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# Development and validation of a sensitive liquid chromatography/tandem mass spectrometry method for the determination of exemestane in human plasma

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ABSTRACT

Exemestane, irreversible steroidal aromatase inhibitor, acts as a false substrate for aromatase enzyme and significantly lowers circulating estrogen concentrations in postmenopausal women with hormone-sensitive breast cancer. A sensitive bioanalytical method was developed and validated to study pharmacokinetics of exemestane. The method was based on liquid–liquid extraction of exemestane with methyl *t*-butyl ether followed by reversed-phase liquid chromatography. Positive electrospray ionization tandem mass spectrometry in multiple reaction monitoring mode was applied for detection of exemestane. Anastrozole was used as internal standard. Calibration curve, fitted to $1/x^2$ weighted linear regression model, was linear in the range of 0.1–40.0 ng/mL. Intra-run precision and accuracy were 1.80–3.17% and 103.4–111.5%, respectively. Inter-run precision and accuracy measured within 3 days were 3.37–4.19% and 101.8–109.6%, respectively. Extraction recoveries of exemestane and internal standard were 79.7–86.2% and 82.9–83.6%, respectively. The method was fully validated and may be applied to pharmacokinetic studies in humans after a single dose administration of 25 mg exemestane tablets.

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## 1. Introduction

Breast carcinoma has become a major health problem affecting as many as one in eight women [1]. It is recognized that approximately one-third of all breast carcinoma are estrogen dependent and regress after estrogen deprivation [2]. Thus, reducing the level of estrogen through inhibition of its biosynthesis by aromatase inhibitor, exemestane remains a valuable treatment of breast carcinoma. Exemestane is metabolised by CYP3A4 and its primary metabolite in plasma is 17-hydroxyexemestane. The concentrations of primary metabolite observed in human plasma are ca. 10 times lower than respective concentrations of parent drug.

According to United States National Institute of Health during the last 2 years over 30 clinical trials were conducted on exemestane and many more are currently in progress, clearly indicating a need for a simple and reliable bioanalytical method for its quantification. The determination of exemestane in human plasma was described using HPLC with UV [3], radioimmunoassay [4] or mass spectrometric detection [5–8]. Simultaneous quantification of exemestane and its main metabolite 17-dihydroexemestane was also reported [6,7]. The described HPLC–UV method [3] is not sensitive enough (lower limit of quantification – LLOQ at 10 ng/mL) for purposes of pharmacokinetic studies in humans after a single dose administration of 25 mg exemestane tablets. Coupling of HPLC and radioimmunoassay techniques [4] enables very low quantification limits (LLOQ at 0.012 ng/mL), nevertheless it also requires extensive sample preparation, which limits method's sample throughput to only 70 samples per week. The first reported mass spectrometric method [5], similarly to HPLC–UV, is not sensitive enough (LLOQ at 1 ng/mL) for the planned study and it includes rather expensive sample preparation by solid phase extraction (SPE). The method reported by Groenewoud et al. [6] does not provide details concerning both HPLC and MS conditions, therefore it is not directly reproducible. The recently published method by Corona et al. [7] is based on simple sample preparation by protein precipitation and requires plasma volume of only 0.1 mL, but the LLOQ was set at 0.2 ng/mL. Depending on mass spectrometer ionization source construction, during the long-term analysis of plasma samples pretreated by protein precipitation, loss of sensitivity caused by endogenous interferences burned in ion source may be observed in some cases. There is also an increased risk of matrix effects occurrence in study samples due to not extensive enough sample clean-up, which may be hardware configuration dependent. The LC–MS/MS method described by Cenacchi et al. [8] presents very attractive LLOQ of 0.05 ng/mL, however, it suffers from tailing peak of exemestane and more expensive sample preparation by SPE, suggesting place for improvement in terms of both chromatographic conditions and sample preparation.

Concerning reported studies, there is no bioanalytical method available with sensitivity of LLOQ ca. 0.1 ng/mL as well as simple

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and LC–MS compatible procedure of sample preparation. Based on our previous experience with LC–MS bioanalytical methods employed in pharmacokinetic studies of clopidogrel [9] and tamsulosin [10], a novel assay for the determination of exemestane using liquid–liquid extraction (LLE) was developed. Presented study, for the first time describes a fully validated LC–MS/MS method suitable for pharmacokinetic studies in humans, which does not require the use of isotope labelled exemestane as an internal standard.

