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Journal of Chromatography B, 855 (2007) 220–227
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a liquid chromatography–tandem mass spectrometry assay for determination of raloxifene and its metabolites in human plasma

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Received 1 February 2007; accepted 4 May 2007
Available online 16 May 2007

Abstract

This paper describes the development and validation of a method for the detection of raloxifene (Ral) and its two glucuronide metabolites, raloxifene-6-glucuronide (M1) and raloxifene-4′-glucuronide (M2), in human plasma samples. Both glucuronides were synthesized enzymatically, purified and used as authentic standards. The assay involves a simple solid phase extraction (SPE) procedure of 0.5 mL of human plasma and subsequent analysis by LC–MS–MS. The recoveries were higher than 71% and chromatographic separation of all the analytes was accomplished in less than 7 min. Linear ranges ($r^2 > 0.99$) were found from 0.200 to 340 μg/L, from 1.600 to 2720 μg/L and from 0.088 to 60.00 μg/L, for M1, M2 and Ral, respectively. The limits of detection achieved were 8, 11 and 6 ng/L for M1, M2 and Ral, respectively. The method presented was successfully applied to a genetic polymorphism study of 47 plasma samples from women taking Evista (raloxifene hydrochloride).

Keywords: Raloxifene hydrochloride; Raloxifene glucuronides; LC/MS/MS; Plasma samples

1. Introduction

Raloxifene hydrochloride is a selective estrogen receptor modulator. It acts as an estrogen agonist in bone and liver and in this way increases bone mineral density and decreases LDL-cholesterol. Along with the bisphosphonates, raloxifene has been recognized as one of the most effective drugs in prevention of osteoporosis in postmenopausal women [1]. In addition, raloxifene was found to increase vertebral mechanical strength independent of change in bone density [2]. The Multiple Outcomes of Raloxifene Evaluation (MORE) clinical trial revealed that raloxifene was as effective as tamoxifene in reducing the risk of invasive breast cancer [3,4]. Raloxifene's mechanism of action on various tissues is still not completely understood. The same applies to its pharmacokinetics. It is known that raloxifene is subjected to an extensive first pass metabolism: 60% of the peroral dose is absorbed and only 2% reaches the systemic circulation [5]. The rest of what is absorbed is conjugated by UDP-glucuronosyltransferases (UGTs) to raloxifene glucuronides, predominantly raloxifene-4′-β-glucuronide (M2) and raloxifene-6-β-glucuronide (M1) [6] (Fig. 1). The glucuronides show little affinity for the estrogen receptors and for bone tissue. Nevertheless, these metabolites should not be overlooked as they can readily reconverted to active raloxifene in various organs, including the liver, lung, spleen, kidney, bone and uterus [7]. The glucuronides are also excreted into the gut where they are cleaved back to raloxifene by bacterial flora. Raloxifene can then be reabsorbed and thereby completes the entero-hepatic cycle and this prolongs its biological half-life to ∼28 h [5]. Until now, there have been no published attempts to explain the quite large inter- and intra-individual variability of its clearance and volume of distribution. Likewise, the impact of highly variable raloxifene levels in plasma, especially with regard to breast cancer prevention and serious thromboembolic events, remains unknown. The complex and not completely understood metabolism, many different actions in different tissues with valuable therapeutic as well as infrequent, but still serious adverse effects, all call for further pharmacological and pharmacokinetic studies. Although a method for the determination of raloxifene in plasma has been published [8] surprisingly no method for

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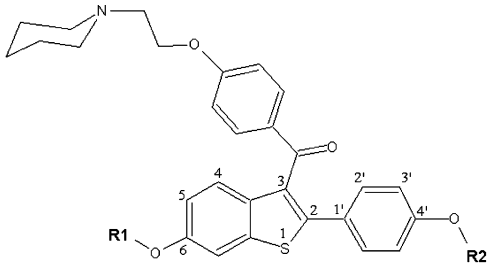
doi:10.1016/j.jchromb.2007.05.004



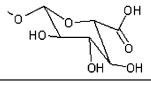
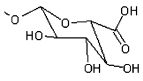
	R1	R2
M1		H
M2	H	
Ral	H	H

Fig. 1. Structure of Ral and its two glucuronides (M1 and M2).

quantification of its metabolites in plasma has been published so far. Zweigenbaum and Henion [8] described a high throughput assay for quantification of tamoxifene, idoxifene and raloxifene in human plasma with LC–MS–MS. With this method, the metabolites of raloxifene were not quantified and the limit of quantification for raloxifene was 38 ng/mL (0.075 μM). This is low enough for high throughput screening assays in drug discovery. However, the maximum plasma concentration after multiple doses of raloxifene reaches only 1.36 (ng/mL)/(mg/kg) [5]. To study raloxifene's metabolism and pharmacokinetics, reliable quantification of raloxifene as well as its metabolites in plasma or serum is needed. Therefore, the aim of this study was to develop and validate a sensitive HPLC/MS/MS method to quantify Ral, M1 and M2 in human plasma. The method developed was successfully applied to a study of the influence of UGT1A1 gene polymorphism on plasma concentrations of Ral, M1 and M2.The starting point of this work was derived from a method developed in our laboratory for quantification of raloxifene in drug quality control studies [9].

