two other conjugation reactions, namely methylation and formation of sulfate esters [3].

To be able to postulate metabolic pathways and kinetics for the biotransformation reactions of drug candidates requires the use of highly selective and sensitive bioanalytical methods. One must be able to identify and quantitate conjugated products in complex biological matrices, and preferably without time-consuming separation of analytes. Identification and quantitation of glucuronides in biological samples is often performed indirectly, by first hydrolysing conjugates with acid [4,5] or enzymes [6,7], because there are few commercial glucuronides available for calibration. However, indirect methods are time-consuming and not always reliable, for example if the hydrolysis is incomplete. Radioactive labelling is a very useful tool in biotransformation studies and frequently utilized [8–10], but it is undesirable in human experiments and not compatible with routine metabolic profiling.

Glucuronides are polar, non-volatile compounds and thus often determined by liquid chromatographic methods. Thermospray ionization is widely used in the analysis of biotransformation products, but the heat used in the ionization process has been shown to cause thermal decomposition of conjugates [11]. Electrospray ionization (ESI) is a mild ionization technique, which has successfully been applied in positive ion mode in the analysis of glucuronides [5,8,12]. However, positive ion ESI provides high sensitivity only for compounds that can be protonated in liquid phase. Because of their acidic character, glucuronides in liquid phase are easily deprotonated and thus are better analysed by negative ion ESI [13]. Owing to the interfering background ions at lower m/z values, moreover, both positive and negative ion ESI-MS may lack selectivity in the analysis of glucuronides at low concentration levels. The selectivity of analysis can be dramatically increased by the use of tandem mass spectrometry, which allows rapid sample preparation and short LC runs, and thus very short total analysis times.

The goal of the present study was to develop a fast and simple LC–ESI-MS–MS method using negative ion mode to the analysis of catechol-type glucuronides in urine. To evaluate the biotransformation and its mechanism, several nitrocatechol-type glucuronides were synthesised at the Department of Pharmacy [14].

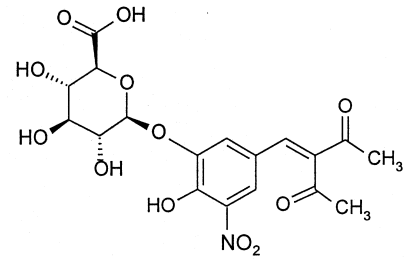
2. Experimental

2.1. Materials

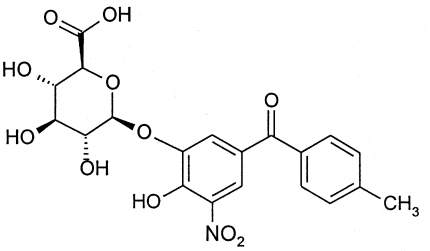
All solvents were HPLC grade. Acetonitrile and methanol were purchased from Rathburn (Walkerburn, UK). Ammonium acetate, ammonium hydroxide and hydrochloric acid were analytical-reagent grade and purchased from Merck (Darmstadt, Germany). 3-O-Glucuronides of nitecapone (NG), tolcapone (TG), *E*- and *Z*-entacapone (EEG, EZG), 3,4-dihydroxy-5-nitrobenzaldehyde (AG, degradation product of EEG, EZG and NG) and 1-O- and 2-O-glucuronides of 4-nitrocatechol (1-O-NKG, 2-O-NKG) were synthesised using liver microsomes from rats pretreated with Aroclor 1254 (RCS-088/Analabs, lot No. K040, North Haven, CT, USA) as catalyst. The reaction mixture contained 50 m*M* phosphate buffer (pH 7.4), 10 m*M* magnesium chloride, 1.2–5 m*M* substrate, 5 m*M* excess uridine 5′-diphosphate glucuronic acid (UDPGA) triammonium salt purchased from Sigma (St. Louis, MO, USA) and 5 m*M* 1,4-saccharic acid lactone (Sigma). The glucuronides were purified by solid-phase extraction (Bakerbond C_{18} Polar Plus) and the chemical structures of the compounds were confirmed by analysis of UV and IR spectra and in fast atom bombardment (FAB) MS, ^{1}H and ^{13}C NMR experiments. *p*-Nitrophenyl β-D-glucuronide from Sigma was used as internal standard (I.S.). Water was purified in a Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Liquid chromatography

