

DEVELOPMENT AND PARTIAL VALIDATION OF A SIMULTANEOUS LC-MS/MS METHOD FOR QUANTIFICATION OF FEZOLINETANT AND ESTRADIOL IN HUMAN PLASMA

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ABSTRACT

A sensitive liquid chromatography–tandem mass spectrometric (LC-MS/MS) approach was created to simultaneously measure Fezolinetant, a non-hormonal neurokinin-3 receptor antagonist, alongside estradiol in human plasma, employing bazedoxifene as the internal standard. Separation of the analytes was accomplished on a Shim-pack GIST C18 column (100 × 2.1 mm, 2 µm) with 2 mM ammonium fluoride in water and methanol, allowing a brief 4.0-min analytical run. Positive electrospray ionization combined with multiple reaction monitoring served as the detection technique. The calibration range spanned 0.1–100 ng/mL and was assessed by means of 1/x²-weighted linear regression. Following the framework set out in ICH M10/US FDA guidance, ten validation characteristics were evaluated: system suitability, selectivity, sensitivity, carryover, linearity, intra- and inter-batch precision and accuracy, extraction recovery, matrix effect, and stability. For system suitability, the area-ratio %CV values were 3.03% for fezolinetant/internal standard and 1.97% for estradiol/internal standard. The lower limit of quantification stood at 0.1 ng/mL, with mean signal-to-noise ratios of 24.78 for fezolinetant and 21.45 for estradiol. The calibration yielded r² values of 0.9964 and 0.9961. Recovery reached approximately 90–93%, and stability accuracy stayed within 95.6–98.8% for fezolinetant and 95.9–98.8% for estradiol. Thus, the method showed satisfactory performance across the ten characteristics that were examined. Nevertheless, dilution integrity, reinjection reproducibility, and ruggedness were not carried out in the source study, and because estradiol is endogenous, a suitable calibration strategy must be confirmed before full validation can be claimed.

Keywords: Fezolinetant; Estradiol; human plasma; LC-MS/MS; bioanalytical method validation; MRM; ICH M10

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neurokinin-3 (NK3) receptor antagonist approved for the treatment of moderate-to-severe vasomotor symptoms associated with menopause. Its mechanism

INTRODUCTION

Fezolinetant is an oral, non-hormonal selective

is based on modulation of the neurokinin B/NK3 receptor pathway involved in hypothalamic thermoregulation.^{1,2,3}

Estradiol is a naturally occurring estrogen and remains an important therapeutic and biomarker analyte in menopausal and reproductive health. Unlike fezolinetant, estradiol is endogenous and is therefore already present in human plasma, creating an additional challenge when conventional drug-free plasma is considered for calibration.^{4,5}

LC-MS/MS is widely used for quantitative bioanalysis because chromatographic separation can be combined with highly selective mass-to-charge transitions. Recent regulatory practice emphasizes assessment of selectivity, sensitivity, calibration performance, accuracy, precision, recovery, matrix effects, carryover, and stability as appropriate to the intended use.^{6,7,8}

The source dissertation identified a gap in simultaneous analytical measurement of fezolinetant and estradiol and developed an LC-MS/MS method using bazedoxifene as internal standard. The present manuscript condenses that experimental work into a journal-style research article, retaining the reported numerical results and explicitly distinguishing the ten validation characteristics that were completed from the three that remained outstanding. The source document also highlights the need to address endogenous baseline estradiol before the method is considered fully justified for routine plasma quantification.