2. Experimental

2.1. Materials

Haloperidol (used as internal standard), raloxifene HCl used for preparation of standard solutions, UDP glucuronic acid (UDPGA), alamethicin, Tris–HCl, polyvinylpyrrolidone K25 (PVP), magnesium chloride and β-glucuronidase *from Helix pomatia* were purchased from Sigma–Aldrich (Steinheim, Germany). Superosomes containing the human recombinant enzymes UGT1A1 and UGT1A10 for production of raloxifene metabolites were purchased from BD Gentest (CA, USA).

The following substances were used for the preparation of the mobile phase: formic acid, acetonitrile, methanol and water. They were all LC–MS grade and purchased from JT Baker (Phillipsburg, NJ, USA).

2.2. Preparation of standards of raloxifene glucuronides

In order to properly quantify raloxifene metabolites, their authentic standards were needed. Commercially they were unavailable, so the only option was to synthesize them in our laboratory. Firstly, we tried the chemical synthesis which was published by Dodge et al. [10]. Unfortunately, the yield was not high enough for characterization of the glucuronides. We decided to perform biochemical synthesis of glucuronide standards from raloxifene using the recombinant human UGT enzymes 1A1 that are also responsible for *in vivo* bioconversion of raloxifene to its glucuronides [11]. Raloxifene was extracted from thoroughly ground Evista tablets (Eli Lilly) with pure methanol. The conditions of enzymatic reaction for bioproduction of raloxifene glucuronides were optimized to give the highest possible yield with respect to the consumption of human recombinant enzymes. The reaction was carried out as follows. 0.2 mg of expressed human UGTs was dispersed in 240 μL of an ice-cold solution containing 250 mM Tris with pH adjusted to 7.45, 40 mM $MgCl_2$ and 125 mg/L of alamethicin. The mixture was briefly vortexed and allowed to stand on ice. After 20 min, 660 μL of aqueous solution containing 390 μM raloxifene and 184 mg/L PVP were added to the mixture. The final methanol content did not exceed 2%. The reaction was preincubated at 37 °C in a thermostated shaker Vortemp 56EVC (Tehtnica, Železniki, Slovenia) for 4 min, then the reaction was initiated by addition of 80 μL of 25 mM UDPGA. The total reaction volume was 1 mL and was allowed to continue for 24 h. The reaction was stopped by addition of 200 μL of acetonitrile, cooled to −20 °C and left on ice for 10 min. The precipitated protein was sedimented by centrifugation at 15,000 × *g* for 10 min at 4 °C. The supernatants from several incubations were collected and deep frozen at −86 °C. The sediment, however, was redispersed in Tris buffer with alamethicin and $MgCl_2$ and was reused in another equal bioproduction step, as just described. This step almost doubled the yield of metabolites.

The supernatants contained small amounts of unreacted raloxifene and its two glucuronides, M1 and M2. The two glucuronides were separated and purified on a preparative HPLC column Eurospher 120 mm × 11 mm C18, 5 μm (Knauer, Berlin, Germany). The liquid chromatograph was an HP 1100 (Agilent, USA), with a modified injector, allowing injections of 2 mL. The mobile phase consisted of 83% 10 mM ammonium acetate with pH adjusted to 4.0 and 17% acetonitrile. The flow rate was 4 mL/min. After 2 min of isocratic elution, the percentage of acetonitrile was increased to 30% in 7 min and then raised to 70% in 5 min and held for 7 min before returning to the initial conditions. UV detector was set to 287 nm and the two UDPGA-dependent peaks (eluting at 10.8 and 12.5 min) were separately collected and combined from several injections and then concentrated at 37 °C by a rotary evaporator (Büchi, Switzerland). The drying was continued in a vacuum dryer (Vakuumska tehnika, Semič, Slovenia) at room temperature until a constant mass was achieved (after approximately 48 h). Both powders were accurately weighed and reconstituted in degassed 50% methanol and deep frozen at −86 °C. Mass spectrometry with positive electrospray ionization was used for identification