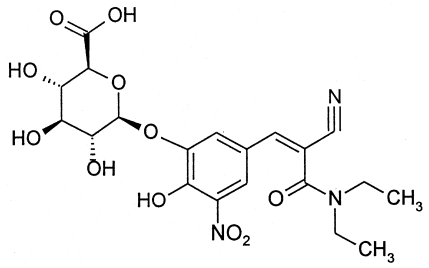
The liquid chromatograph used in the method development was a Perkin-Elmer series LC 200 pump equipped with a Perkin-Elmer series 200 autosampler and with a Perkin-Elmer diode array detector 235C connected via a PENelson 600 series



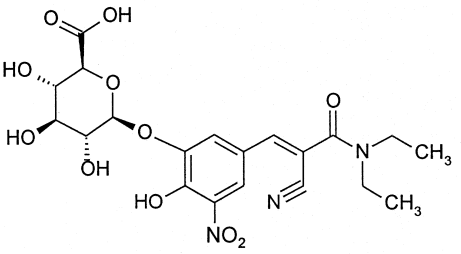
Nitecapone glucuronide (NG)



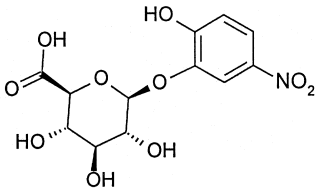
Tolcapone glucuronide (TG)



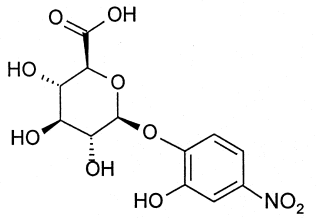
Z-entacapone glucuronide (EZG)



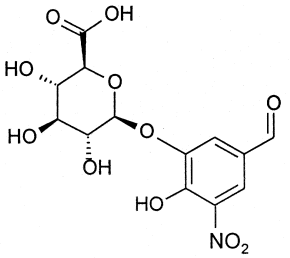
E-entacapone glucuronide (EEG)



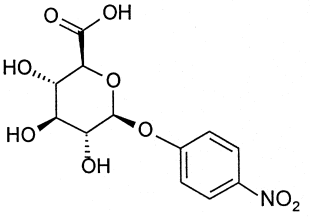
4-nitrocatechol-2-O-glucuronide (2-O-NKG)



4-nitrocatechol-1-O-glucuronide (1-O-NKG)



3,4-dihydroxy-5-nitrobenzaldehyde-3-O-glucuronide (AG)



4-nitrophenyl glucuronide (PNPG)

Fig. 1. Structures of nitrocatechol glucuronides.

LINK (Norwalk, CT, USA) to PE Nelson Division's Turbochrom software. The guard column was 1 cm× 4.6 mm I.D. packed with 5 μm Inertsil ODS-2 stationary phase (GL Sciences, Tokyo, Japan), and the analytical column was 15 cm×3.9 mm I.D. packed with 5 μm Waters Symmetry C_{18} stationary phase (Waters, Milford, MA, USA). The isocratic LC mobile phase consisted of 10 m*M* ammonium acetate adjusted to pH 7.0 with ammonium hydroxide and acetonitrile (10–20%), flow-rate was 1 ml/min. The composition of the mobile phase was optimized separately for each glucuronide. Mobile phases were filtered and degassed before use. Diode array detection was set at 365 nm for entacapone glucuronide isomers, 4-nitrocatechol-2-O-glucuronide and nitecapone glucuronide, at 340 nm for tolcapone glucuronide and at 335 nm for 4-nitrocatechol-1-O-glucuronide. LC separations were performed at room temperature. These LC conditions were also used in LC–MS analysis. However, the liquid chromatograph used in LC–MS was an HP 1090 equipped with a Rheodyne (Cotati, CA, USA) injector with a 5-μl loop. The eluate flow from the column (1 ml/min) was split 1:100 with a pneumatic splitter and 10 μl/min were delivered to the electrospray source of a mass spectrometer. A micro syringe pump (Harvard Apparatus, USA) equipped with a Rheodyne injector was used in the recording of ESI mass spectra and in the optimization of ion source and MS–MS parameters. The samples in acetonitrile–water (15:85) were injected via a 100-μl loop at a flow-rate of 10 μl/min.