## 2. Experimental

### 2.1. Chemicals and reagents

Exemestane, 6-methylenandrostan-1,4-diene-3,17-dione, reference standard, was purchased from the Haorui Pharma-Chem Inc., Edison, NJ, USA.

Anastrozole, 2-[3-(2-cyanopropan-2-yl)-5-(1,2,4-triazol-1-ylmethyl)phenyl]-2-methylpropanenitrile, the internal standard (IS), was prepared at Pharmaceutical Research Institute in Warsaw, Poland.

Methanol, acetonitrile (both of MS grade) and formic acid (analysis grade) were purchased from Merck, Darmstad, Germany. Methyl *t*-butyl ether (HPLC grade) was purchased from Sigma Aldrich, Steinheim, Germany. Ammonium solution 25% (analysis grade) was supplied by Chempur, Piekary Slaskie, Poland. Purified water from Milli-Q system, Millipore, Molsheim, France, was used throughout the study.

### 2.2. Instrumentation

LC–MS/MS analysis was performed using Quattro Micro API triple quadrupole mass spectrometer equipped with an electrospray ion source (Waters, Manchester, UK). The HPLC system was Alliance 2695 series liquid chromatograph with a gradient pump, an autosampler with a cooler, a column oven and a vacuum degasser (Waters, Milford, MA, USA). The data was processed using MassLynx version 4.1 software (Waters, Manchester, UK).

### 2.3. Chromatographic conditions

The chromatographic separation from endogenous compounds was performed on Symmetry C18 column (50 mm × 2.1 mm, 3.5 μm, Waters, Milford, MA, USA), which was preceded by a C18 guard column (4 mm × 2 mm, Phenomenex, Torrance, CA, USA). Gradient elution was applied with 0.1% aqueous formic acid (Phase A) and acetonitrile (Phase B). The following gradient program was used: 0–7 min: 20 → 55% B (linear), 7–8 min: 55% B, 8–8.1 min: 55 → 90% B, 8.1–9 min: 90% B, 9–9.1 min: 90 → 20% B, 9.1–10.0 min: 20% B. The flow rate of the mobile phase was set at 0.4 mL/min. The column and the autosampler were maintained at 30 ± 5 °C and 20 ± 5 °C, respectively.

### 2.4. Mass spectrometric conditions

The Quattro Micro API triple quadrupole mass spectrometer was equipped with an electrospray ionization source (ESI) and it was operated in the positive ion detection mode in the multiple reaction monitoring mode (MRM). Transitions 297 → 121 *m*/*z* and 294 → 225 *m*/*z* were monitored for exemestane ($C_{20}H_{24}O_2$, molecular weight: 296.40 u, exact mass: 296.18 u) and IS ($C_{17}H_{19}N_5$, molecular weight: 293.36 u, exact mass: 293.16 u), respectively. The desolvation temperature was 350 °C with nitrogen gas flow of 800 L/h. The capillary voltage was 1.1 kV and the source temperature was 110 °C. The cone voltage of 22 V for exemestane and 30 V for IS was selected. The optimized cell collision energy was 19 eV for both exemestane and IS. Argon was used as a collision gas.

### 2.5. Preparation of standards and quality control samples

Calibration standards contained exemestane at following concentration levels: 0.1, 0.3, 5.0, 10.0, 15.0, 20.0, 30.0, and 40.0 ng/mL. Quality control (QC) samples contained exemestane at three concentration levels: 0.3, 15.0 and 30.0 ng/mL. The highest calibration standard (40.0 ng/mL) was prepared by spiking blank human plasma with appropriate volume of working solution of exemestane (10 μg/mL in 50% aqueous acetonitrile). Other calibration standards and QC samples were prepared by appropriate dilutions with blank human plasma and stored below −14 °C. Sodium citrate was used as an anticoagulant.

