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Determination of raloxifene and its glucuronides in human urine by liquid chromatography–tandem mass spectrometry assay

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ABSTRACT

A selective, sensitive, accurate and precise liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for determination of raloxifene and its three glucuronides: raloxifene-6-β-glucuronide (M1), raloxifene-4′-β-glucuronide (M2), raloxifene-6,4′-diglucuronide (M3) in urine samples is presented in this paper. To our knowledge the developed analytical method is the first fully validated method capable of simultaneous determination of raloxifene and its glucuronides in real urine samples. Moreover, for the first time a method for determination of raloxifene diglucuronide in relevant biological samples was introduced. Metabolites were obtained by a bioconversion process of raloxifene to its glucuronides using the microorganism *Streptomyces* sp. and were used as standards for validation. Urine samples were introduced to a simple solid phase extraction prior to the analysis by LC–MS/MS. The method was linear in a wide range with high determination coefficient ($r^2 > 0.997$). The limits of quantification achieved were 1.01, 1.95, 2.83 and 4.69 nM for raloxifene, M1, M2 and M3, respectively. The recoveries were higher than 92.5%, the accuracy was within 100 ± 8.8% and the precision was better than 12% for all compounds. The developed method was successfully applied to the real urine samples and showed to be appropriate for use in further research of still not completely discovered raloxifene pharmacokinetics. Furthermore, the presented method could also serve for a potential application in anti-doping analysis.

1. Introduction

Raloxifene, a selective estrogen receptor modulator, is approved worldwide for the prevention and treatment of postmenopausal osteoporosis and it is also indicated for the prevention of breast cancer in postmenopausal women. Raloxifene significantly reduces the risk for vertebral fractures [1] but has no effect on nonvertebral fracture risk [2]. Raloxifene therapy is associated with a reduced risk of invasive breast cancer in postmenopausal women irrespective of the presence or absence of risk factors [3]. Furthermore, raloxifene was shown to significantly lower the incidence of coronary events in postmenopausal women younger than 60 years [4]. Like tamoxifene, in men, raloxifene also significantly increases serum testosterone levels albeit to a lower extent [5]. Therefore, raloxifene is included in the list of drugs prohibited by the World Anti-Doping Agency (WADA) [6].

After rapid absorption, raloxifene undergoes extensive first pass metabolism: 60% of administered dose is absorbed and only 2% reaches the systemic circulation [7]. The rest represents raloxifene-4′-β-glucuronide (M2), raloxifene-6-β-glucuronide (M1) [8] and raloxifene-6,4′-diglucuronide (M3) (Fig. 1) [9]. The glucuronides exhibit minimal binding to estrogen receptor but they should not be overlooked as they can be readily reconverted to active raloxifene in various organs [9]. Raloxifene undergoes the enterohepatic cycle and this prolongs its biological half-life to 28 h [7]. Raloxifene is primarily excreted in feces and less than 0.2% is excreted unchanged in urine. Less than 6% of administered dose of raloxifene is recovered in urine in form of glucuronides [7]. Raloxifene exhibits the quite high inter- and intra-individual variability of its clearance and volume of distribution [9]. To support additional pharmacokinetic studies and to explain the reasons for variability, appropriate analytical methods need to be developed. Furthermore, since raloxifene can be abused in sports, selective and sensitive methods for its detection and quantification in urine are needed.

Abbreviations: WADA, World Anti-Doping Agency; M1, raloxifene-6-β-glucuronide; M2, raloxifene-4′-β-glucuronide; M3, raloxifene-6,4′-diglucuronide; RAL, raloxifene; HAL, haloperidol; DMSO, dimethyl sulfoxide; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MS, mass spectrometer; ME, matrix effect; IS, internal standard; CV, coefficient of variation; TFA, trifluoroacetic acid; FA, formic acid; QC, quality control; LOD, limit of detection; LLOQ, lower limit of quantification; SPE, solid phase extraction; MRM, multiple reactions monitoring mode; SRM, selected reaction monitoring mode; EMV, electron multiplier voltage.

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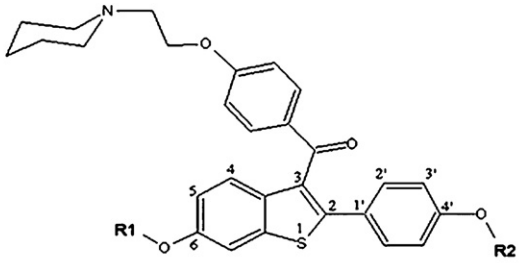


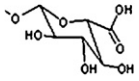
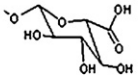
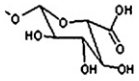
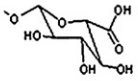
	R1	R2
RAL	H	H
M1		H
M2	H	
M3		

Fig. 1. Structure of raloxifene and its three glucuronides.

A LC–MS/MS method for the determination of raloxifene in plasma has been published by Zweigenbaum and Henion [10] with the limit of quantification for raloxifene 12.7 nM (6 μg/L). Additionally, our research group developed a method for detection of raloxifene and its metabolites M1 and M2 in plasma samples with limits of quantification, 0.18 nM (0.088 μg/L), 0.31 nM (0.20 μg/L) and 2.46 nM (1.6 μg/L), respectively [11]. Few methods for determination of raloxifene in urine have been published recently by Kang et al. [12], Kumar et al. [13] and Mazzarino et al. [14] with limit of quantification for raloxifene 21.1 nM (10.0 μg/L), 42.2 nM (20.0 μg/L) and 63.0 nM (30.0 μg/L), respectively, but no method for quantification of its metabolites in urine has been found. The methods of Kang et al. [12] and Mazzarino et al. [14] has required preceding hydrolysis of raloxifene glucuronides to raloxifene what is an additional quite time consuming step in sample preparation.

