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A validated HPLC-MS/MS method for the quantification of systemic mifepristone after subcutaneous application in mice†

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Mifepristone (RU486, MIF) is a synthetic steroidal hormone with progesterone and glucocorticoid receptor antagonistic characteristics. MIF is commonly used for pharmalogical abortions, but also for the treatment of endometrial and endocrine disorders. The goal of the study was to establish and validate a targeted HPLC-MS/MS method for the quantification of MIF and one of its active metabolites metapristone (MET) in plasma after subcutaneous implantation of slow-release MIF pellets in female BALB/c mice. Additionally, we aimed to apply the analytical method to tissue of several organs to understand the tissue-specific distribution of both analytes after release into systemic circulation. Sample preparation comprised a simple liquid–liquid extraction with diethylether and required 100 µl of plasma or homogenates of approximately 50 mg of tissue. The presented HPLC-MS/MS method showed high sensitivity with baseline separation of MIF, MET, and the internal standard levonorgestrel within a run time of only 8.0 minutes and comparable limits of quantification for plasma and tissue homogenates ranging from 40 pg ml^{-1} to 105 pg ml^{-1} for MIF and MET. The presented study is suitable for murine plasma and tissues and can be easily applied to human samples.

Introduction

The synthetic steroidal hormone mifepristone (RU486, MIF) is a progesterone receptor as well as a glucocorticoid receptor antagonist.[1] MIF is now widely approved as a first-line drug for emergency contraception and medical management of early pregnancy termination.[2,3] Additional applications in clinical routine are the symptomatic treatment of endometriosis and endocrine disorders such as Cushing's disease.[4,5] In addition to the aspects mentioned above, there are reports that demonstrate an antiproliferative and antimetastatic effect of MIF on different cancer cell lines *in vitro*.[6,7] Moreover, several clinical trials have investigated the efficacy of MIF in humans, underpinning its potential role beyond traditional treatments for various cancer types.[8–11]

After oral administration, MIF is metabolized into three biologically active metabolites, namely *N*-monodemethyl mifepristone (RU42633, metapristone, MET), *N*-didemethyl mifepristone (RU42848), and hydroxylated mifepristone (RU42698), with MET being the most predominant metabolite.[12,13] The high affinity of MIF for progesterone and glucocorticoid receptors and the stable levels of the metabolites in the blood suggest a combined biological effect of MIF and its metabolites.[13]

MIF is usually administered orally in single or multiple doses up to 800 mg per day.[14,15] Drug-loaded implants, representing an alternative for long-term treatment of gynecological diseases such as endometriosis, have been tested in preclinical studies.[16,17] Such drug delivery systems not only allow a steady release of the drug, but also avoid the hepatic first-pass elimination effects of the analyte itself.[16]

Targeted high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has been commonly used for the qualitative and quantitative analyses of biological specimens.[18,19] Advantages such as low time consumption, superior analytical sensitivity and specificity, and the possibility to

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measure over a wide concentration range often override traditional assays such as immunoassays.[20] We aimed to establish and test a robust targeted HPLC-MS/MS method to quantify MIF and its active metabolite MET in plasma after slow-release, custom-made pellets were implanted under the skin of female BALB/c mice. Furthermore, the analytical method was applied to tissue samples of different organs to ultimately trace the analytes after release into systemic circulation and also to elucidate the tissue-specific distribution of the analytes.

Results

Method validation

Product ion scan experiments of MIF, MET, and the internal standard (ISTD) levonorgestrel (LNG) in 50/50 (vol/vol) methanol/water containing 0.1% formic acid (FA) were used to optimize mass transitions for Selected Reaction Monitoring (SRM). Product ion scan spectra for MIF and MET are shown in Fig. 1. We used the fragments produced upon collision-induced dissociation (CID) with *m*/*z* 372.3 and *m*/*z* 358.3 as quantifier transitions, as well as fragments with *m*/*z* 134.2 and *m*/*z* 120.2 as qualifier transitions for MIF and MET, respectively. Product ion scans of the ISTD also provided meaningful spectra (data not shown, see Table 3 for parameters). The retention times of MIF, MET, and LNG were 3.45, 3.20, and 5.06 minutes, respectively. A representative overlay of Extracted Ion Chromatograms (EICs) for MIF, MET, and LNG is shown in Fig. 2.