### 2.6. Sample preparation

An aliquot of human plasma (0.5 mL) was mixed with 50 μL of IS working solution (20 ng/mL of IS in 50% acetonitrile). Aqueous ammonia (0.1 mL of 25% solution) was added and the solution mixed well. Methyl *t*-butyl ether (2.5 mL) was added and the mixture was shaken for 5 min on a vibrax mixer at 1800 rpm. The sample was centrifuged for 5 min at 3500 rpm at 4 ± 2 °C and frozen below −50 °C for 10 min. The organic layer was transferred to a glass tube and evaporated to dryness under a stream of nitrogen in a water bath at 40 ± 2 °C. The residue was reconstituted in 150 μL of 50% aqueous acetonitrile and shaken on a vibrax mixer for 1 min at 1400 rpm. The solution was transferred to an autosampler vial and 30 μL of this solution was injected into the LC–MS/MS system.

## 3. Results

### 3.1. Method development

Initial experiments conducted on a single quadrupole mass spectrometer (LCMS-2010, Shimadzu, Duisburg, Germany) suggested that an atmospheric pressure chemical ionization (APCI) allows more efficient ionization of exemestane than ESI and therefore provides better sensitivity of bioanalytical method. In our hands the method based on a single quadrupole mass spectrometer detection was found to be not sensitive enough to perform pharmacokinetic studies in humans, where LLOQ of 0.1 ng/mL was required. Therefore, further experiments were performed on a tandem mass spectrometer.

Optimization of component dependent parameters for APCI and ESI ionization sources was carried out by direct infusion of standard solution into the mobile phase. Exemestane standard solution at 10 μg/mL in 50% aqueous acetonitrile was infused at a flow rate of 10 μL/min. Then a standard solution of IS at 10 μg/mL in 50% aqueous acetonitrile was infused using ionization conditions selected for exemestane.

As reported previously [7,8,11], under optimized ionization conditions in the positive APCI mode, the precursor ion spectrum of exemestane showed that the molecular ion $[M+H]^+$ of *m*/*z* 297 was the most abundant. Contradictory to the initial experiments performed on a single quadrupole LC–MS, better sensitivity on tandem mass spectrometer was obtained using ESI ion source. This observation, discussed previously by Corona et al. [7], could be explained by a different construction of APCI source in Shimadzu and Waters instruments. The optimized MS parameters are described in Section 2.4. The full-scan first quadrupole mass spectrum and respective product-ion mass spectrum for exemestane are presented in Figs. 1 and 2, respectively. The $[M+H]^+$ of *m*/*z* 297 was the most abundant peak on MS1 spectrum of exemestane

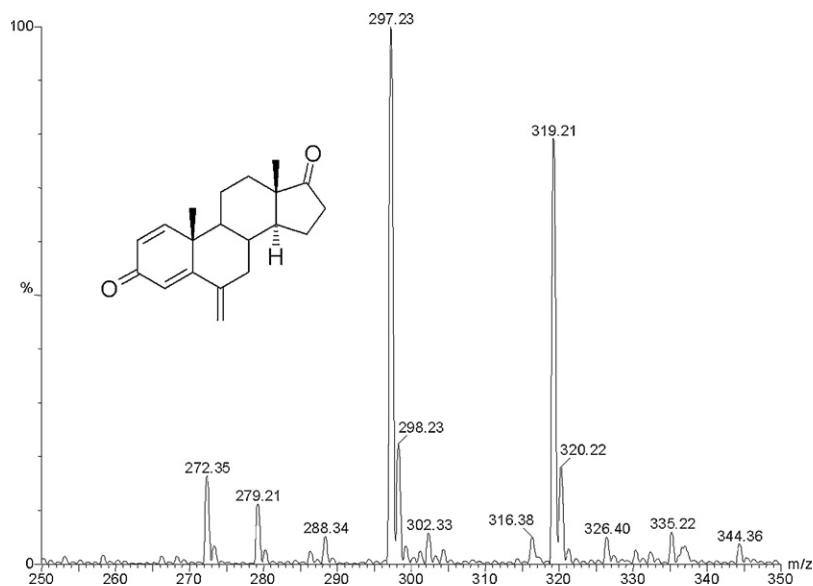


**Fig. 1.** Full-scan first quadrupole mass spectrum (MS1) of exemestane in positive ESI mode.

(Fig. 1) and significant peak of sodium adduct $[M+Na]^+$ of $m/z$ 319 was also observed.