The authors were contacted by control laboratories with request to adopt their already published method [11] to urine samples. Therefore, our aim was to develop a method for detection and quantification of raloxifene, M1, M2 and M3 in urine that could serve as a doping test for raloxifene abuse and can also be used for examination of still not completely discovered raloxifene pharmacokinetics. Previously published method for quantification of raloxifene, M1 and M2 in plasma was used as a basis for the development of a new LC–MS/MS method. Suitability of the developed and validated method was confirmed on urine samples of postmenopausal women treated with raloxifene.

2. Experimental

2.1. Materials

Bacterial cell line (*Streptomyces* sp.—ATCC 55043) was obtained from American Tissue Culture Collection (ATCC). Soluble starch was purchased from Kemika (Zagreb, Croatia), glucose from Fluka (Buchs, Switzerland), N-Z amine Type A, $CaCO_3$, soybean flour, raloxifene hydrochloride (RAL), haloperidol (HAL), dimethyl sulfoxide (DMSO), β-glucuronidase from *Helix pomatia*, trifluoroacetic acid (TFA), ammonium acetate and ammonium formiate from Sigma–Aldrich Chemie (Deisenhofen, Germany), yeast extract from BD (CA, USA), KCl from Scharlau (Barcelona, Spain) $MgSO_4{\cdot}7H_2O$ from Alkaloid (Skopje, Macedonia), $FeSO_4{\cdot}7H_2O$ from Merck (NJ, USA) and molasses from Healthy Food Brands Ltd (Wellingborough, UK). Formic acid (FA), acetonitrile and methanol were all LC–MS grade and were purchased from JT Baker (Philipsburg, NJ, USA).

2.2. Production of raloxifene glucuronide standards

2.2.1. Biotransformation of raloxifene to its glucuronides

As raloxifene glucuronides M1, M2 and M3 were not available; a biotransformation of raloxifene to its glucuronides was accomplished. The biotransformation of raloxifene was made by a modification of the method that was published by Lilly Research Laboratories [15].

Bacterial cell line (*Streptomyces* sp.) was cultured according to manufacturer's protocol and stored in 1 mL aliquots at −86 °C. For each bioconversion an aliquot was thawn on ice and transferred into a baffled 500 mL Erlenmeyer flask containing 50 mL vegetative medium. Vegetative medium contained (per litre): 10 g soluble starch, 5 g glucose, 2.5 g N-Z amine Type A, 2.5 g yeast extract, 0.5 g $CaCO_3$, 0.2 mg KCl, 0.2 mg $MgSO_4{\cdot}7H_2O$, and 0.004 mg $FeSO_4{\cdot}7H_2O$. pH of the media was adjusted to 7.0–7.5 with 1 M HCl or 1 M NaOH before autoclaving. Bacterial culture was grown in vegetative medium for 17 h at 30 °C on rotary shaker at 150 rpm.

1 mL of cell culture was transferred into a baffled 500 mL Erlenmeyer flask containing 50 mL bioconversion medium. Bioconversion medium contained (per litre): 25 g glucose, 15 g soybean flour, 3.0 g molasses, 2.5 g $CaCO_3$, 1.0 g N-Z amine Type A, 0.2 mg KCl, 0.2 mg $MgSO_4{\cdot}7H_2O$, and 0.004 mg $FeSO_4{\cdot}7H_2O$. pH of the media was adjusted to 7.2–7.5 with 1 M HCl or 1 M NaOH before autoclaving. Bacterial culture was grown in bioconversion medium for 1 h at 30 °C on rotary shaker at 150 rpm before addition of bioconversion substrate. Substrate was raloxifene (60 mg of powder of raloxifene hydrochloride or a tablet containing 60 mg of raloxifene hydrochloride). Bacterial culture was further grown at 30 °C on rotary shaker at 150 rpm for 72 h. 1 mL samples were aseptically harvested every 12 h for monitoring of the bioconversion process.

Table 1
The gradient elution program for semi-preparative chromatographic separation of raloxifene and its metabolites. Mobile phase A = 0.1% FA in water, mobile phase B = 98% acetonitrile, 2% water.

Time [min]	Mobile phase B [%]	Flow [mL/min]
0.5	5	4
1	7	5
5	12	6
8	14	6
8.5	15	3
13.5	23	3
17.5	45	3
18	45	3
18.5	45	5
19.5	5	5
21.9	5	5

At 72 h almost all raloxifene was converted to its two monoglucuronides and diglucuronide and the bioconversion was stopped.

2.2.2. Purification of raloxifene glucuronides

The cultured broth was centrifuged at 10,000 × *g* for 10 min to separate the bacteria from the production medium. Afterwards, the supernatant was treated with methanol in 1:1 (v/v) ratio, and left to stand overnight at −20 °C. The precipitated impurities were separated by another centrifugation at 10,000 × *g* for 10 min. The methanol was removed by a rotating evaporator at 40 °C.

Further purification of raloxifene glucuronides consisted of two steps: firstly, the supernatant was subjected to a simple solid phase extraction (SPE) procedure and secondly, the eluates were separated and purified on a semi-preparative HPLC, followed by evaporation and lyophilisation.

The SPE clean-up was performed on several 60 mg/3 mL Strata-X cartridges (Phenomenex, CA, USA), which were activated by 3 mL of methanol and equilibrated with 1 mL of 0.1% TFA in water, then 30 mL of diluted culture supernatant (1:1 (v/v) with water containing 0.2% TFA) were applied to the cartridges, followed by washing with 3 mL of 10% methanol in water. Afterwards, the cartridges were dried under vacuum for 10 min and the products were eluted with 3 mL of 1:1 (v/v) mixture of methanol and acetonitrile containing 2% formic acid.

The eluates were dried under a stream of nitrogen (TurboVap LV, Zymark, Portland, USA), reconstituted in 10% acetonitrile, filtered through a 0.22 μm filter and transferred to preparative HPLC vials.

The semi-preparative HPLC consisted of a modified Agilent 1100 system equipped with an Agilent fraction collector (Agilent Techologies, Santa Clara, USA). The injection volume was 380 μL. The chromatographic separation was performed on a 100 mm × 10 mm C18 Onyx Semi-prep monolythic column (Phenomenex, Torrance, USA) using a gradient elution (Table 1) at 40 °C with UV-detection at 287 nm.