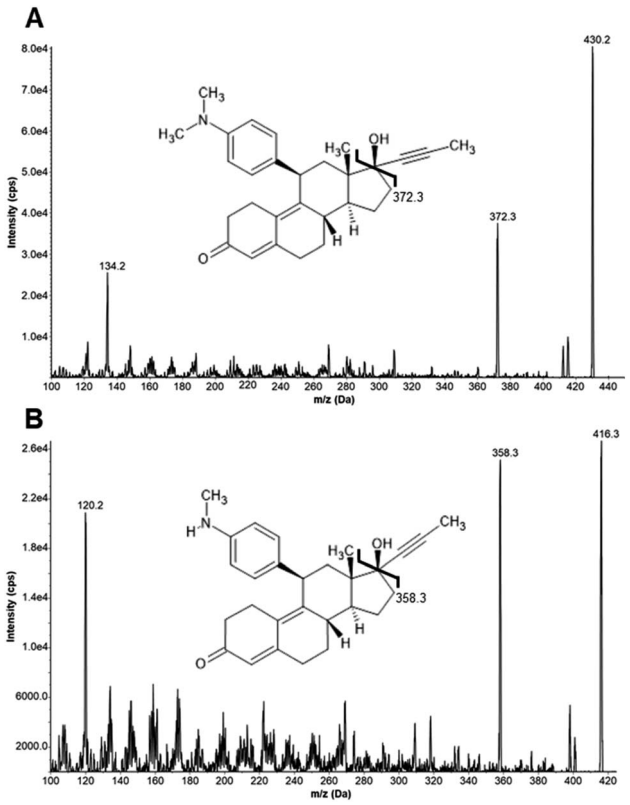

Fig. 1 MS/MS product ion scan of MIF (A) and MET (B) at collision energies of 30 and 25 in positive ion mode. MIF: mifepristone, MET: metapristone.

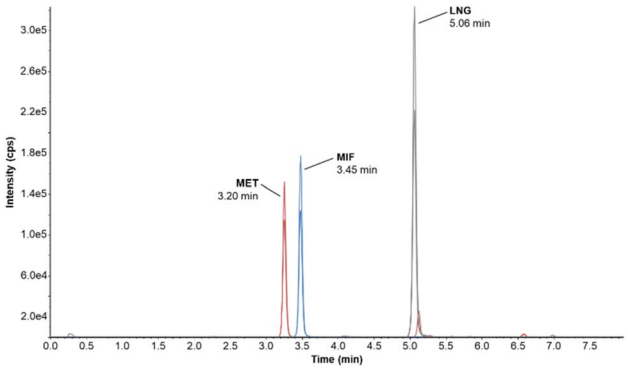

Fig. 2 Overlay of representative extracted ion chromatograms (EICs) of MIF and MET (calibrant containing 10 ng ml^{-1} MIF and MET, with LNG as internal standard). MIF: mifepristone, MET: metapristone, LNG: levonorgestrel.

Method selectivity was demonstrated by the analysis of six extracted double blank human plasma samples without added ISTD. No interference was observed in any of the tested samples at the respective retention times (data not shown). Additionally, six extracted blank human plasma samples with added ISTD were analyzed, and again, no interfering peaks were observed (data not shown). Measurements of blank mouse and human plasma showed no difference with regard to potential interferences throughout the duration of the analysis (data not shown).

Subsequent solvent injections following the highest calibrator had minimal carry-over, with less than 0.5% in comparison to the directly preceding highest calibrator. During the validation process, the average ion ratio of quantifier and qualifier for calibrators and QC samples in plasma was 0.692 (95% C.I.: 0.672–0.711) for MIF, 0.770 (95% C.I.: 0.735–0.805) for MET, and 0.692 (95% C.I.: 0.689–0.696) for LNG. The mean ion ratio of quantifier and qualifier for calibrators and QC samples in neat solvent solution were 0.710 (95% C.I.: 0.697–0.724) for MIF, 0.770 (95% C.I.: 0.752–0.787) for MET, and 0.691 (95% C.I.: 0.688–0.693) for LNG.