and purity determination of both metabolites (Varian 1200L, Walnut Creek, CA, USA). Purity was determined by subjecting both glucuronides to enzymatic hydrolysis (incubation volume 1 mL, 25,000 units of β-glucuronidase in 100 mM Tris buffer at pH 5.0 and incubation for 5 h at 37 °C). The resulting free raloxifene was quantified by a calibration curve generated using the authentic standard. Both metabolites showed a purity of more than 98.9% and gave identical MS/MS signals (parent *m/z* 650 fragmenting to 474 daughter, which corresponds to protonated raloxifene without the glucuronic acid moiety), confirming that the two substances are structural isomers. The actual position of glucuronic acid in both peaks was deduced from previous work by Kemp et al. [11], who found that the human recombinant UGT1A1 forms predominantly M1 with respect to M2. Therefore, the larger peak was concluded to be M1 and the smaller M2. This assignation was further confirmed by incubating raloxifene with UGT1A10, for which it is known from the literature data to produce almost exclusively M2 [6,11].

2.3. Preparation of standard solutions

10.72 mg of M1 and 5.20 mg of M2 were accurately weighed and dissolved in 50 mL 50% methanol to obtain concentrations of M1 and M2 of 214.4 and 104.0 mg/L, respectively. 10.00 mg of raloxifene standard was also dissolved in 50% methanol to obtain a concentration of 100.00 mg/L. These primary stock solutions were used to prepare a combined aqueous standard stock solution that contained 6.80, 54.40 and 1.20 mg/L of M1, M2 and Ral, respectively. This concentration ratio of M1:M2:Ral was chosen so that that the spiked plasma samples would closely resemble the real plasma samples from patients. The standard stock solution was aliquoted and kept deep frozen at −86 °C and was used to prepare nine fresh working spiking solutions each day. The stock solution of the internal standard, haloperidol, was prepared similarly by dissolving the free base in 50% methanol and then by dilution with bidistilled water to 100 μg/L. Separately, from different weighings, primary stock solutions, a standard stock solution and working spiking solutions were prepared and used for quality control samples (QCs).

2.4. Preparation of calibration and quality control samples

Each of nine plasma calibration samples was prepared by spiking 475 μL of human plasma with 25 μL of working spiking solution. The concentrations of M1, M2 and Ral ranged from 0.200 to 340.0 μg/L, from 1.600 to 2720 μg/L and from 0.035 to 60.00 μg/L, respectively. The quality control samples (Table 1) were prepared in the same way, except the volumes were 20 times larger and only at three levels, low (QC-L), medium (QC-M) and high (QC-H). The QCs were aliquoted and stored at −86 °C.

2.5. Sample preparation

Twenty-five microliters of internal standard solution (haloperidol, 100 μg/L) was added to each plasma sample of 500 μL. The samples were subjected to a solid phase extraction (SPE) procedure using Strata X 60 mg columns (Phenomenex, CA, USA) on Rapidtrace (Zymark, MA, USA). Before the samples were loaded, the SPE columns were sequentially conditioned with 2 mL of methanol and 2 mL of water. The columns were washed sequentially with 2 mL water and 2 mL of 5% methanol in water, followed by drying with nitrogen gas for 2 min (280 kPa). The elution was performed with 3 mL of a mixture of formic acid, methanol and acetonitrile (2:48:48, v/v/v). The eluants were dried in a stream of nitrogen at 40 °C in a Turbovap apparatus (Zymark, MA, USA). The dried samples were reconstituted with 170 μL of reconstitution solvent, made of acetonitrile, water and formic acid (9.95:89.95:0.1, v/v/v) and transferred to autosampler vials with inserts.

Table 1
Concentrations of M1, M2 and raloxifene (Ral) in quality control samples (QC-L, QC-M, QC-H) and LOD and LOQ achieved

	M1 [μg/L]	M2 [μg/L]	Ral [μg/L]
LOD	0.008	0.011	0.006
LOQ	0.200	1.60	0.088
QC-L	0.500	4.00	0.088
QC-M	10.0	80.0	1.76
QC-H	340	2720	60.0

2.6. Chromatographic conditions

Chromatographic separation was performed on a ProStar 210 liquid chromatograph (Varian) using a Luna C18(2), 50 mm × 2.0 mm column with an installed guard column (Phenomenex, CA, USA) at 50 °C. The injection volume was 25 μL. Mobile phase A was 0.1% formic acid in acetonitrile and mobile phase B was 0.1% formic acid in water. The separation required gradient elution (presented in Table 2). The 100% organic solvent was not necessary for the analyte separation, but this step greatly improved the reproducibility of MS detector response because late eluting lipophilic contaminants such as lecithin [12] were successfully eluted from the column after each run and did not cause ion suppression of the detector response in the next run (data not shown).