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several steps in order to achieve good resolution and symmetric peak shapes in as short measurement time as possible. A number of different chromatographic columns were tested, including Zorbax SB-Phenyl 150 mm × 2.1 mm, 5 μm (Agilent Technologies); Gemini C6-Phenyl 50 mm × 2.1 mm, 3 μm (Phenomenex) and Symmetry C18 50 mm × 2.1 mm, 3.5 μm (Waters). The mixture of acetonitrile and 0.1% aqueous acetic acid was used as the initial mobile phase [3,7,8,11,12]. The results suggested that required sensitivity could not be achieved in these conditions. Therefore, other mixtures were tested, including aqueous solutions of ammonium acetate and formic acid. In order to further improve the sensitivity of the bioanalytical method, isocratic elution was replaced with several gradient programs.

It was found that the acceptable resolution of exemestane and IS peaks could be obtained with the gradient elution of acetonitrile and 0.1% aqueous formic acid on a Symmetry C18 column (50 mm × 2.1 mm, 3.5 μm, Waters). The exemestane was eluted at 5.4 min and IS at 3.4 min (Fig. 3). The optimized LC parameters are described in Section 2.3.

The complete chromatographic resolution of exemestane and IS excluded the risk of competition between the molecules at the ionization source as well as between the respective ions in a collision cell. The resolution led to better repeatability of exemestane ionization and fragmentation, especially in the case of higher concentrations of exemestane in plasma samples. Moreover, the optimized gradient program provides a better protection of the column from the accumulation of hydrophobic interfering compounds that might have been extracted from plasma samples.

As mentioned in the introduction, LLE is a technique of choice for sample preparation applied to LC–MS determination of small molecules in biofluids. Although LLE is more time consuming than