The fractions containing raloxifene glucuronides were separately collected in glass containers and analyzed for identity and purity on LC–MS/MS. Triple quad mass spectrometer Agilent 6460 was operated in positive electrospray ionization mode (Agilent Techologies, Santa Clara, USA). Full-scan (*m*/*z* 100–900) and also product ion scan spectra (*m*/*z* 650 for M1 and M2; and *m*/*z* 826 for M3) for all three glucuronides were recorded. The chromatographic conditions and other MS settings were the same as used in LC–MS/MS analysis of urine samples (Section 2.5).

After identification and confirmation of purity, fractions were evaporated and lyophilised. Because of small amounts of produced metabolites the lyophilisates were reconstituted in DMSO and the concentrations of stock solutions were determined according to the calibration curve of raloxifene authentic standard after incubation of each metabolite with β-glucuronidase (2000 units/mL of β-glucuronidase in 50 mM ammonium acetate at pH 5.0 and incubation for 5 h at 37 °C). The concentrations of metabolite stock solutions were determined in three parallels for each metabolite and confirmed at three different dilutions of stock solutions. The identity and purity of metabolites in stock solution was again confirmed by LC–MS/MS.

Table 2
Concentrations of RAL, M1, M2 and M3 in calibration curve (SU) and quality control samples (QC) along with LOD and LOQ achieved.

	RAL [nM]	M1 [nM]	M2 [nM]	M3 [nM]
SU1	0.25	1.95	2.83	4.69
SU2	0.51	3.91	5.66	9.38
SU3	1.01	7.81	11.3	18.8
SU4	2.03	15.7	22.7	37.5
SU5	4.06	31.3	45.3	75.0
SU6	8.12	62.5	90.6	150
SU7	16.6	125	181	300
SU8	32.5	250	363	600
SU9	65.0	500	725	1200
SU10	130	1000	1450	2400
SU11	260	2000	2900	4800
LOD	0.017	0.024	0.033	0.039
LLOQ	1.01	1.95	2.83	4.69
QCl	2.2	5.56	8.06	13.3
QCm	21.7	83.4	121	200
QCh	130	1000	1450	2400

2.3. Preparation of standard and quality control samples

Stock solutions of raloxifene species were prepared in DMSO at concentrations of 2.174, 2.074, 1.567 and 0.401 mM for RAL, M1, M2 and M3, respectively. Stock solution of HAL was prepared in methanol at a concentration of 100 mg/L and diluted to 1 mg/L with 50% methanol in water. Eleven urine calibration standards were prepared by spiking the appropriate standard solution to 0.5 mL of blank urine. Spiked concentrations of raloxifene species in calibration curve (SU) and quality control (QC) samples are presented in Table 2.

2.4. Urine sample preparation

25 μL of internal standard solution (HAL, 1 mg/L), 500 μL of water (MilliQ) and 100 μL of 1% TFA was added to each 500 μL of urine sample. The samples were subjected to a solid phase extraction using Strata-X 30 mg/1 mL (Phenomenex, Torrance, USA) on Visiprep™ SPE Extraction Vacuum Manifold (Supelco, Bellefonte, USA). Before the transfer of prepared urine sample to the SPE cartridge, the cartridge was conditioned with 1 mL of methanol and equilibrated with 1 mL of 0.1% TFA in water. After loading the cartridge with prepared sample, it was washed sequentially with 1 mL of water and 1 mL of 10% methanol followed by drying under vacuum for 10 min. The sample was then eluted from the cartridge with 1 mL of 2% FA in acetonitrile:methanol = 1:1 (v/v). The eluted sample was dried in a stream of nitrogen at 45 °C in a Caliper Turbo Vap LV apparatus (Zymark, Portland, USA) and reconstituted with 170 μL of reconstitution solvent (4.5 mM ammonium formiate in methanol:water = 1:1 (v/v)). The reconstituted sample was transferred to autosampler vials with inserts and subjected to LC–MS/MS analysis.

2.5. Liquid chromatography–tandem mass spectrometry conditions

The Agilent 1290 Infinity liquid chromatographic system (Agilent Techologies, Santa Clara, USA) equipped with vacuum degasser, binary pump, autosampler, thermostat and 6460 Triple Quad Mass Spectrometer (Agilent Techologies, Santa Clara, USA)

Table 3
The MRM and collision energy characteristics for optimal quantification of raloxifene, its glucuronides and haloperidol (IS).

	MRM *m/z* transitions	Collision energy [eV]
M3	826 → 474	40
M2	650 → 474	28
M1	650 → 474	28
RAL	474 → 112	32
HAL (IS)	376 → 165	32

was used for the development and validation of analytical method and to analyze the urine samples.

Chromatographic separation was performed by using a Kinetex 50 mm × 2.1 mm column coupled with an In-Line filter KrudKatcher Ultra HPLC 0.5 μm and a guard column C18 (2) 4 mm × 2 mm (Phenomenex, Torrance, USA) at 50 °C. The mobile phase consisted of 0.1% FA in water (mobile phase A) and 100% acetonitrile (mobile phase B). The flow rate was set at 1 mL/min and the separation required gradient elution. The elution started with 10% of mobile phase B for 0.25 min, and then the elution continued with the following gradient: 10–15–20–30–50–50–10% of mobile phase B in 0.25–0.50–0.90–1.90–1.95–2.50–2.60 min. The run time was 3 min. The volume of injection was 0.5 μL and after each injection, the sampling needle was washed with 0.1% FA in isopropanol:water = 40:60 (v/v). During the analyses the autosampler temperature was kept at 4 °C. Before entering the MS the output flow was split in the ratio of 1:1 (v/v), where half of the flow entered the MS and half was driven to waste. The MS was also additionally protected by using a flow-diverter valve which let the flow to enter the MS only between 0.7 and 2.5 min, otherwise the flow was directed to waste.