The output flow from the column was split in the ratio of 1:5. One part of the flow entered the MS detector; five parts were passed to the photo diode array detector. The MS detec-

Table 2
Gradient employed for successful separation of raloxifene metabolites

Time [min]	*A* [%]	Flow [mL/min]
0.00	10	0.5
0.25	10	0.5
5.00	31	0.5
6.00	63	0.5
7.00	100	0.5
8.00	100	0.8
10.0	100	0.8
12.0	10	0.8
12.5	10	0.5
16.0	10	0.5

tor was further protected from sample contaminants by using a flow-diverter valve which was programmed to let the flow from the splitter enter the MS only between 3.9 and 6.3 min. At all other times, the flow was diverted to waste and the spray needle was protected from drying by infusing a make-up flow of 50% methanol with an infusion pump at 50 μL/min (11 Plus pump, Harvard Apparatus, MA, USA). Flow diversion allowed the plate and ESI chamber to be cleaned less often. Moreover, this improved the reproducibility of the detector response because less hydrophilic (eluting near the solvent peak) as well as less lipophilic contaminants (eluting late) were allowed to enter the spray chamber.

2.7. Mass spectrometry conditions

LC/MS/MS analysis was performed on a Varian 1200L triple-quadrupole LC–MS (Varian). The mass detector was coupled to a ProStar 210 liquid chromatograph by an electrospray ionizer (ESI), operated in the positive mode. Capillary, plate, lenses, quadrupoles and detector voltages were all optimized to allow the highest possible signal transduction for raloxifene and the lowest noise. Signal optimization was performed by a constant infusion of 50 μg/L raloxifene solution in 40% acetonitrile at a rate of 80 μL/min. The pressure of the drying gas was 150 kPa and the temperature was 400 °C. The pressure of the collision gas (pure argon) was 0.200 Pa. The mass spectrometer was used in the multiple reaction monitoring mode (MRM) (Table 3). Both quadrupoles Q1 and Q3 were set at unit mass resolution and the scan time was 1 s. Instrument control, data acquisition and quantification were performed by a Varian MS Workstation, Ver. 6.5.

2.8. Method validation parameters and procedures

2.8.1. Specificity, linearity and limits of detection and quantification

Specificity was determined by analyzing plasma samples from six different lots and subjecting them to the same sample preparation and analytical procedure. The presence or absence of any interfering peaks at the retention times of analytes or the internal standard was evaluated.

In order to assess the linearity of the detector response, a series of plasma calibration samples were prepared as described previously in Section 2.4. On 3 days of validation, three standard calibration curves containing nine non-zero calibrators were prepared and analyzed by linear regression in the concentration range from 0.200 to 340 μg/L, from 1.600 to 2720 μg/L and from 0.088 to 60 μg/L, for M1, M2 and Ral, respectively. A correlation coefficient of more than 0.99 was set as acceptable, otherwise the calibration run would have been rejected. Back calculation of the concentration was made for each calibration sample. The detection limit was set as the dilution showing a signal-to-noise ratio of more than 3. The limit of quantification was not determined based on a signal-to-noise ratio (although this ratio exceeded 10), but was set as the lowest standard on the calibration curve that exhibits acceptable accuracy and precision (deviation from the nominal value of less than ±20%) [13].

Table 3
The MRM used for the quantification of raloxifene and its glucuronides and haloperidol as an internal standard (IS)

Analyte	MRM m/z + transition	Collision energy [eV]
M1	650 → 474	−18
M2	650 → 474	−18
Raloxifene	474 → 112	−23
Haloperidol (IS)	376 → 165	−25

2.8.2. Accuracy and precision

Accuracy and precision were determined from the calibration curve and detector responses from six replicates of each quality control sample (QC-L, QC-M and QC-H) on each of the 3 days of validation.

Within-day precision was calculated as the coefficient of variation of analysis of six replicate QC-L, QC-M and QC-H samples. For between-day precision, analyses of the same six replicate samples at low, medium and high concentration levels were performed on 3 different days over a period of 2 weeks. Accuracy was deemed acceptable when the calculated concentration was within ±15% of the nominal concentration, except at the limit of quantification where it should not deviate more than ±20%. Similarly, precision was acceptable when the coefficient of variation of replicates was smaller than ±15%, except at the LOQ, where it should not exceed ±20% [13].

2.8.3. Recovery and matrix effects

The samples for recovery determination were prepared at three concentration levels in six replicates by spiking the analytes to blank plasma before extraction. The recovery reference samples were prepared by spiking the reconstitution solvent with the same amounts of working spiking solution as used for the plasma samples. Plasma samples were extracted with the described SPE method, while the recovery reference samples were left unextracted. Both types of samples were immediately analyzed by LC–MS–MS and the recovery was calculated as a ratio between the detector response of extracted plasma samples and the response of recovery reference samples.