2. MATERIALS AND METHODS

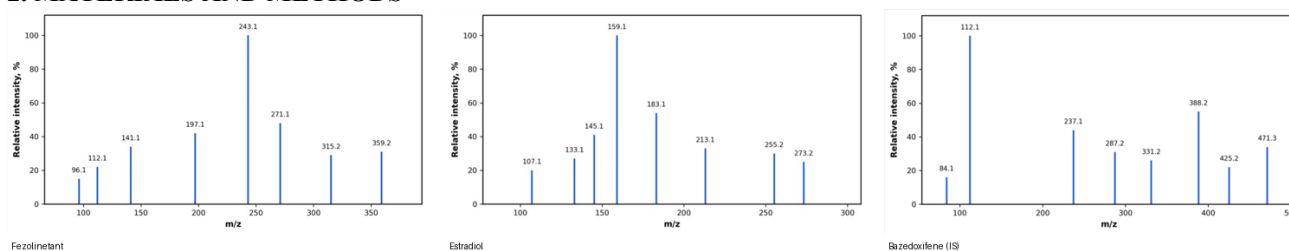


Figure 1. Product-ion mass spectra of (A) fezolinetant, (B) estradiol, and (C) bazedoxifene.

2.4 Calibration and quality-control design

Calibration standards covered 0.1, 0.2, 0.5, 1, 2, 5, 10, 25, 50 and 100 ng/mL. Quality-control levels were LLOQ (0.1 ng/mL), LQC (0.3 ng/mL), MQC (55 ng/mL), and HQC (80 ng/mL). A fixed internal-standard response was incorporated into calibration, QC and unknown samples. The source study used $1/x^2$ -weighted least-squares linear regression of analyte/internal-standard area ratio versus nominal concentration.

2.5 Plasma sample preparation

The validated workflow used liquid–liquid extraction of screened human plasma followed by

2.1 Analytes, internal standard and biological matrix

Fezolinetant and estradiol were selected as the analytes and bazedoxifene was used as the internal standard. Drug-free human K2EDTA plasma was obtained from Bionees, Bengaluru, and screened across individual donor lots before use. The source study used ten plasma lots for selectivity assessment and six lots for matrix-effect evaluation.

2.2 Instrumentation and chromatographic conditions

The analytical platform comprised a UHPLC system coupled to a triple-quadrupole mass spectrometer operated in positive electrospray ionization and multiple reaction monitoring (MRM) mode. Chromatographic separation used a Shimadzu Shim-pack GIST C18 column (100×2.1 mm, $2 \mu\text{m}$). The mobile phases were 2 mM ammonium fluoride in water (A) and methanol (B). The reported gradient ranged from 45 to 95% B, with a flow rate of 0.4 mL/min, column temperature of 40°C , injection volume of $5 \mu\text{L}$, and total run time of 4.0 min. Retention times were approximately 1.35 min for fezolinetant, 1.75 min for bazedoxifene, and 2.48 min for estradiol.

2.3 Mass spectrometric detection

Positive ESI was selected for the three components. The monitored precursor/product-ion transitions were m/z $359.2 \rightarrow 243.1$ for fezolinetant, $273.2 \rightarrow 159.1$ for estradiol, and $471.3 \rightarrow 112.1$ for bazedoxifene. The reported source-dependent settings were applied according to the optimized method.

concentration and reconstitution before LC-MS/MS analysis. The source document reports dichloromethane as the extraction solvent during the optimized method-development workflow and describes subsequent chromatographic analysis of the prepared extracts. Detailed operational preparation steps are retained in the dissertation source and are not repeated here.

2.6 Validation procedure

Validation was structured around the US FDA/ICH M10 framework. The completed characteristics were system suitability, selectivity/specificity, sensitivity at the LLOQ,

injector carryover, linearity, intra-batch precision and accuracy, inter-batch precision and accuracy, extraction recovery, matrix effect, and stability. The source study did not complete dilution integrity, reinjection reproducibility, or ruggedness; these are therefore not presented as validated characteristics in this manuscript.^{6,8}

2.7 Data analysis

For calibration, back-calculated concentrations, mean accuracy, standard deviation and %CV were determined. Precision was expressed as %CV and accuracy as the percentage of nominal concentration. The source study applied the acceptance windows of $\pm 15\%$ for routine levels and $\pm 20\%$ at the LLOQ, together with corresponding precision limits.^{6,8}