2.3. Mass spectrometry

The mass spectrometer was a Quattro II tandem quadrupole mass spectrometer (Micromass, Altrincham, UK) with a Micromass MassLynx data system. The electrospray interface was a standard Micromass pneumatically assisted ESI source operating with a capillary voltage of 2.8 kV and temperature of 70°C. The operating parameters were tuned manually during direct infusion of a 10 μg/ml solution of a glucuronide. The cone voltage was optimized by recording mass spectra at voltages between 5 and 70 V. The cone voltage is the voltage difference between the lenses in the atmospheric ion source. The mass spectrometer was operated in negative ion mode.

The collision conditions in tandem mass spectrometry were optimized by recording product ion spectra of deprotonated molecules of the compounds at collision energies between 5 and 40 eV. The scan range in the recording of product ion spectra was from m/z 50–500 (3 s/scan). The maximum intensity of a product ion chosen for single reaction monitoring (SRM) in LC–ESI-MS–MS analysis was obtained at a collision energy of 30 eV and at a collision gas (argon) pressure of 5×10^{-4} mbar. In LC–ESI-MS–MS runs, the resolution of quadrupoles one and three was tuned to unit resolution. When the internal standard (I.S.) and analyte were fully separated by LC the m/z values of precursor and product ions were monitored successively during the LC run. In cases where full separation of the I.S. and analyte was not obtained, the m/z values of precursor and product ions were monitored scan by scan.

2.4. Samples

Standard solutions of analytes were prepared by dissolving an appropriate amount of each compound in urine to give a concentration of 0.1 mg/ml. The internal standard, *p*-nitrophenyl glucuronide, was prepared in concentration 0.3 mg/ml. Spiked samples were prepared by adding appropriate amounts of standard solutions and 0.5 ml of internal standard solution to urine collected from healthy volunteers. Total volume of the sample was 5.0 ml. The solutions were stored at −70°C.

Urine samples were prepared by solid-phase extraction (SPE) using Waters Sep-Pak Plus C_{18}-cartridges preconditioned with 1.0 ml of methanol and 1.0 ml of 0.05 *M* hydrochloric acid in 2% methanol. Urine sample (1.0 ml) was acidified (pH 1, pH paper) with 50 μl of 1 *M* hydrochloric acid and transferred to the SPE cartridge and washed with 1.0 ml of 0.002 *M* hydrochloric acid and 1.0 ml of water. Analytes were eluted from the cartridge with 1.5 ml of methanol and dried in vacuum. Samples were diluted to 1.0 ml with water and filtered (Millipore HV 0.45 μm, Nihon Millipore, Yonezawa, Japan). Five μl of the filtrate was injected into the LC column. Calibration graphs were constructed for both entacapone glucuronide isomers and tolcapone

glucuronide from spiked urine samples of six different concentrations (1–60 μg/ml) each including 30 μg/ml of internal standard. Graphs were constructed by plotting peak–area ratios of compounds to the internal standard as a function of concentration. Repeatability of the method and that of the instrumentation were tested with both entacapone glucuronide isomers at a concentration level 10 μg/ml.

3. Results and discussion

LC analysis of glucuronides has usually been performed by ion-pair chromatography [6,15] or reversed-phase chromatography under acidic conditions [16–18]. The use of negative ion ESI demands that the analytes are already negatively charged in the eluent. Ammonium acetate buffer solution at pH 7.0 was chosen as eluent to confirm the ionization of the acidic glucuronides. Ammonium acetate was selected as buffer species because it easily volatilizes and is thus appropriate to the electrospray ionization. Under these buffer conditions, compounds were found to exhibit moderate retention under reversed-phase conditions with eluent containing 10–20% of acetonitrile. The retention of glucuronide conjugates increased with the concentration of ammonium acetate, and the most appropriate concentration (10 m*M*) was selected from concentrations of 2–20 m*M*. Acetonitrile was preferred over methanol as organic solvent because peak shapes and peak separation were superior.