In the development of every quantitative LC/ESI/MS method, the matrix effect should be thoroughly assessed [14,15]. Five different blank plasma lots were used to evaluate whether different plasma matrices could suppress or enhance the signal of the internal standard or any of the analytes. For each of the three concentration levels, five 500 μL aliquots of each plasma lot were extracted and then spiked with the analytes for subsequent analysis. The corresponding peak areas were compared to the responses of analytes spiked to the neat reconstitution solvent, at the same concentration level. The matrix effect was then calculated as a ratio of the former to the latter and multiplied by 100% [15].

2.8.4. Stability

The stability was evaluated by analyzing QC samples and comparing the concentrations found to the nominal values.

2.8.4.1. Stock solution stability. The stock solutions were aliquoted and kept at −86 °C and five replicates were thawed on three different occasions, the last after 1 month, appropriately diluted with the reconstitution solvent and immediately analyzed by LC–MS–MS.

2.8.4.2. Long-term sample stability. Six quality control samples at low and high levels (QC-L and QC-H) were kept in the deep freezer at −86 °C, thawed after 1 month, extracted and analyzed by LC–MS–MS.

2.8.4.3. Short-term sample stability or bench-top stability. Similar to the long-term sample stability testing, six quality control samples at low and high concentration levels were analyzed after 6 h at room temperature (25 ± 3 °C).

2.8.4.4. Autosampler stability. Six replicates of quality control samples at low and high concentration levels were extracted, dried, reconstituted and left in the thermostated autosampler for 8 h at 4 °C and then injected and quantified.

2.8.4.5. Freeze–thaw stability. Three replicates of quality control samples at medium concentration level (QC-M) were stored at −86 °C. Thawing was performed at room temperature, followed by freezing for 24 h. The samples were subjected to two more freeze–thaw cycles before being extracted and analyzed by LC–MS–MS.

2.9. Study design

A total of 47 postmenopausal women patients with osteoporosis were enrolled in the study. Informed consent was obtained from each individual and the study was approved by the Slovenian Ethics Committee for Research in Medicine (Nr. 27/02/07). The patients were treated for 6 months with 60 mg raloxifene per day and were followed in the Maribor General Hospital (Department for Diabetology and Endocrinology), Slovenia. Blood samples were collected in the steady state, 4–6 h after administration. After centrifugation, samples were stored at −85 °C until analysis.

2.10. Statistical analyses

A two-tailed Student's *t*-test was used to compare the M1, M2 and Ral levels in subjects with different UGT1A1 genotypes. In order to confirm equal variances, a prior *F*-test was performed.

3. Results and discussion

3.1. Method validation

3.1.1. Specificity, selectivity, linearity, LOD and LOQ

The chromatograms of samples from two patients presented in Fig. 2a and b show good resolution, no detectable matrix