3. RESULTS AND DISCUSSION

3.1 Optimization and chromatographic performance

Method development involved optimization of chromatographic separation, mass-spectrometric response, extraction strategy and regression model. Methanol produced sharper chromatographic responses than the alternative organic modifier evaluated in the source study. Among aqueous

additives, 2 mM ammonium fluoride improved estradiol response and reproducibility while maintaining suitable responses for fezolinetant and bazedoxifene. The final sub-2- μm C18 method separated the three components within 4.0 min, with retention times of 1.35, 1.75 and 2.48 min, respectively.⁹

3.2 System suitability and selectivity

Six consecutive injections of a standard mixture produced mean peak areas of 268,003 for fezolinetant, 227,826 for estradiol and 250,541 for bazedoxifene. The corresponding peak-area %CV values were 2.75%, 2.17% and 0.65%, while analyte/internal-standard area-ratio %CV values were 3.03% and 1.97%. These values were below the reported $\leq 5\%$ criterion.

Selectivity was evaluated across ten independent blank plasma lots. Maximum interference was 3.46% at the fezolinetant retention time and 4.10% at the estradiol retention time; internal-standard interference did not exceed 0.048% of the internal-standard response. Thus, all screened lots remained below the reported 20% analyte and 5% internal-standard thresholds.

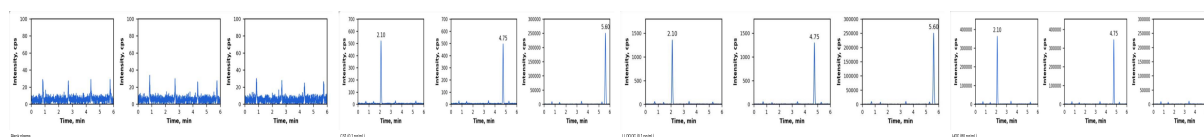


Figure 2. Representative LC-MS/MS chromatograms from the source study: (A) blank human plasma, (B) CS1 at 0.1 ng/mL, (C) LLOQ QC at 0.1 ng/mL, and (D) HQC at 80 ng/mL.

3.3 Sensitivity and carryover

At the 0.1 ng/mL LLOQ, mean signal-to-noise ratios were 24.78 for fezolinetant and 21.45 for estradiol, exceeding the reported minimum criterion of 5. Peak-area %CV values were 6.98% and 8.60%, respectively. Injector carryover after the ULOQ was 0.20% for fezolinetant, 0.23% for estradiol and 0.08% for bazedoxifene, all within the stated acceptance limits.

3.4 Linearity and calibration performance

Analyte	Regression equation	r^2	Accuracy range	%CV range
Fezolinetant	$y = 0.02046x - 0.000731$	0.9964	95.06–100.60%	1.71–5.98%
Estradiol	$y = 0.01728x - 0.000612$	0.9961	95.20–98.52%	2.21–5.00%

Table 1. Calibration regression and performance summary for fezolinetant and estradiol.

3.5 Intra- and inter-batch precision and accuracy

Within a single analytical batch, fezolinetant showed mean accuracies of 98.19%, 96.91%, 95.71%

The calibration range of 0.1–100 ng/mL was fitted using $1/x^2$ -weighted linear regression. For fezolinetant, the pooled regression equation was $y = 0.02046x - 0.000731$ with $r^2 = 0.9964$. For estradiol, the equation was $y = 0.01728x - 0.000612$ with $r^2 = 0.9961$. Back-calculated accuracy across the ten calibration levels ranged from 95.06–100.60% for fezolinetant and 95.20–98.52% for estradiol, with %CV ranges of 1.71–5.98% and 2.21–5.00%, respectively.

and 99.96% at LLOQ, LQC, MQC and HQC, respectively. The corresponding %CV values were 7.12%, 6.89%, 1.48% and 2.99%. Estradiol showed

mean accuracies of 90.38%, 102.75%, 94.12% and 97.62%, with %CV values of 8.23%, 4.96%, 4.09% and 2.21%, respectively.