Average ion ratio of quantifier and qualifier in plasma samples from mice was 0.718 (C.I.: 0.665–0.771) for MIF, 0.900 (C.I.: 0.845–0.954) for MET, and 0.735 (C.I.: 0.727–0.743) for LNG.

Average ion ratio of quantifier and qualifier in tissue samples from mice was 0.718 (C.I.: 0.710–0.731) for MIF, 0.750 (C.I.: 0.698–0.802) for MET, and 0.742 (C.I.: 0.728–0.756) for LNG. Evaluation of the greenness and practicality of the validated method achieved final scores of 0.6 (S-Fig. 1A) and 65.0 (S-Fig. 1B), respectively.

Linearity, limit of detection and limit of quantification. Across six independent measurements, all standard curves showed acceptable linearity. The single regression lines had *r*-values of ≥0.989 ($1/\chi$ weighing, average $r = 0.997$) for calibrants in plasma and ≥0.997 ($1/\chi$ weighing, average $r = 0.999$) for calibrants in neat solvent solution over the concentration range from 0.039 to 40.0 ng ml^{-1}. Overall linearity for both analytes, MIF and MET, was indicated by coefficients of determination of $r^2 \geq 0.977$ (average $r^2 = 0.993$) for plasma and $r^2 \geq 0.995$ (average $r^2 = 0.999$) for neat solvent calibrants, respectively.

Table 1 Intra-day and time-dependent intermediate trueness and precision for MIF and MET in spiked plasma and neat solvent samples[a]

		Plasma							Neat solvent solution	
			Intra-day			Inter-day			Inter-day	
Analyte	QC level	Target value, mean area (cps)	Mean area (cps)	Trueness (%)	CV (%)	Mean area (cps)	Trueness (%)	CV (%)	Mean area (cps)	CV (%)
Mifepristone (MIF)	Low	5.0×10^3	5.8×10^3	116	11	7.4×10^3	148	25	6.4×10^3	8
	High	1.2×10^6	1.6×10^6	133	1	1.7×10^6	142	34	1.6×10^6	9
Metapristone (MET)	Low	4.6×10^3	3.6×10^3	78	10	4.5×10^3	98	34	5.0×10^3	9
	High	9.2×10^5	1.1×10^6	120	2	1.0×10^6	109	39	1.3×10^6	7

[a] Mean, trueness and CV were calculated using independent measurements of $n = 6$. MIF: mifepristone, MET: metapristone.

We obtained comparable levels for the lower limit of quantification (LLOQ) and limit of detection (LOD) for MIF and MET in both, plasma and neat solvent solution, respectively. The LLOQ and LOD in plasma for MIF was 0.040 ng ml^{-1}, and 0.013 ng ml^{-1}, 0.045 ng ml^{-1} and 0.015 ng ml^{-1} in neat solvent solution. On the other hand, MET had greater LLOQs and LODs in the corresponding matrices. LLOQ and LOD for MET were 0.096 ng ml^{-1} and 0.032 ng ml^{-1} in plasma, and 0.105 ng ml^{-1} and 0.035 ng ml^{-1} in neat solvent solution.

Intra-day, time-dependent intermediate precision and trueness. Results derived from intra-day and time-dependent intermediate (*i.e.*, inter-day) experiments are summarized in Table 1. The overall intra-day precision (*i.e.*, coefficient of variation (CV) in %) of six independent plasma QC samples spiked with low and high concentrations of MIF and MET ranged from 1 to 11% for MIF and 2 to 10% for MET. The CV for time-dependent intermediate precision in plasma ranged from 15% to 19% for MIF, whereas MET showed a higher degree of imprecision with CVs between 34% and 39%. Time-dependent intermediate imprecision for neat solvent solutions were lower for both, MIF and MET, compared to plasma with CVs ranging from 7% to 9%.