The use of selected buffer solution provided good ionisation efficiency in negative ESI due to the acidic character of glucuronides. Good sensitivity in positive ion ESI would be expected in acidic solutions only for glucuronides that include a basic group. Pneumatic-assisted nebulization was used in ESI, since the eluent used in LC–ESI-MS–MS analysis contained large amounts of water (80–90%). The high surface tension and evaporation heat of water interfere with ion evaporation and droplet disintegration and may lead to an unstable ionization process and reduced sensitivity.

Operating parameters for recording ESI-MS spectra were tuned manually to maximum intensity of the deprotonated molecule. Cone voltage is an important parameter for adjusting the amount of fragmentation

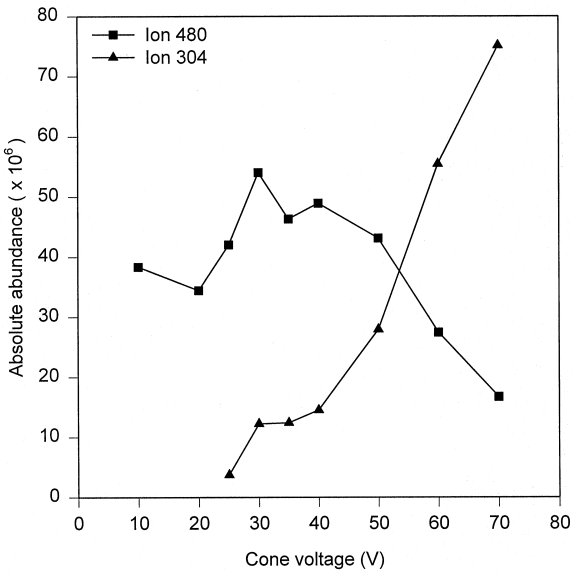


Fig. 2. Effect of cone voltage to the fragmentation of *E*-entacapone glucuronide. The ions *m*/*z* 480 and *m*/*z* 304 are $[M-H]^-$ and $[M-Glu]^-$, respectively.

of the deprotonated molecule, and Fig. 2 shows the effect of this parameter on the fragmentation of *E*-entacapone glucuronide. The fragment ion (*m*/*z* 304) formed by loss of the glucuronide moiety from the deprotonated molecule (*m*/*z* 480) appeared at a cone voltage of 25 V, and its abundance increased noticeably when the cone voltage was increased from 25 to 70 V. As shown in Fig. 2, the total abundance of ions and thus the sensitivity in multiple ion monitoring does not decrease at high cone voltages. The reliability of identification with ESI-MS can be increased by using high cone voltages, since at high voltages fragment ions will be generated and multiple ion monitoring can be used. The maximum intensity of the deprotonated molecule (precursor ion) was obtained at a cone voltage of 30 V for MS–MS analysis.

The ESI mass spectra recorded at a cone voltage of 30 V are presented in Table 1. All spectra showed abundant deprotonated molecules, a fragment ion $[M-Glu]^-$ formed by the loss of the glucuronide moiety as a neutral species, leaving ether oxygen and negative charge with the drug moiety, and a weak sodium adduct ion. The ion $[M-Glu]^-$ was the base peak in the spectrum of 3,4-dihydroxy-5-nitrobenzal-

Table 1
ESI mass spectra and MS–MS spectra of glucuronides studied

Compound	*M*	MS spectra (*m*/*z*) (cone voltage 30 V)			MS–MS spectra (*m*/*z*) (collision energy 30 eV)	
		$[M-2H+Na]^-$	$[M-H]^-$	$[M-Glu]^-$	Precursor ion	Product ion
AG	359	380 (15.5)	358 (79.9)	182 (100.0)	358 (3.0)	182 (100.0)
EEG	481	502 (8.2)	480 (100.0)	304 (22.6)	480 (5.0)	304 (100.0)
EZG	481	502 (5.3)	480 (100.0)	304 (32.5)	480 (6.9)	304 (100.0)
NG	441	462 (1.9)	440 (100.0)	264 (17.7)	440 (4.1)	264 (100.0)
1-O-NKG	331	352 (0.6)	330 (100.0)	154 (62.3)	330 (1.4)	154 (100.0)
2-O-NKG	331	352 (1.3)	330 (100.0)	154 (15.2)	330 (1.4)	154 (100.0)
TG	449	470 (2.5)	448 (100.0)	272 (9.4)	448 (2.0)	272 (100.0)