Across five independent batches (n = 30 per QC level), inter-batch %CV values were 0.58%, 0.69%, 0.89% and 0.51% for fezolinetant and 0.54%, 0.63%,

0.36% and 0.91% for estradiol at LLOQ, LQC, MQC and HQC. Corresponding mean accuracies were 97.89%, 97.15%, 97.74% and 98.29% for fezolinetant and 97.21%, 97.02%, 97.99% and 97.06% for estradiol.

Analyte	QC level	Mean accuracy (%)	Precision (%CV)
Fezolinetant	LLOQ	98.19	7.12
Fezolinetant	LQC	96.91	6.89
Fezolinetant	MQC	95.71	1.48
Fezolinetant	HQC	99.96	2.99
Estradiol	LLOQ	90.38	8.23
Estradiol	LQC	102.75	4.96
Estradiol	MQC	94.12	4.09
Estradiol	HQC	97.62	2.21

Table 2. Intra-batch precision and accuracy summary for fezolinetant and estradiol.

3.6 Extraction recovery and matrix effect

Extraction recovery was consistent across QC levels. Fezolinetant recovery averaged 90.69%, 90.78% and 91.99% at LQC, MQC and HQC, respectively. Estradiol recovery averaged 90.19%, 93.05% and 92.33%, while bazedoxifene recovery averaged 91.23%. The source data therefore indicate reproducible extraction performance across the tested concentration levels.

Matrix effect was assessed using six plasma lots. The reported individual matrix factors for fezolinetant ranged from 0.873 to 0.949 and for estradiol from 0.887 to 0.915; internal-standard-normalized matrix factors ranged from 0.903–0.993 for fezolinetant and 0.930–0.977 for estradiol. The source summary reports normalized matrix-factor values of 0.966 and 0.953 for fezolinetant and estradiol, respectively, with variability within the stated acceptance criterion.

Analyte / level	Mean recovery (%)	SD	%CV
Fezolinetant LQC	90.69	1.42	1.56
Fezolinetant MQC	90.78	1.94	2.13
Fezolinetant HQC	91.99	1.55	1.69
Estradiol LQC	90.19	1.90	2.11
Estradiol MQC	93.05	0.63	0.68
Estradiol HQC	92.33	1.98	2.15
Bazedoxifene IS	91.23	0.84	0.92

Table 3. Extraction recovery of fezolinetant, estradiol, and bazedoxifene.

3.7 Stability

Stability was evaluated at LQC and HQC under bench-top, autosampler, freeze–thaw and long-term storage conditions. For fezolinetant, reported accuracy ranged from 95.60% to 98.78%; for estradiol, it ranged

from 95.93% to 98.79%. No progressive degradation pattern was observed across the tested conditions. These findings support the stability of both analytes under the conditions evaluated in the source study.

Condition	Fezolinetant accuracy (%)	Fezolinetant %CV	Estradiol accuracy (%)	Estradiol %CV
Bench-top, 6 h	95.89–97.13	3.57–3.74	96.83–98.03	3.38–3.53
Autosampler, 24 h	96.43–96.48	2.61–2.96	95.93–98.79	3.21–3.25

Freeze–thaw, 3 cycles	96.58–98.22	2.82–4.35	97.50–98.30	2.67–4.27
Long-term, 30 days	95.60–98.78	3.25–4.46	98.45–98.62	3.40–3.59

Table 4. Stability performance of fezolinetant and estradiol in human plasma.