Stability. To further investigate possible degradation processes due to diverse sample storage conditions, stability experiments using samples at two different QC levels stored at 4 °C, −20 °C, and −80 °C for up to 42 days were performed. Short-term stability (*i.e.*, 12 hours) experiments covered autosampler stability at 4 °C, while evaluation of long-term stability was carried out for processed samples stored at −20 °C and −80 °C after 28 and 42 days (Table 2).

Both analytes were stable for short-term storage up to 24 hours at 4 °C (*i.e.*, autosampler stability) in processed samples.

Table 2 Results of stability tests of processed spiked plasma samples under different storage conditions[a]

			Mifepristone (area/area ISTD)		Metapristone (area/area ISTD)	
Condition	Storage time	QC level	Recovery (%)	CV (%)	Recovery (%)	CV (%)
4 °C						
Autosampler stability	24 hours	Low	102	25	101	27
		High	96	49	98	46
−20 °C						
Freeze/thaw stability	28 days	Low	67[b]	17	41[b]	9
		High	134	27	83	19
Processed sample stability	28 days	Low	123[b]	28	66[b]	26
		High	93	35	55	31
	42 days	Low	127[b]	39	35[b]	15
		High	98	12	65	30
−80 °C						
Processed sample stability	28 days	Low	160	25	103	22
		High	157	22	138	23
	42 days	Low	147	57	110	32
		High	81	47	64	37

[a] Mean percentage recovery compared to reference values on day 0 using independent measurements of $n = 4$–6. ISTD: internal standard.
[b] Indicates samples below LOQ for the respective QC level.

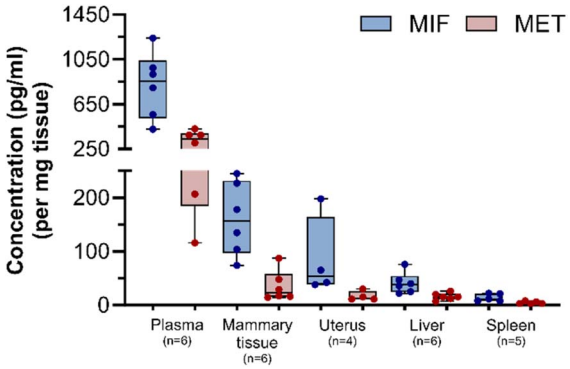

Fig. 3 Concentration of MIF and MET in plasma and across different tissues in female BALB/c mice at the termination day (111 days after subcutaneous 3 mg MIF pellet implantation). MIF: mifepristone, MET: metapristone, $n = 4–6$.

Stability was found to be 67% and 134% for MIF as well as 41% and 83% for MET for low and high QC samples after three repeated freeze/thaw cycles within 28 days compared to the first analytical run, respectively. MIF showed acceptable stability in processed samples regardless of sample storage conditions for up to 42 days at both low and high QC levels, respectively. Interestingly, MIF accumulated when stored as processed sample. In general, MET showed a higher degree of instability than MIF in processed samples and was more prone to degradation. Recoveries for MET were lower than for MIF in processed samples stored at −20 °C or −80 °C. Especially, some samples spiked with low QC levels stored at −20 °C were below the LOD.

Robustness. Furthermore, we monitored and evaluated the effect on method performance after applying variations, such as changes in eluent composition or column temperature, to the method. The method appeared to be robust during the application of changes to the initial method, as the retention times of the analytes showed only minimal shifts upon changes (±0.1–0.2 min, data not shown).

Application of the validated method

After method validation, six plasma samples and 21 samples derived from different murine tissues were processed to ultimately trace and quantify MIF and MET levels. Mice were treated with 3 mg of subcutaneously implanted MIF pellets. Concentrations of MIF and MET in plasma, mammary glands, uterus, liver, and spleen from MIF-treated animals are shown in Fig. 3. The highest levels of MIF were found in plasma, with mean plasma concentrations of 820 ± 295 pg ml^{-1} for MIF and 301 ± 119 pg ml^{-1} for MET. The levels of MIF and MET in tissues upon continuous release were highest in fat-rich organs like mammary glands, with lower amounts detected in the uterus and liver, and the lowest levels in the spleen.