The chromatographic system was coupled to 6460 Triple Quad Mass Spectrometer with Jet Stream electrospray ionization (Agilent Techologies, Santa Clara, USA) operated in the positive mode. Instrument parameters were set as follows: drying gas temperature 275 °C, drying gas flow 5 L/min, nebulizer 45 PSI, sheath gas temperature 320 °C, sheath gas flow 11 L/min, capillary entrance voltage 4000 V, nozzle voltage 1000 V, delta EMV 200 V. Both quadropoles Q1 and Q3 were set at wide mass resolution and the dwell time was 50 ms. Instrument control, data acquisition and quantification were performed by MassHunter Workstation software B.03.01 (Agilent Technologies, Torrance, USA). Automated procedure using Agilent Optimizer software to optimize multiple reactions monitoring (MRM) transitions and fragmentor voltage (200 V for all compounds) was used. The settings for quantification using MRM are presented in Table 3.

2.6. Method validation

The method was validated according to the FDA guidance on bioanalytical method validation [16], except for parameters recovery and matrix effect (ME) that were evaluated according to Matuszewski [17,18]. The urine samples were quantified using the ratio of the peak area of each analyte to that of IS.

For the determination of the selectivity of our method, six different sources of female urine were subjected to the same sample preparation and analytical procedure for the investigation of potential matrix interferences. A comparison of these chromatograms with those obtained after spiking blank urine with RAL, M1, M2, M3 and HAL (IS) ascertained that endogenous substances do not interfere with the assay. The presence or absence of any interfering peaks at the retention times of analytes or IS was evaluated.

The *recovery* of the method was determined by comparing the ratio of analyte responses obtained for standards spiked into blank urine before extraction and analyte responses obtained for standards spiked after extraction of blank urine at three concentration levels (QCl, QCm, QCh) on three parallels.

As it was suggested by Matuszewski [18] a determination of a relative *matrix effect* is much more important than the determination of absolute ME in the evaluation and validation of bioanalytical method in biofluids. The relative ME was checked in two ways. Firstly, the relative ME was evaluated on six different sources of urine for all tested analytes at three concentration levels (QCl, QCm, QCh) and three parallels by comparing the ratios of responses of raloxifene species to IS in different lots of urine by the post extraction spike method. Relative ME was expressed as a coefficient of variation (CV%) at each concentration level for each analyte. Secondly, on the basis of three concentration levels, slopes were calculated for each urine source. For the method to be considered reliable and free from the relative ME, the calculated coefficient of variation of determined slopes in different sources of matrices should not exceed 4% [17].

The *limit of detection* (LOD) was determined by spiking blank urine with low concentrations of raloxifene species to the concentration that achieved the signal-to-noise ratio of more than three.

The *lower limit of quantification* (LLOQ) was determined as the lowest standard on the calibration curve that reached precision better than 20% and accuracy from 80% to 120%.

The *linearity* was determined on the basis of eleven standard urine solutions with concentrations of M1, M2 and M3 presented in Table 2. In the case of RAL only nine calibration standards (SU3–SU11) were used. A non-weighted linear regression was applied to calculate the slopes and the intercepts of the calibration lines constructed as the ratio of analyte to IS response versus analyte concentration. The calibration curves were divided into two concentration ranges for all four analytes because of the relatively large concentration range. A determination coefficient of more than 0.99 was set as acceptable.

To determine intra- and inter-day *precision*, the QC samples at three levels were prepared in five replicates per day for three consecutive days. Intra-day precision was calculated for each day as CV% of five replicates. The inter-day precision was determined as a CV% of the mean values computed for three consecutive days. Precision was acceptable when the CV% was better than 15%, except at the LLOQ, where it should be less than 20%.

The *accuracy* was determined as percent ratio of the analyte concentration calculated from the calibration line versus nominal analyte concentration at three concentration levels (QCl, QCm, QCh) on five replicates. Accuracy was acceptable, when the determined concentration reached from 85% to 115% of nominal concentration, except for LLOQ, where the interval should be from 80% to 120%.

The *stability* was evaluated on three replicates of QCm and QCh samples. Four types of stability were investigated: freeze–thaw, short-term, long-term and post-preparative stability. Freeze–thaw stability was determined after three freeze–thaw cycles. When testing the short-term stability, urine samples were left for 24 h on the bench at room temperature, followed by preparation of the sample and analysis. For the determination of long-term stability, urine samples were stored at −86 °C for one month. Post-preparative stability was determined by re-injection of prepared sample after 18 h in autosampler. The stability was then evaluated by comparing the concentration found to the nominal values.

2.7. Analysis of patient samples

Urine samples were obtained from postmenopausal women with osteoporosis treated with 60 mg of raloxifene hydrochloride daily. For determination of RAL, M1, M2 and M3 a first morning urine was used. The urine was aliquoted to polypropylene tubes

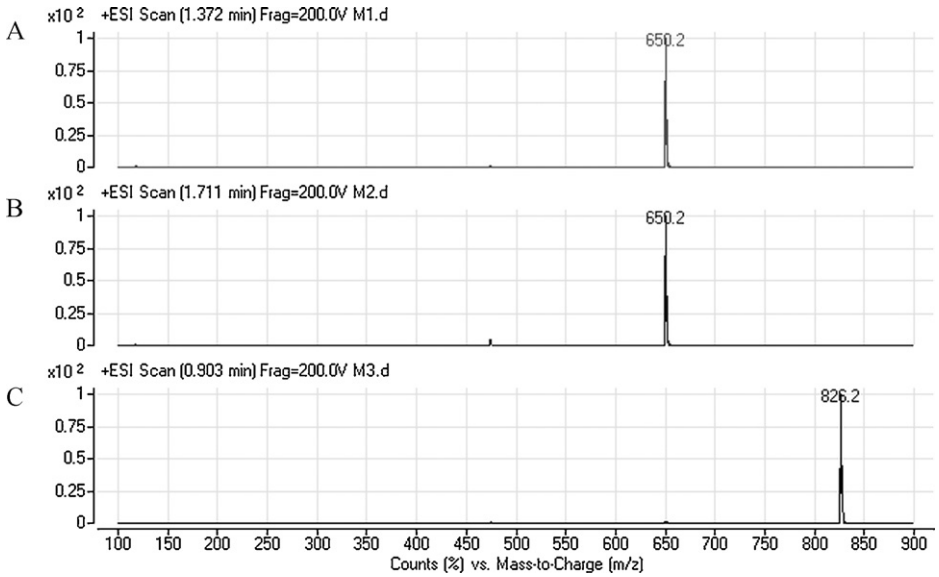


Fig. 2. Full scan mass spectra of M1 (trace A), M2 (trace B) and M3 (trace C) confirming the identity and purity of each glucuronide.

and stored at −86 °C until analysis. The samples were prepared as described in Section 2.4 and then subjected to LC–MS/MS analysis.