M, molecular mass; $[M-Glu]^-$, fragment ion formed by the loss of glucuronide moiety, intensity in parentheses.

dehyde glucuronide (AG). 4-nitrocatechol-1-O-glucuronide (1-O-NKG) produced more abundant $[M-Glu]^-$ than 4-nitrocatechol-2-O-glucuronide (2-O-NKG), indicating higher stability of the deprotonated molecule of 2-O-NKG than that of 1-O-NKG. It follows that these regional isomers can be distinguished on the basis of ESI-MS spectra recorded at a cone voltage of 30 V. The abundances of $[M-Glu]^-$ in the spectra of the *E*- and *Z*-entacapone glucuronides are only slightly different and these isomers cannot be reliably distinguished on the basis of ESI-MS spectra. Although ESI-MS offers good potential for the analysis of glucuronides, background ions may cause problems. ESI-MS spectra included several interfering ions at low mass range, which disturbed the analysis at low concentrations. To improve the reliability of the analysis, tandem mass spectrometry was preferred in the study of glucuronide metabolites.

The deprotonated molecule was chosen as the precursor ion in tandem mass spectrometric analysis. The most abundant product ion formed in the collisionally induced dissociation was $[M-Glu]^-$ (Table 1). The effect of collision energy on the formation of $[M-Glu]^-$ is demonstrated with *Z*-entacapone and tolcapone glucuronides in Fig. 3. For both compounds the maximum absolute abundance of the product ion was achieved at the collision energy 30 eV, which provided maximum sensitivity in single reaction monitoring (SRM) in LC–ESI-MS–MS analysis. At energies below 30 eV $[M-Glu]^-$ was the only product ion, but at higher energies the number of product ions increased. The MS–MS spectra of 1-O-NKG and 2-O-NKG recorded at low collision energies showed that the deprotonated ion of 1-O-NKG dissociated with lower collision energies than that of 2-O-NKG (Fig. 4), indicating and confirming the conclusion made with MS spectra (Table 1) that the deprotonated ion of 2-O-NKG is more stable than that of 1-O-NKG. The spectra of *Z*- and *E*-entacapone glucuronides were very similar and these isomers cannot be distinguished by MS–MS (Table 1).

The monitored precursor and product ions, retention times and eluent compositions used in LC–ESI-MS–MS analysis are presented in Table 2. The

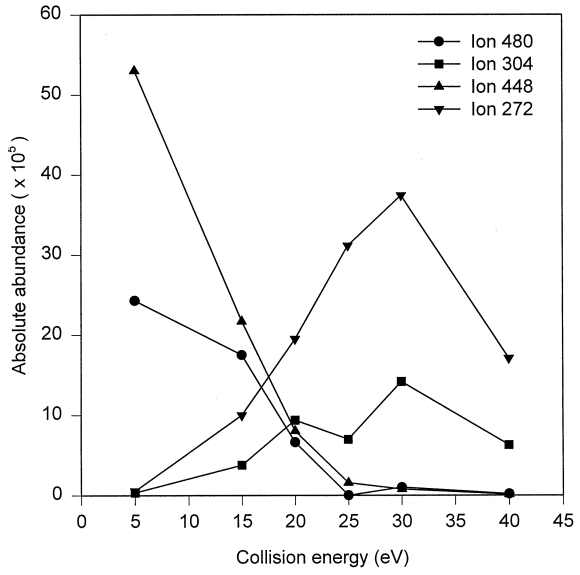


Fig. 3. Effect of collision energy on the absolute abundances of $[M-H]^-$ (precursor) and $[M-Glu]^-$ (product) ions of *Z*-entacapone (*m*/*z* 480→*m*/*z* 304) and tolcapone (*m*/*z* 448→*m*/*z* 272) glucuronides.