Discussion

Beside the use of MIF as an abortifacient and contraceptive drug in women, several studies have demonstrated the antiproliferative effects in breast cancer cells *in vitro*, but also in experimental models and in human breast tissue after (low-dose) MIF treatment.[21,22] A study by Poole *et al.* reported that treatment of Brca1/p53-deficient mice with MIF delayed or even prevented breast cancer formation.[23]

We hereby established a robust HPLC-MS/MS method for the quantification of MIF and one of its active metabolites, MET, in plasma after subcutaneous MIF pellet implantation in female BALB/c mice. Furthermore, the analytical method was applied to different tissue types, such as mammary glands, uterus, liver, and spleen, to ultimately trace the analytes after release in the systemic circulation.

We chose positive ESI ionization mode to yield extensive fragmentation patterns at an acceptable level of chemical noise. We used the most abundant fragment ions at *m*/*z* 372.3, *m*/*z* 358.3 and *m*/*z* 245.2 as quantifier ions for MIF, MET, and LNG as ISTD, respectively. Our method proved high selectivity, as no endogenous or exogenous substances compromised the ability of the method to specifically capture MIF, MET, and the ISTD.

Several complex sample preparation procedures have been described for the extraction of MIF and derived metabolites from plasma, which require larger sample volumes, which were not feasible in our preclinical setting.[24,25] To keep sample preparation as straightforward as possible, we used a liquid–liquid extraction (LLE) protocol with diethylether as the extraction solvent for plasma and tissue homogenates, as described earlier by Homer *et al.*[26] The data obtained with LLE and described herein resulted in reliable detection of the analytes, and, therefore, solid phase extraction was omitted.

Reported LODs and LLOQs for MIF with LLE were 30 and 50 pg on column,[26] respectively. The LLOQ of our method for MIF (40 pg ml^{-1}) and MET (96 pg ml^{-1}) in plasma was approximately five-to ten-fold lower than reported for MIF and its metabolites, with a LOQ of 0.5 ng ml^{-1} in maternal whole blood after pharmacological abortion.[27] The LOQ in a neat solvent solution for MIF and MET in tissue was similar as determined for plasma. A targeted method for the analysis of 11 contraceptive progestins and four steroids (*i.e.*, progesterone, testosterone, androstenedione and cortisol) in plasma by high-resolution LC-MS reported LOQs ranging from 2.4 to 78.1 pg ml^{-1}, which are in line with our data.[19] Therefore, we conclude that the sensitivity of our approach is within the range of earlier reports for comparable analytes.

Stable isotopically labelled (SIL) ISTDs are chemically identical to the compound and are normally the preferred ISTD option for any quantitative assessment by mass spectrometry. However, there are also several reports using structural analogs as additional variants for ISTDs.[28,29] We implemented the structural analog LNG as an ISTD. LNG shows a great structural similarity and exhibits comparable physico–chemical properties as the two analytes of interest. There are some reasons for the implementation of a SIL ISTD instead of a structural analog, such as higher degrees of accuracy, precision, and post-preparation stability.[30] The overestimation of MIF in processed samples could be attributed to the implemented structural analog instead of a SIL compound as ISTD. Reasons for an overestimation of an analyte could be a higher degradation rate

of the analogous ISTD compared to the SIL variant or the possible elimination of glucuronide-conjugates during storage, which might result in higher levels of MIF. Implementation of deuterated MIF as an ISTD could potentially correct for the overestimation of the compound in processed samples after long-term storage.

The method validation involved QC levels spiked with low and high concentrations of MIF and MET. Stability experiments indicated lower recoveries for QC material, particularly spiked with low levels of MET. These lower recoveries in MET may not only be the consequence of the low spiked concentrations of MET, which were to be expected borderline to the LOQ. In general, MET showed a higher degradation rate at prolonged storage in processed samples compared to MIF, which showed moderate stability when stored at −20 °C and −80 °C, suggesting immediate measurements after sample preparation and no long storage from sample collection to analysis.

Comparing the intermediate precision of plasma and neat solvent solutions showed a larger matrix effect in plasma, leading to lower CVs (in %).