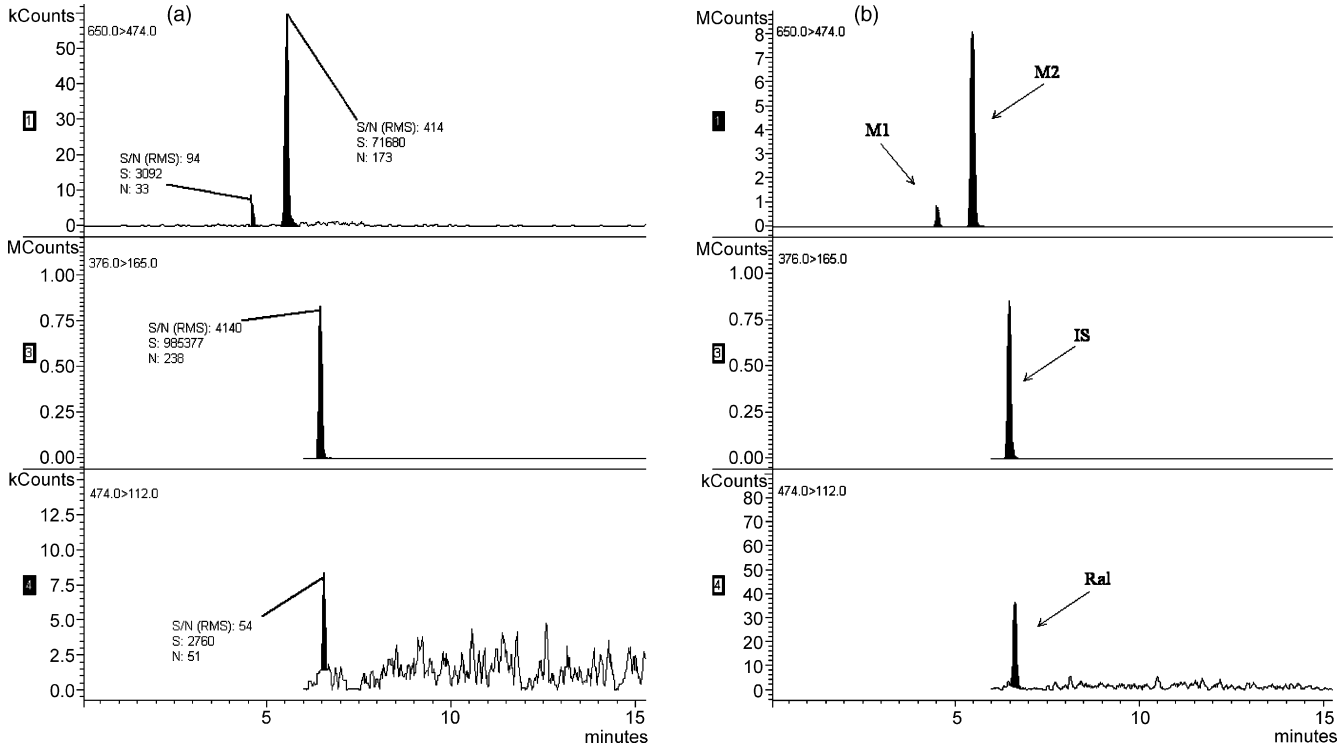


Fig. 2. (a and b) LC–MS–MS multiple reaction monitoring chromatograms of plasma samples from two patients on raloxifene therapy representing the two extremes from the low and high concentration range. Traces 1, 3 and 4 represent mass transitions of the two glucuronides, internal standard and Ral, respectively. Sample from patient A contained 0.21, 2.33 and 0.13 μg/L of M1, M2 and Ral, respectively. For patient B, the measured concentrations of M1, M2 and Ral were 55.4, 147 and 5.86 μg/L, respectively. The relatively high signal-to-noise ratio (shown on chromatogram a) allows simple peak integration and reliable quantification.

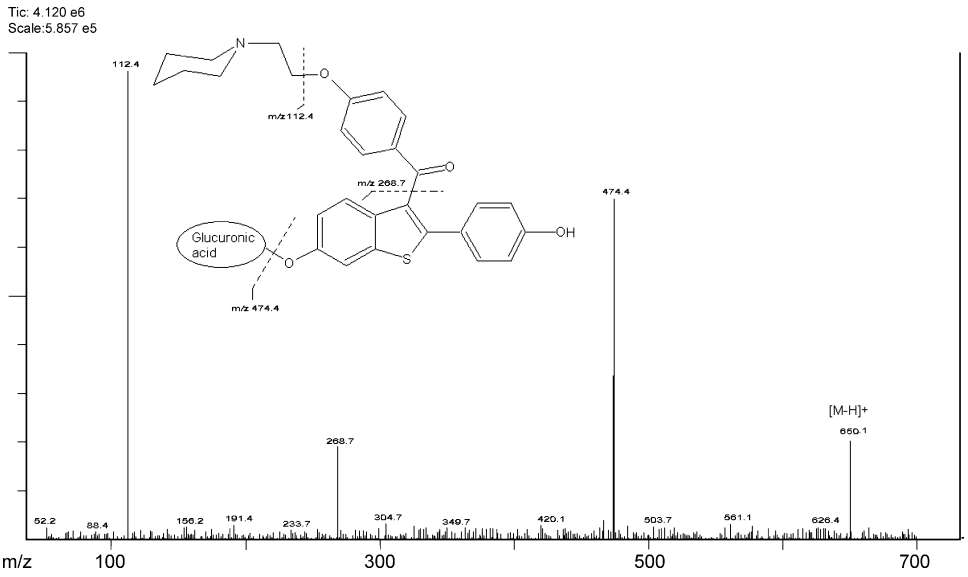


Fig. 3. The fragmentation pattern of raloxifene glucuronide M1. The same fragmentation can be observed for M2, because both produce the same daughter ions, *m/z* 474, 269 and 112. Likewise, the fragmentation of Ral is very similar except that there is no *m/z* 650 signal.

interference and sensitive detector response over a wide concentration range. Specificity was confirmed by the absence of any peaks at the retention times of the analytes and haloperidol in processed blank plasma samples from six different lots. Some ion-cross talk was observed between M1, M2 and Ral channels. This is hardly surprising, because after the cleavage of the glucuronic acid moiety, raloxifene glucuronides actually produce raloxifene molecular ions (Fig. 3). When the concentrations of glucuronides are very high, some of the resulting raloxifene is cleaved further, producing raloxifene fragments (*m/z* 112) and these can be recorded as small peaks on the raloxifene trace earlier in the chromatogram. In the authors' opinion, however, this is not of significant concern because the glucuronides are well separated from raloxifene chromatographically and do not interfere with its integration. Moreover, to avoid any confusion, the LC–MS software was set not to record any peaks in the raloxifene trace (474 → 112) much earlier than its actual retention time (Fig. 2). The limits of detection and quantification achieved are shown in Table 1.