3. Results and discussion

3.1. Production of raloxifene glucuronide standards

Appropriate standards were needed for the successful development and validation of LC–MS/MS method. Because M1, M2 and M3 were commercially unavailable, we decided to prepare them in our laboratory. Firstly we tried the biochemical synthesis of glucuronide standards as it is described in Trontelj et al. [11], but with this method the preparation of M3 was not possible.

Therefore, the biotransformation of raloxifene was attempted by a modification of the method that was published by Lilly Research Laboratories [15] and turned out to be successful.

After described purification steps of bioconversion broth, the identity and purity of each raloxifene glucuronides was evaluated. The resulting chromatograms made in full scan mode confirmed the identity and purity of raloxifene glucuronides, giving only one chromatographic peak at retention times 1.372, 1.711 and 0.903 min for both monoglucoronides and diglucoronide, respectively. Full scan spectra of each peak showed strong molecular ions at m/z 650, 650 and 826 for M1, M2 and M3, respectively, with no background noise (Fig. 2). Additionally, the product ion scan showed the same mass spectra for M1 and M2 (fragmentation of parent m/z 650 to m/z 474 and 112 what corresponds to fragmentation of raloxifene without the glucuronic acid moiety (m/z 176)) and confirms that M1 and M2 are structural isomers (Fig. 3). The actual position of M1 and M2 in chromatogram was determined on the basis of literature data and our previous work [8,11,19]. Our previous work refers to the incubation of raloxifene with recombinant UGT1A10, which produces only M2. Based on that fact the retention time for M2 was determined and the not appearing peak in this incubation thereafter corresponds to M1. Product ion spectra confirmed the M3 structure by two subsequent m/z 176 neutral loses from the parent pseudomolecular ion (m/z 826), giving fragments of monoglucuronide (m/z 650) and of raloxifene (m/z 474) and additional m/z 112 fragment of raloxifene (Fig. 3).

Because the produced amount of glucuronides was low and difficult to accurately weight, the products were reconstituted in DMSO and the concentration of each solution was determined by incubation with β-glucuronidase. At described conditions conversion of glucuronides to raloxifene with β-glucuronidase was complete. The determined concentrations of stock solutions were as follows: 2.074 mM for M1, 1.567 mM for M2 and 0.401 mM for M3. Based on obtained results the purity of all three metabolites was confirmed.

3.2. Method validation

3.2.1. Selectivity

The chromatograms of spiked human urine (LLOQ) with analytes and IS and chromatogram of a patients urine are presented in Fig. 4. Retention times for RAL, M1, M2, M3 and HAL were 2.104, 1.426, 1.780, 0.973 and 2.110 min, respectively. Selectivity was confirmed by the absence of any peaks in each of the selected reaction monitoring mode (SRM) chromatograms of each analyte and IS at their retention times in processed blank urine samples from six different sources. Beside no matrix interferences, the chromatograms in Fig. 4 also show good resolution and separation of all raloxifene species with good symmetry and sharpness of peaks. However, cross-talk interference between SRM transitions of glucuronides and raloxifene was detected, because all three glucuronides form the same fragment m/z 474 → 112 (Fig. 3) but due to good chromatographic separation of peaks this should not be problematic. However, no cross-talk was observed for co-eluting peaks of RAL and HAL (Fig. 5). Nevertheless, to avoid any confusion, the LC–MS/MS software was set to collect data in two segments. In the first segment data for glucuronides and in the second segment, that starts earlier than raloxifene peak in raloxifene trace (m/z 474 → 112), the data for RAL and HAL were collected.

3.2.2. Matrix effect and recovery

The evaluation of ME on bioanalytical LC–MS/MS methods in biological fluids is a very important and sometimes overlooked aspect of assay validation. The current FDA Guidance for Industry and Bioanalytical Method Validation clearly indicate the need to assess matrix effect of LC–MS/MS methods but no guides to demonstrate the presence or absence of ME are suggested [16]. Therefore the well recognised post-extraction spike method was used for assessment of ME. As it was proposed by Matuszewski the demonstration of the absence of a "relative" ME is even a more important parameter than the evaluation of absolute ME [18]. This aspect of the ME assessment is highly relevant for the development of selec-

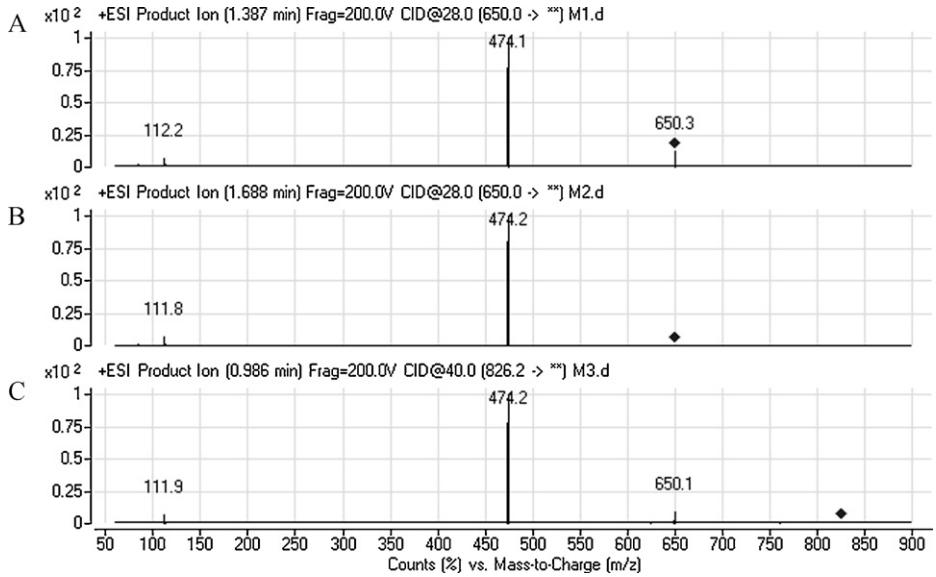


Fig. 3. Product ion scan that shows fragmentation pattern of raloxifene monoglucuronides M1 (trace A), M2 (trace B) and raloxifene diglucuronide M3 (trace C).