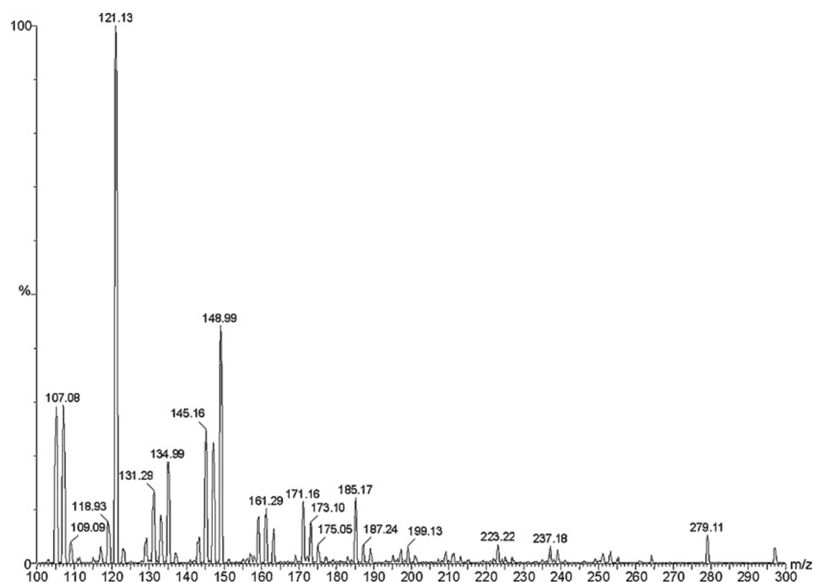


**Fig. 2.** Product-ion mass spectrum (MS2) of exemestane $[M+H]^+$ of $m/z$ 297 in positive ESI mode.

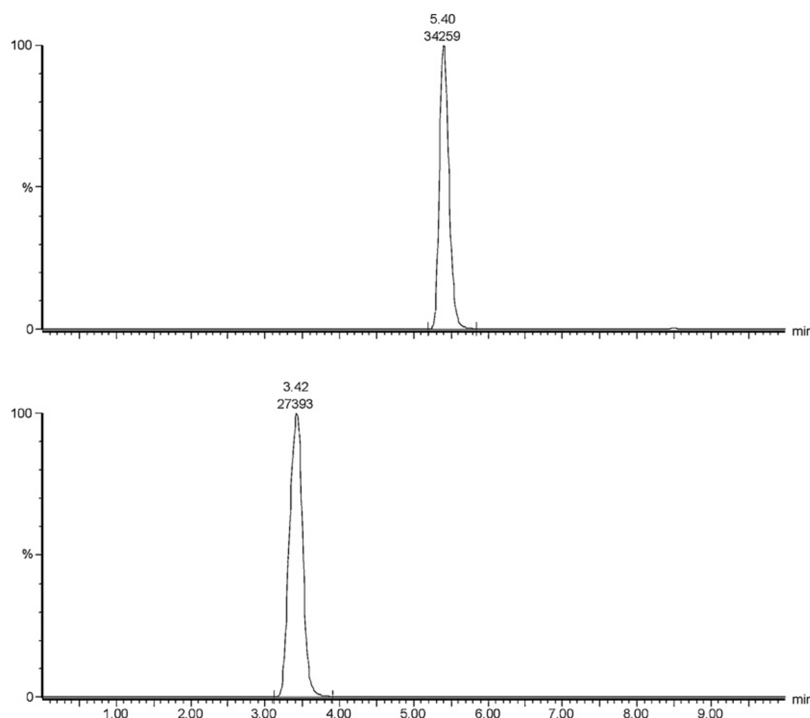


**Fig. 3.** LC–MS/MS chromatogram of standard solution of the highest concentration of exemestane and IS in MRM mode: transitions 297 → 121 $m/z$ and 294 → 225 $m/z$ for exemestane (top) and IS (bottom), respectively.

protein precipitation, it provides better sample clean-up and therefore the risk of potential matrix effects is significantly decreased. Moreover, LLE is less expensive than SPE. During the development of sample preparation method the following organic solvents were used: methylene chloride, ethyl acetate and methyl *t*-butyl ether. Because anastrozole is a week base, it was decided to alkalize aqueous phase for initial experiments with sodium hydroxide (0.1–1.0 M) or ammonia solution. The obtained results suggested that the combination of 25% aqueous ammonia and methyl *t*-butyl ether provides stable instrument response and decreased extraction of interfering compounds resulting in reduction of number of additional peaks observed on MS chromatograms. Finally, the optimization of organic solvent volume lead to the conclusion that 2.5 mL of methyl *t*-butyl ether is sufficient to obtain high recovery of exemestane and IS from 0.5 mL plasma sample.

It is generally accepted that stable isotope labelled substances are preferred as the internal standards, because of their ability to correct ionization variability caused by endogenous and exogenous compounds. However, in the case of exemestane determination, it surprisingly seems that anastrozole gives better precision of measurements (RSD < 4.2%) than application of $^{13}$C-3 exemestane as IS described by Corona et al. (RSD < 7.8%) [7] and Cenacchi et al. (RSD < 9.6%) [8]. The difference may not be statistically significant, but better precision proves that anastrozole, although of a different chemical structure then exemestane, is an appropriate internal standard. Moreover, it is less expensive alternative for the use of labelled exemestane.

### 3.2. Method validation

The validation parameters were defined according to the respective regulatory guidance [13]. The study was performed in compliance with the principles of Good Laboratory Practice (GLP). For the calculation of precision, accuracy, calibration curve parameters and selected stability results, a normal distribution of measurements was assumed. The outliers were detected with Dixon *Q*-test ($\alpha = 0.05$) and discarded from the calculations of stability as well as the calibration curve parameters. The statistical analysis of stability included the comparison of two sets of experimental data, assumed log-normal distribution of measurements' results and it was based on the application of confidence intervals (C.I.) [14,15]. The construction of C.I. depends on variance equality, therefore *F*-Snedecor test at $\alpha = 0.01$ was applied for the testing of the hypothesis on variance equality.

#### 3.2.1. Limits of detection (LOD) and quantification (LLOQ)

The limit of detection (LOD) was 0.025 ng/mL and the LLOQ was 0.1 ng/mL (Fig. 4). The accuracy for LLOQ was 108.5% (RSD = 2.92%, $n = 6$) within 1 day and 109.9% (RSD = 3.70%, $n = 6$) within 3 days.

#### 3.2.2. Selectivity and carry-over

The selectivity test, in which blank human plasma samples from six different sources were analyzed, showed no peaks near the retention time of exemestane and IS. The carry-over experiment, in which blank human plasma samples were analyzed right after highest concentration calibration standards (procedure repeated 6 times), showed no peaks influencing quantification.

#### 3.2.3. Linearity

The calibration curve was linear in the range 0.1–40.0 ng/mL regarding the peak area ratio of exemestane to IS versus the nominal concentration of exemestane. The curve was obtained by a weighted linear regression analysis with $w = 1/x^2$ chosen according to the minimum sum of percentage relative errors [16]. The values of regression parameters for the curve, described by the equation: $y = ax + b$, where: $a = 0.039$, $b = 0.003$ and $r = 0.9938$ ($n = 6$). All regression parameters were statistically significant ($\alpha = 0.05$, $df = n - 2$).