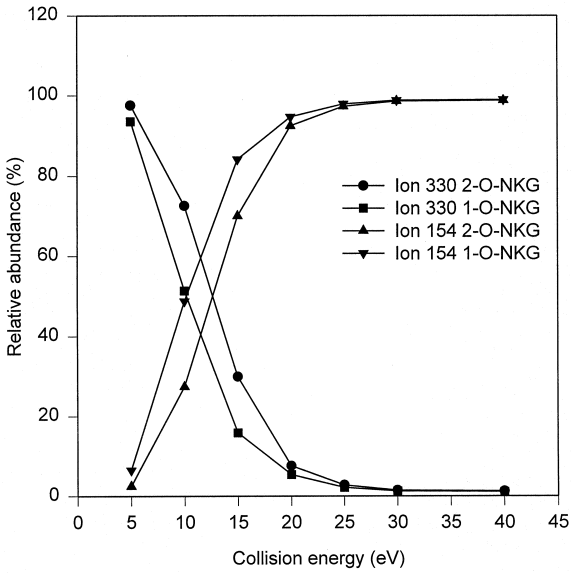


Fig. 4. Effect of collision energy on the relative abundances of $[M-H]^-$ (m/z 330, precursor) and $[M-Glu]^-$ (m/z 154, product) ions of 4-nitrocatechol-1-O-glucuronide and 4-nitrocatechol-2-O-glucuronide.

new method provides analysis within 4 min, allowing high sample throughput in metabolic studies. The full separation of internal standard and analyte by LC was obtained with all the glucuronides studied except with nitecapone glucuronide. However, the full separation is not necessary since the separation can be carried out efficiently by MS–MS. Since *Z*- and *E*-entacapone glucuronides produce similar MS–MS spectra, it was necessary to separate them by LC. The analysis of these isomers by LC–MS–MS is shown in Fig. 5. In the same way it was possible to separate 4-nitrocatechol-1-O-glucuronide and 4-nitrocatechol-2-O-glucuronide by LC. The retention times in six successive injections made with *E*- and *Z*-entacapone and tolcapone glucuronides were within 5 s, indicating good repeatability of the LC method.

To evaluate the applicability of the new method to quantitative analysis, we studied limits of detection, repeatability of the method and the instrument, and linearity. The limits of detection ($S/N=3/1$) were determined to be 0.1–0.2 μg/ml when 5 μl of the spiked urine were used for the analysis (5–10 pg of glucuronide introduced to ESI-MS–MS). Detection limits can easily be lowered by concentrating urine samples in the pretreatment procedure or by injecting a larger amount of sample.

Repeatability of the method was tested with six parallel, separately prepared *E*- and *Z*-entacapone glucuronide samples (10 μg/ml) and repeatability of the instrument was studied by injecting the same sample five times. These studies gave excellent results considering that the samples were in biological matrices. Repeatability of the method was 2.56% for *E*-entacapone glucuronide and 2.57% (relative standard deviation) for *Z*-entacapone glucuronide, and repeatability of the instrument was 1.52% for both compounds. The use of internal standard proved necessary since repeatability of the peak areas was not so good. At 10 μg/ml the relative standard deviation for the peak area of *E*-entacapone glucuronide was 8.47% and that for *Z*-entacapone glucuronide 8.46% ($n=6$). Linearity of the method was studied with three compounds, the two entacapone glucuronide isomers and tolcapone glucuronide, using spiked urine samples in the concentration range of 1–60 μg/ml. The results

Table 2
Monitored precursor and product ions, retention times and eluent compositions used in LC–ESI-MS–MS analysis

Compound	Precursor ion m/z	Product ion m/z	Retention time (min)	Retention time of I.S. (min)	Eluent composition (buffer[a], acetonitrile)
EEG	480	304	3.64	2.08	85:15
EZG	480	304	2.86	2.08	85:15
NG	440	264	2.61	2.67	90:10
1-O-NKG	330	154	2.92	2.66	90:10
2-O-NKG	330	154	2.10	2.66	90:10
TG	448	272	2.76	1.67	80:20

Internal standard: precursor ion m/z 314, product ion m/z 138.
[a]10 m*M* ammonium acetate solution pH 7.0.