tive LC–MS/MS method. In our method development a significant ME was noticed for raloxifene and its metabolites. To minimize or eliminate this effect, different actions were taken as described by Van Eeckhaut et al. [20] and Kloepfer et al. [21]. To lower the ME, firstly, sample preparation method was modified. After washing the SPE cartridge with 1 mL of water, 1 mL of 10% methanol was used to wash the cartridge more efficiently. Secondly, smaller injection volume was used (0.5 μL instead of 20 μL or more as seen in other methods), what was enabled by use of one of the most sensitive LC–MS/MS on the market. Thirdly, the eluent flow entering the ESI interface was reduced by post-column splitting in ratio 1:1. The results of relative ME for each analyte are presented in

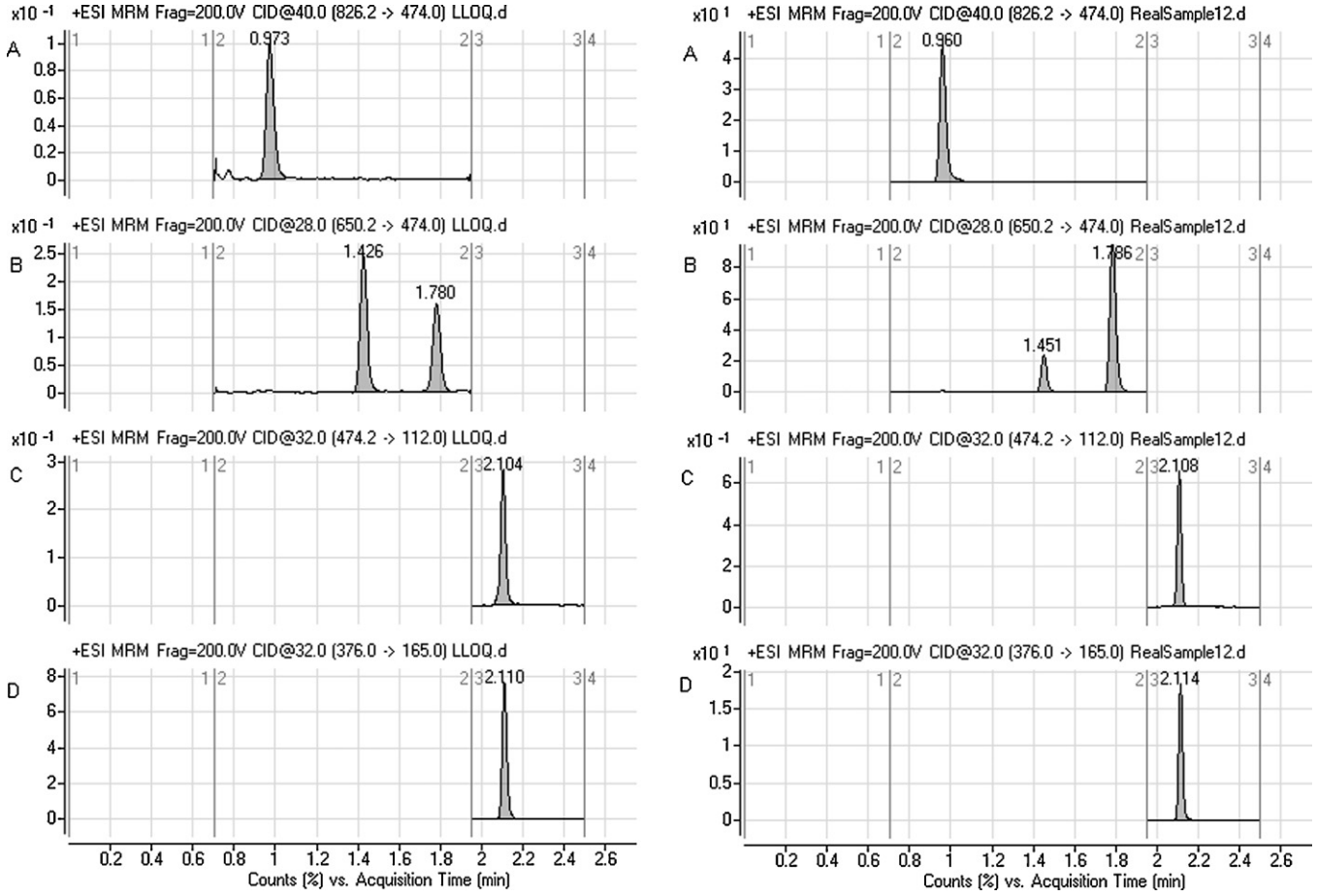


Fig. 4. The LC–MS/MS chromatograms of a urine spiked with standards at LLOQ (left) and of urine sample from a patient receiving raloxifene (right). Trace A represents mass transition for M3, trace B for M1 and M2, trace C for RAL and trace D for HAL.