The calibration curves obtained had to be divided into two concentration ranges for all three analytes because of the relatively large concentration difference between the lowest and the highest calibrator; for example, M1 ranged from 200 ng/L up to 340 μg/L, which is a 1700-fold difference. The method showed good linearity over the entire concentration range, exhibiting a correlation coefficient (r^2) of 0.99 or higher (Table 4).

3.1.2. Accuracy and precision

The within-day and between-day precision and accuracy are within acceptable limits and are presented in Tables 5–7 for M1, M2 and Ral, respectively.

3.1.3. Recovery and matrix effect

The recoveries of M1 and M2 at low, medium and high levels ranged from 91 to almost 100% (Table 8). The recovery of Ral, however, was somewhat lower: 71, 82 and 85% at low, medium and high levels, respectively.

The different plasma matrices did not have significant effects on the analyte signals. The matrix effects for M1, M2 and Ral ranged from 96 to 99%, from 94 to 100% and from 93 to 110%, respectively, with SD of less than 10%. The matrix effect for the internal standard was 87% and was very reproducible (SD < 1%). The figures relatively close to 100% with low standard deviation show that the matrix effect is low and reproducible, and would not interfere with the assay.

3.1.4. Stability

The stock solutions were found to be stable for at least 1 month at −86 °C. The changes in signal intensity after storage were less than 4% for M1, M2, Ral and haloperidol. All the QC samples stored at −86 °C were found to be stable for at least 1 month. With all the analytes, the average accuracy dropped by less than 5%. After 8 h in the autosampler, an increase in signal

Table 4
Linear regression data for M1, M2 and Ral for lower and higher concentration ranges

Analyte	Low concentration				High concentration			
	Range [μg/L]	Slope, $\times 10^{-3}$	Intercept, $\times 10^{-3}$	r^2	Range [μg/L]	Slope, $\times 10^{-3}$	Intercept, $\times 10^{-3}$	r^2
M1	0.200–2.00	2.62	1.67	0.999	2.00–340	3.11	2.09	0.999
M2	1.60–16.0	4.27	−1.83	0.993	16.0–2720	9.22	−4.25	1.000
Ral	0.088–0.353	5.42	5.53	0.991	0.353–60.0	3.19	6.48	0.998

Table 5
Within-day and between-day precision and accuracy for M1

	Sample	Nominal concentration [μg/L]	Mean concentration found [μg/L]	Precision [CV%]	Accuracy bias [%]	n
Within-day	LLOQ	0.200	0.192	5.9	4.0	5
	QC-L	0.500	0.510	5.3	2.0	5
	QC-M	10.0	9.9	6.8	−1.0	5
	QC-H	340	336	3.2	0.1	5
Between-day	LLOQ	0.200	0.196	8.7	−2.0	15
	QC-L	0.500	0.521	5.4	4.2	15
	QC-M	10.0	10.6	6.1	6.0	15
	QC-H	340	343	3.3	0.9	15

Table 6
Within-day and between-day precision and accuracy for M2

	Sample	Nominal concentration [μg/L]	Mean concentration found [μg/L]	Precision [CV%]	Accuracy bias [%]	n
Within-day	LLOQ	1.60	1.56	3.7	−2.5	5
	QC-L	4.00	4.01	4.5	0.3	5
	QC-M	80.0	80.6	8.7	0.8	5
	QC-H	2720	2744	2.1	0.9	5
Between-day	LLOQ	1.60	1.55	6.5	−3.1	15
	QC-L	4.00	4.05	5.2	1.3	15
	QC-M	80.0	80.0	6.8	0.0	15
	QC-H	2720	2717	2.7	−0.1	15

of up to 6% was observed. On the other hand, plasma samples standing at room temperature for 6 h did show a moderate drop in concentration. The short-term stability was still considered acceptable, as the decrease was less than 7%. The freeze–thaw stability was also acceptable, even though the average fall in the concentration of M1 in QC-H samples after three freeze–thaw cycles was 9%. A summary of the stability tests for M1, M2 and Ral is presented in Table 8.