#### 3.2.4. Accuracy and precision

The results of the intra-run (within 1 day, $n = 6$) and inter-run (within 3 days, $n = 6$) accuracy and precision for three QC levels are presented in Table 1. For each QC level the accuracy was within the

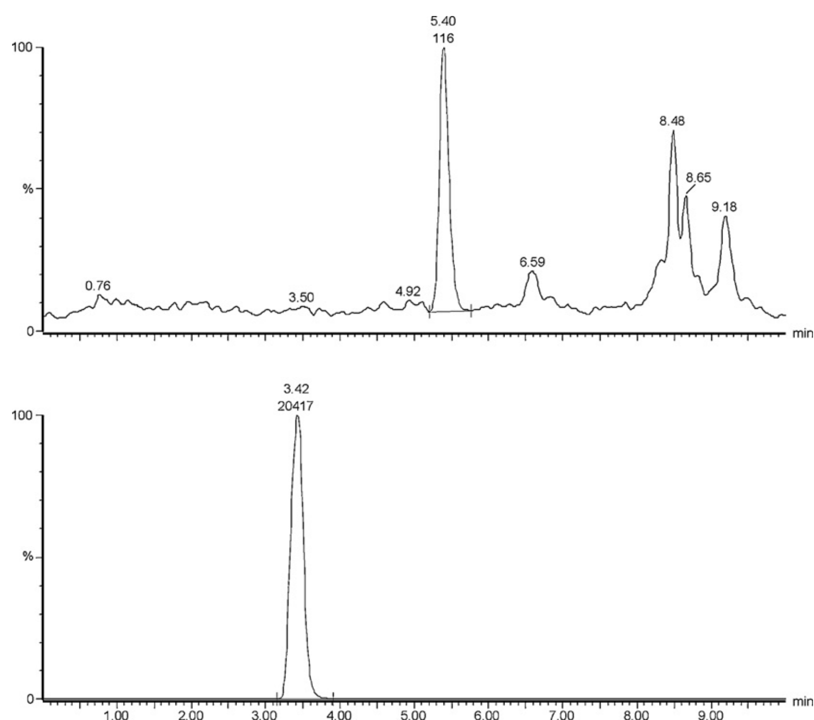


**Fig. 4.** LC–MS/MS chromatogram of blank human plasma spiked with 0.1 ng/mL of exemestane and working concentration of IS in MRM mode: transitions 297 → 121 *m/z* and 294 → 225 *m/z* for exemestane (top) and IS (bottom), respectively.

**Table 1**
Intra-run and inter-run accuracy and precision of exemestane determination.

| Nominal concentration (ng/mL) | Mean observed concentration (ng/mL) | Accuracy (%) | Precision (%) |
|---|---|---|---|
| Intra-run ($n$ = 6) | | | |
| 0.3 | 0.33 | 111.5 | 1.80 |
| 15.0 | 16.31 | 108.8 | 3.01 |
| 30.0 | 31.02 | 103.4 | 3.17 |
| Inter-run ($n$ = 6) | | | |
| 0.3 | 0.33 | 109.6 | 3.37 |
| 15.0 | 15.72 | 104.8 | 4.19 |
| 30.0 | 30.53 | 101.8 | 3.51 |

acceptance criteria of 85–115% and the precision was within the acceptance criteria <15%.

*3.2.5. Stability*

The results of the stability tests: autosampler, freeze and thaw, short-term and preliminary long-term at two storage temperatures are presented in Table 2. For each concentration level 90% C.I. for the mean stability met the acceptance criteria falling within the range of 85–115%. The respective stability tests confirmed the stability of exemestane in the stock and working solutions.

*3.2.6. Recovery, matrix effect and process efficiency*

To determine the extraction recovery of exemestane for each QC level, blank human plasma samples from six different sources were spiked with both exemestane and IS then extracted immediately before LC–MS/MS analysis (post-extraction spiked plasma samples). The comparison of the peak areas determined in the pre- and post-extraction spiked plasma samples indicated the extraction recovery of exemestane and IS. The extraction recovery of exemestane was stable across the studied concentration range and did not influence recovery of IS (Table 3).