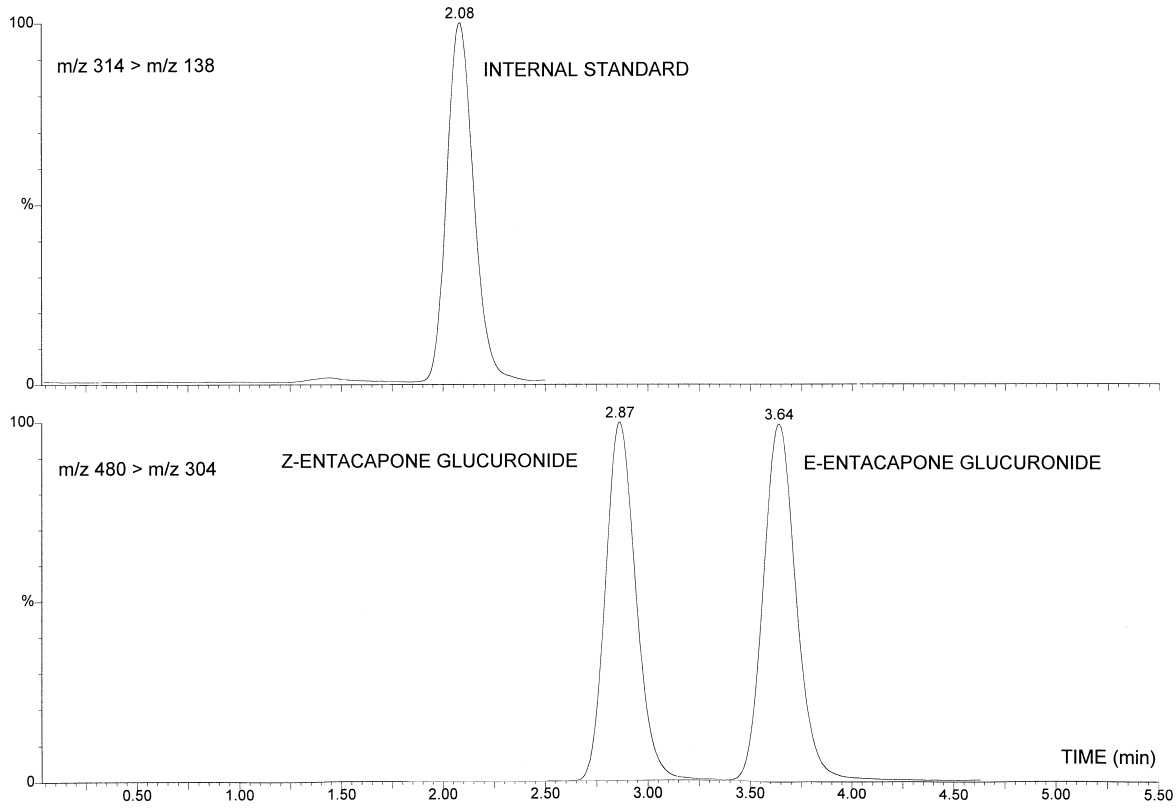


Fig. 5. Chromatogram obtained from the LC–ESI-MS–MS analysis of *E*-entacapone and *Z*-entacapone glucuronides (10 μg/ml).

show excellent linearity of the method with correlation coefficients better than 0.9985 (Table 3).

4. Conclusions

The LC–ESI-MS–MS in negative ion mode provides a rapid, sensitive and accurate method for the analysis of several nitrocatechol-type glucuronides in urine samples. The method can be applied also for other types of glucuronides, since the main fragment ion is shown to be a negatively charged drug molecule. The method is easy to automate being highly suitable for metabolic studies, where large number of samples must be analysed with minimal sample preparation.

Table 3
Regression data from the linearity study of EEG, EZG and TG ($n=6$)

Compound	Slope	Intercept	Correlation coefficient
EEG	0.3097	−0.0867	0.9997
EZG	0.2769	−0.1566	0.9998
TG	0.3631	−0.1385	0.9985

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