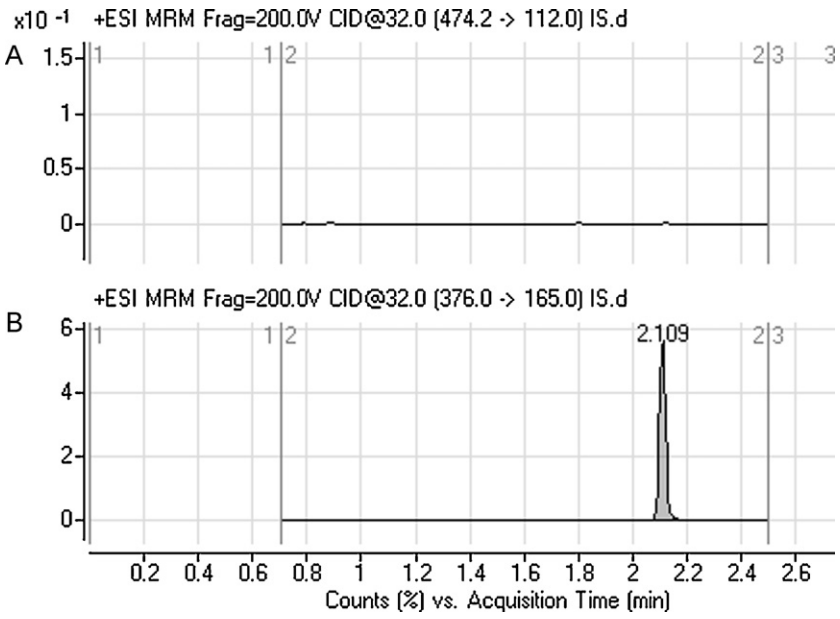


Fig. 5. The LC–MS/MS chromatogram presenting internal standard (HAL) injected alone and monitoring response at the RAL channel. Trace A represents mass transition for RAL, trace B for HAL.

Table 4
Recovery and relative matrix effect (ME) data for RAL, M1, M2 and M3.

	RAL	M1	M2	M3
Recovery (%)	95.9–101.6	96.0–100.4	96.6–99.7	92.5–100.2
Relative ME (%)	3.7–7.4	2.9–5.4	3.5–4.2	3.9–5.0
Slope CV (%)	3.2	2.6	3.6	3.4

Table 4. Relative ME expressed as CV(%) obtained from six different urine sources at three concentrations (QC) ranged between 2.9 and 7.4% for all analytes and is comparable with inter-day precision (Table 6) of QC samples prepared from single urine source. Variability of standard line slopes in different sources of biofluids (precision of standard line slope expressed as CV (%)) may also serve as a good indicator of relative ME. Absence or insignificant ME was confirmed also by this parameter as all analytes had coefficient of variation of slopes under suggested limit of 4% (Table 4).

Evaluation of recovery on three concentration levels at three parallels for all analytes revealed values presented in Table 4. Recovery was for all analytes very high and reproducible and ranged from 92.5% and up to 101.6%. Additionally, on the basis of high recovery values of all glucuronides it could be assumed that the raloxifene glucuronides are stable during the process of sample preparation.

3.2.3. Linearity, limit of detection and lower limit of quantification

After the division of calibration curves into two concentration ranges for all analytes, the linearity was achieved in the following concentration range: RAL ranged from 1.01 to 260 nM, M1 from 1.95 to 2000 nM, M2 from 2.83 to 2900 nM and M3 from 4.68 to 4800 nM (Table 5). The achieved linearity (r^2) was in all cases higher than 0.997.

Achieved LOD and LLOQ values for the developed LC–MS/MS method are presented in Table 2. The method showed to be sensitive enough for determination of RAL, M1, M2 and M3 in urine of postmenopausal patients treated with raloxifene. The LLOQ for metabolites could be achieved even at lower concentrations but due to their much higher concentrations compared to parent raloxifene there was no need to go lower.

3.2.4. Accuracy and precision

The intra- and inter-day accuracy and precision of the method for RAL, M1, M2, M3 are presented in Table 6 and are within acceptable limits.

Additionally, the performance of the LC–MS/MS instrument was tested. Precision expressed as CV (%) after six injections of the same sample was 0.5%, 0.9%, 0.5%, 0.8% for RAL, M1, M2 and M3, respectively.

3.2.5. Stability

All stability results are summarized in Table 7. There was no deviation from time-zero for RAL, M1, M2 and M3 in urine at any concentration level for three freeze–thaw cycles. Also short-term stability (24 h) turned out to be appropriate. Stability showed to be acceptable after 1 month storage on −86 °C for all analytes. No significant deviation was also observed when the prepared samples were left in autosampler for 18 h.

Table 5
Linearity data for RAL, M1, M2 and M3.

	Low concentration range				High concentration range			
	Range [nM]	Slope, $\times 10^{-3}$	Intercept, $\times 10^{-3}$	r^2	Range [nM]	Slope, $\times 10^{-3}$	Intercept, $\times 10^{-3}$	r^2
RAL	1.01–16.0	11.330	−2.665	0.9972	16.0–260	14.996	−16.183	0.9984
M1	1.95–62.5	21.086	−5.417	0.9996	62.5–2000	20.200	176.400	0.9967
M2	2.83–90.6	17.006	−0.694	0.9993	90.6–2900	17.431	34.904	0.9996
M3	4.69–150	9.189	0.043	0.9997	150–4800	9.073	68.397	0.9998

Table 6
Presentation of intra- and inter-day accuracy and precision of the method for RAL, M1, M2, M3.

	Nominal concentration [nM]	Intra-day			Inter-day		
		Mean concentration found[a] [nM]	Precision [CV%]	Accuracy[b] [%]	Mean concentration found[a] [nM]	Precision [CV%]	Accuracy[b] [%]
RAL							
QCl	2.17	2.29	6.3	105.3	2.36	12.0	108.8
QCm	21.70	23.01	4.8	106.0	22.12	10.7	102.0
QCh	130.00	132.63	0.8	102.0	132.30	4.0	101.8
M1							
QCl	5.56	5.51	3.0	99.1	5.72	3.8	102.8
QCm	83.35	86.26	4.2	103.5	86.58	2.3	103.9
QCh	1000.00	1029.98	1.8	103.0	1019.31	1.4	101.9
M2							
QCl	8.06	8.21	2.8	101.8	8.04	2.3	99.7
QCm	120.85	122.67	2.5	101.5	117.14	1.2	96.9
QCh	1450.00	1463.55	2.2	100.9	1450.63	0.8	100.0
M3							
QCl	13.33	14.14	3.5	106.1	13.71	2.9	102.9
QCm	200.00	203.42	1.4	101.7	198.61	0.4	99.3
QCh	2400.00	2441.39	2.3	101.7	2427.21	0.5	101.1

[a] Back-calculated concentrations of analytes in urine.
[b] (Mean concentration found/nominal concentration) × 100.