The influence of the matrix effects on method reliability was studied according to Matuszewski et al. [17]. The relative matrix effect was studied at three QC levels and calculated based on RSD

**Table 3**
The extraction recovery of exemestane and IS.

| Nominal concentration (ng/mL) | Post-extraction spiked (mean ± SD) | Pre-extraction spiked (mean ± SD) | Recovery (%) |
|---|---|---|---|
| Peak area of exemestane ($n$ = 6) | | | |
| 0.3 | 440 ± 5 | 350 ± 13 | 79.7 |
| 15.0 | 17,462 ± 363 | 14,572 ± 358 | 83.5 |
| 30.0 | 33,712 ± 1013 | 29,056 ± 830 | 86.2 |
| Peak area of IS ($n$ = 6) | | | |
| 20.0[a] | 29,736 ± 670 | 24,746 ± 1039 | 83.2 |
| 20.0[b] | 30,826 ± 423 | 25,761 ± 817 | 83.6 |
| 20.0[c] | 31,081 ± 1031 | 25,770 ± 556 | 82.9 |

[a] Plasma samples containing 0.3 ng/mL of exemestane.
[b] Plasma samples containing 15.0 ng/mL of exemestane.
[c] Plasma samples containing 30.0 ng/mL of exemestane.

**Table 2**
The stability of exemestane in plasma samples expressed as 90% confidence intervals ($n$ = 6).

| Stability | Autosampler | Freeze and thaw | Short-term | Preliminary long-term | Preliminary long-term |
|---|---|---|---|---|---|
| Temperature | Room temperature | ≤−14 °C | Room temperature | ≤−14 °C | ≤−65 °C |
| Time | 24 h | 3 cycles | 4 h | 10 days | 10 days |
| 0.3 ng/mL | 103.6–109.7% | 92.5–98.4% | 89.1–102.3% | 89.4–95.3% | 93.8–100.1% |
| 15.0 ng/mL | 97.6–103.9% | 89.2–93.9% | – | – | – |
| 30.0 ng/mL | 101.9–107.7% | 93.7–98.7% | 95.7–105.3% | 100.6–105.5% | 100.5–107.9% |

**Table 4**
The influence of the matrix effects ($n = 6$).

| Nominal concentration (ng/mL) | Absolute ME (%) | Relative ME (%) |
|---|---|---|
| 0.3 | 96.0 | 2.84 |
| 15.0 | 97.9 | 2.88 |
| 30.0 | 89.4 | 3.30 |

of the exemestane/IS peak area ratios in the post-extraction spiked plasma samples prepared using six different sources of plasma. The calculated RSD was less than 15% (Table 4), so it was concluded that the matrix effects do not influence method reliability.

The process efficiency, which depends on both extraction recovery and absolute matrix effect, was calculated based on the comparison of the exemestane and IS peak areas in the pre-extraction spiked plasma samples to that of the respective standard solution. The results were consistent for all QC levels and ranged from 76.5% to 83.1%.

## 4. Discussion and conclusions

The described method shows the broad range of linearity, i.e. 0.1–40.0 ng/mL as well as simple and LC–MS compatible sample preparation procedure based on the liquid–liquid extraction. The symmetry of peaks was improved comparing to previously reported methods. The optimized sample preparation, chromatographic and MS conditions, including the use of anastrozole as an internal standard instead of labelled exemestane, lead to improvement of measurement precision. Among previously reported LC–MS/MS methods, only the one developed by Cenacchi et al. [8] was more sensitive (LLOQ = 0.05 ng/mL, plasma volume 0.5 mL), however, it employed sample preparation using SPE on 96-well plates, which requires specific equipment and is more expensive than the procedure proposed in the presented work. The method by Corona et al. [7] was characterised by higher LLOQ = 0.02 ng/mL, but required only 0.1 mL of plasma.

The developed bioanalytical method for exemestane determination in human plasma was fully validated, proving its reliability and robustness. The detailed study in plasma samples and in solutions, based on the application of confidence intervals, confirmed the stability of exemestane in the conditions used during the bioanalytical part of pharmacokinetic studies. The method might be applied to pharmacokinetic studies in humans, e.g. after a single oral administration of 25 mg exemestane tablets.

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