3.2. Application of the method to real samples

The assay developed was applied to a study of genetic polymorphism testing in a sample of 47 postmenopausal women with osteoporosis, taking a daily dose of 60 mg raloxifene. The aim of the study was to determine whether genetic polymorphisms in the UGT gene could play a role in the pharmacokinetics of raloxifene. The glucuronidation efficiency of various UGT polymorphic variants could affect the clearance of raloxifene and thereby its plasma concentrations. Furthermore, variable levels of metabolites might also be a consequence of different glucuronidation activities. The genetic study is still under way, but the plasma samples analyzed ($n = 47$) indicated a large inter-individual variability in the levels of M1, M2 and Ral. In the assayed samples, the mean concentration levels found (with CV%) were 43.6 μg/L (81%), 204 μg/L (79%) and 1.97 μg/L (63%) for M1, M2 and Ral, respectively. The mean concentration level of raloxifene found is in accordance with values reported in the literature [5]. The level of inter-individual variability, however, was somewhat higher, 63% compared to 37–52% from literature data. This could be a consequence of the heterogeneity of the patients enrolled and of the fact that the frequency of the homozygous genotype, UGT1A1 *28/*28, was higher (22%) in the selected sample than reported (∼11%), in the European population [16]. The presence of UGT1A1 *28 polymorphism, leading to a reduced glucuronidation activity, was found to significantly elevate the total concentration of raloxifene and its metabolites ($p < 0.05$). At first glance, it is difficult to understand why the *28/*28 genotype did not significantly increase only the concentration of Ral, and lower the levels of metabolites in the blood. One has to take into account, however, that the glucuronides are far better substrates for efflux transporters in the intestine and in the liver than their aglycones [17,18]. Therefore,

Table 7
Within-day and between-day precision and accuracy for Ral

	Sample	Nominal concentration [μg/L]	Mean concentration found [μg/L]	Precision [CV%]	Accuracy bias [%]	n
Within-day	LLOQ	0.088	0.093	9.2	−5.7	5
	QC-M	1.764	1.773	11.0	−0.5	5
	QC-H	60.0	58.6	4.0	−2.3	5
Between-day	LLOQ	0.088	0.089	13.4	1.2	15
	QC-M	1.764	1.747	9.0	−1.0	15
	QC-H	60.0	60.1	4.9	0.2	15

Table 8
Summary of stability tests [%] for M1, M2 and Ral

	M1	M2	Ral
Long term[a]	95.2–97.6	97.6–99.4	96.8–98.1
Short term[b]	92.9–94.2	96.9–97.9	96.2–98.7
Autosampler[c]	104.4–105.7	104.1–106.3	100.8–105.1
Freeze–thaw[d]	91.0–93.9	92.9–95.1	93.5–97.1

The stability was evaluated in three or six replicates of low and high QC plasma samples.
[a] 1 month at −86 °C, $n = 6$.
[b] 6 h at room temperature, $n = 6$.
[c] 8 h at 4 °C, $n = 6$.
[d] Three freeze–thaw cycles, $n = 3$.

a reduced glucuronidation activity leads to a decreased excretion of raloxifene metabolites and to an accumulation of total raloxifene in the body.

4. Conclusion

The LC/MS/MS method developed in this work proved to be sensitive, specific, accurate and precise. It offers the simultaneous determination of raloxifene and its two glucuronides, M1 and M2. The method development required quite some effort, especially because the glucuronide standards had to be synthesized, purified and characterized. The sample preparation includes a relatively simple solid phase extraction step with simultaneous concentration of the analytes from the samples and requires no derivatization. The analysis of raloxifene in plasma was also a challenging task because of its very low concentration; on average, its maximal plasma concentration (c_{max}) reaches only 1.36 (μg/L)/(mg/kg) [5]. The limits of quantification achieved in plasma were 0.088 μg/L for Ral, 0.200 μg/L for M1 and 1.6 μg/L for M2. The method presented was shown to reliably detect raloxifene down to $c_{max}/18$, which allows pharmacokinetic studies to be performed for at least 4.1 half-lives after reaching the c_{max}. The developed method was successfully applied to a study of the effects of genetic polymorphism on pharmacokinetics of raloxifene in a sample of 47 women taking 60 mg raloxifene daily. Moreover, the method described could easily be adapted to various other biological samples as it covers a broad concentration range (1700-fold difference), has a good precision, accuracy and high recovery. In addition, the implementation of LC–MS–MS multiple reaction monitoring provides the needed specificity for confident analysis of raloxifene and its metabolites in complex matrices.

Without a doubt, the described method will facilitate further research in this interesting field, especially in the light of the recently discovered capability of raloxifene to prevent invasive breast cancer among postmenopausal women.

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