Table 7
Stability data for RAL, M1, M2 and M3 in urine samples.

	RAL	M1	M2	M3
Autosampler	100.5–101.9	99.9–105.8	101.8–103.3	104.4–105.0
Short-term	100.2–102.7	97.9–101.8	98.9–100.9	97.5–102.9
Freeze–thaw	97.9–98.6	98.8–99.8	98.4–99.5	98.8–100.3
Long-term	95.2–95.4	96.3–98.2	94.8–97.3	98.5–98.9

3.3. Analysis of patient samples

The assay proved appropriate for the quantification of raloxifene, M1, M2 and M3 in the urine of postmenopausal women treated with 60 mg of raloxifene daily. In the assayed samples, the mean concentration levels found (with standard deviation) were 4.5 ± 1.7, 106.9 ± 84.9, 1187.8 ± 839.0 and 1185.8 ± 668.0 nM for RAL, M1, M2 and M3, respectively. It was not possible to make a comparison of obtained concentrations of raloxifene species with other published methods [12–14] because the glucuronides were not measured and the determined LLOQ of raloxifene was too high for quantification of raloxifene in real urine samples. However, a comparison of obtained urine concentrations of RAL, M1 and M2 with their plasma concentrations [11] showed that in both matrices the concentrations of glucuronides are much higher than concentration of RAL, moreover the concentration of M2 is in both matrices several times higher than concentration of M1. For M3 there are currently no available data because we implemented for the first time a method for quantification of raloxifene diglucuronide in urine or plasma. The developed method for simultaneous determination of RAL, M1, M2 and M3 could be also applied to other matrices and therefore appropriate for use in further research of still not completely discovered raloxifene pharmacokinetics.

Because raloxifene is also on the list of prohibited substances published by WADA [6] another application of this method could be anticipated. Advantage of our method lies in the fact that determination of glucuronides is more convenient compared to examination of raloxifene due to extensive metabolism of raloxifene to its metabolites [9]. The additional advantages of developed method compared to published methods [12,14] are lower LOD for raloxifene and less time needed for one analysis due to absence of the step for hydrolysis of raloxifene glucuronides to raloxifene. As it was found out on real urine samples, the concentration of parent raloxifene in urine is as expected very low and therefore the determination of its glucuronides, that are present in much higher concentration level, is much more reliable. Although assayed urine samples were derived from postmenopausal women, we assume that they do not differ significantly from drug abusers in sports, who probably take raloxifene for longer periods of time for the attainment of the wished effect. Furthermore, presuming linear pharmacokinetics, 3.125% of drug would still remain in the body after five elimination half lives after the last dose of raloxifene what corresponds to more than five days. This is a safe assumption since the terminal parts of the log-linear concentration curves for raloxifene and its glucuronides are essentially parallel because of the constant interconversion of raloxifene and its glucuronides [7]. By considering this approximation the concentration of raloxifene glucuronides M1, M2, and M3 in urine would drop to approximately 3, 37 and 37 nM, respectively (Table 8), which is still above the quantification limit of our method. Moreover, a simple calculation reveals that the

Table 8
Elimination of RAL, M1, M2 and M3 to urine.

Multiple of $t_{1/2}$	0	1	2	3	4	5	6	...	14	15
% in the body	100	50	25	12.5	6.25	3.125	1.565	...	0.0061	0.0031
M1 [nM]	106.9	53.45	26.73	13.36	6.681	3.341	1.670	...	0.0065	0.0033
M2 [nM]	1187.8	593.9	297.0	148.5	74.24	37.12	18.56	...	0.0725	0.0362
M3 [nM]	1185.8	592.9	296.5	148.2	74.11	37.06	18.53	...	0.0724	0.0362
RAL [nM]	4.5	2.25	1.125	0.5625	0.2813	0.1406	0.0703	...	0.0003	0.0001

$t_{1/2}$: elimination half life (raloxifene $t_{1/2}$ is 28 h).

presented method would still be able to detect the most abundant raloxifene glucuronides in urine (M2, M3) even after 14 half lives or more than 16 days after the last dose.

4. Conclusions

To our knowledge the developed analytical method is the first fully validated method capable of simultaneous determination of raloxifene and its metabolites in real urine samples. Moreover, for the first time a method for determination of raloxifene diglucuronide (M3) in relevant biological samples was introduced. The developed LC–MS/MS method provides a sensitive, specific, accurate and precise method for quantification of raloxifene and its three glucuronides in human urine. The limit of quantification for determination of raloxifene in urine (1.01 nM) achieved with the developed method was lower than by any of the previously published methods [12–14] and consequently appropriate for the analysis of real urine samples, because expected concentration of raloxifene in urine is very low. The limits of quantification achieved for metabolites were 1.95, 2.83 and 4.69 nM for M1, M2 and M3, respectively, and are more than sufficient for the quantification of glucuronides as their concentration in urine turned out to be quite high. Short LC–MS/MS run time of the developed method enables analysis of many samples in a short time. However, the development of this method required a lot of effort, because all the metabolites had to be synthesized, characterised and purified.

Applicability of the method was confirmed on urine samples of women receiving raloxifene. In the assayed samples quite high concentrations of metabolites with mean values of 107, 1188 and 1186 nM for M1, M2 and M3, respectively, were found. While mean concentration of raloxifene (4.5 nM) was as expected very low.

The method is undoubtedly appropriate for use in further research of still not completely discovered raloxifene pharmacokinetics. Although a newly developed method for determination of raloxifene and its glucuronides has been developed for use in pharmacokinetic studies, it can also be used as a potential application in anti-doping analysis.

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