3.8 Overall validation status and methodological limitation

The source study completed ten of the thirteen validation characteristics identified in its ICH M10-based plan, and the completed characteristics met the stated acceptance criteria. Importantly, the method should be described as partially validated rather than fully validated because dilution integrity, reinjection reproducibility and ruggedness were not executed. In addition, estradiol is endogenous; therefore, confirmation of baseline estradiol in the screened plasma lots and selection/justification of a surrogate-matrix or surrogate-analyte strategy remain important before routine quantitative application.^{6,8}

This distinction is particularly relevant for publication: the numerical validation results are strong for the characteristics assessed, but they do not support a claim of complete ICH M10 compliance. The manuscript therefore limits its conclusions to the parameters actually demonstrated.

4. CONCLUSION

A rapid LC-MS/MS method was developed for simultaneous quantification of fezolinetant and estradiol in human plasma using bazedoxifene as internal standard. The optimized method provided a 4.0-min chromatographic run, a 0.1–100 ng/mL calibration range, satisfactory selectivity, low carryover, good recovery, consistent matrix behavior, and acceptable intra- and inter-batch accuracy and precision. Stability was also demonstrated across the tested handling and storage conditions. The method is a useful analytical platform for further investigation of comparative exposure of a non-hormonal vasomotor-symptom therapy and estradiol. Before the assay can be described as fully validated, the three outstanding validation characteristics and the endogenous-baseline/calibration issue for estradiol should be addressed.

5. DECLARATIONS

Ethics statement

The source dissertation states that drug-free human K2EDTA plasma was commercially obtained and screened for analytical interference. No individually identifiable participant data were used in the reported analytical work. The dissertation does not provide a separate institutional ethics approval number; this should be clarified with the laboratory/institution before journal submission if required by the target journal.

Conflict of interest

The authors should confirm and insert the applicable conflict-of-interest declaration before submission.

Funding

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applicable funding declaration before submission.

Data availability

The validation results are reported in the manuscript tables; the underlying tabulated dataset can be supplied as supplementary material if requested.

Author contributions

The dissertation identifies Balusu Charu Hasini as the student researcher and T. V. Sowjanya as the supervisor/project guide. A formal contribution statement (conceptualization, methodology, investigation, data analysis, writing and supervision) should be confirmed by the authors before submission.

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REFERENCES

- U.S. Food and Drug Administration. VEOZAH (fezolinetant) tablets, for oral use: highlights of prescribing information. Silver Spring, MD: FDA; 2023.
- Cucinella L, Tedeschi S, Memoli S, Cassani C, Martini E, Nappi RE. Pharmacokinetic evaluation of fezolinetant for the treatment of vasomotor symptoms caused by menopause. *Expert Opin Drug Metab Toxicol*. 2024;21(2):105-113. doi:10.1080/17425255.2024.2416046.
- Shaukat A, Mujeeb A, Shahnoor S, Nasser N, Khan AM. Veozah (fezolinetant): a promising non-hormonal treatment for vasomotor symptoms in menopause. *Health Sci Rep*. 2023;6(10):e1610. doi:10.1002/hsr2.1610.
- Ferreira MS, Arruda AMM, Pepi GT, et al. High sensitivity method validated to quantify estradiol in human plasma by LC-MS/MS. *J Chromatogr B*. 2017;1064:109-114. doi:10.1016/j.jchromb.2017.09.005.
- National Center for Biotechnology Information. PubChem compound summary: estradiol, CID 5757. Bethesda, MD: NCBI.
- Gniazdowska E, Kajda-Szczśniak M, Buch P. ICH M10 guideline – a harmonized global approach to bioanalysis. *Prospects Pharm Sci*. 2023.
- Vazvaei-Smith F, et al. ICH M10 bioanalytical method validation guideline—1 year later. *AAPS J*. 2024.
- U.S. Food and Drug Administration. Bioanalytical method validation guidance for industry. Silver Spring, MD: FDA; 2018.
- Thorsteinsdottir U, et al. Design of experiments for development and optimization of a liquid chromatography coupled to tandem mass spectrometry bioanalytical assay. *J Mass Spectrom*. 2021.