After full validation, the analytical method was effectively applied to samples derived from a preclinical study. MIF is normally administered orally at different doses, and MIF could be detected for at least 4 days in humans after one single administration.[31] After oral administration, plasma concentrations of the active metabolite MET are normally higher compared to the parent drug MIF.[31] We determined the bioavailability of MIF upon subcutaneous pellet implantation in female BALB/c mice. This approach allows a steady, low-dose-release of MIF for long-term treatments of any progesterone-dependent diseases. MIF pellet implantation led to its metabolization into MET and to a systemic distribution *via* the blood stream to the organs, with the highest MIF levels in the mammary glands followed by the uterus and liver, whereas the spleen showed the lowest levels of MIF and MET. These observed differences in abundancies of MIF and MET support the assumption that different organs have different capabilities to accumulate MIF, and that MIF accumulates at the desired side of action, namely mammary tissue and uterus, when used as a therapeutic option in breast cancer and endometriosis or even as a potential breast cancer prevention strategy.

To the best of our knowledge, no study has assessed MIF levels in tissues after subcutaneous implantation. Taken together, the validated analytical method enables a fast and simultaneous detection of MIF and MET in plasma and tissue homogenates. Further experiments attributed to MIF's anti-cancer properties in a preclinical, disease-related setting are currently under investigation.

Experimental

Materials

Mifepristone (MIF, RU486), levonorgestrel (LNG, internal standard (ISTD)) and metapristone (MET, monodemethylmifepristone, RU42633) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Axon Medchem (Groningen, Netherlands), respectively. Water, acetonitrile, methanol, and ethanol absolute (>99.7%) as well as formic acid (FA, ≥99%) were obtained from VWR International (Rosny-sous-Bois, France). Diethylether (>99.9%) was purchased from Sigma-Aldrich. All chemicals and solvents were HPLC-MS grade and were used without any further purification.

The study was conducted in accordance with the local legislation and institutional requirements. Left-over plasma samples from healthy, female volunteers were used for the validation procedure. Written informed consent was obtained from all volunteers.

Methods

Preparation of calibrants, quality control material, and sample preparation. Stock solutions (1 mg ml^{-1}) of MIF, MET, and LNG were prepared by dissolution in ethanol and stored at −20 °C. Six calibrants with concentrations of MIF and MET (0.039, 0.156, 0.625, 2.5, 10.0, and 40.0 ng ml^{-1}) and three quality control (QC) samples (0.098, 1.5 and 25 ng ml^{-1}) were prepared by serial dilution in human blank plasma. All calibrants and QC samples were aliquoted and stored at −80 °C before use. Samples were processed on the day of measurement.

Regarding MIF and MET quantification in tissue samples and the lack of appropriate matrix calibrants, neat standard solutions were used as calibrants (0–40.0 ng ml^{-1}) and QC samples (0.098, 1.5 and 25 ng ml^{-1}).

Sample preparation consisted of a liquid–liquid extraction protocol as described by Homer *et al.*[26] For this purpose, 2 μl of LNG (1 μg ml^{-1}) as an internal standard was added to 100 μl plasma, vortexed briefly before the addition of 900 μl diethylether. After vortexing for 1 minute, all samples were centrifuged at 21 000×*g* for 10 minutes at 4 °C. Then, 850 μl of clear supernatant was transferred to a new tube and dried at room temperature under nitrogen. Dried residues were dissolved in 50 μl of 20/80 (vol/vol) acetonitrile/water containing 0.1% FA, vortexed for 1 minute, and centrifuged at 21 000×*g* for 10 minutes at 4 °C. Upper phases were transferred to glass vials and stored at 4 °C until injection.

Mifepristone extraction from tissues. Mammary, liver, uterus and spleen tissues from female BALB/c mice (described below) were homogenized with ice-cold methanol by using an Ultra-Turrax (IKA, Steifen im Breisgau, Germany). Frozen mammary, liver and spleen tissue were homogenized in 6 μl methanol per 1 mg tissue, and uterus samples were homogenized in 12 μl methanol per 1 mg tissue. After complete homogenization, tissue homogenates were centrifuged at 2600×*g* for 10 minutes at 4 °C followed by another centrifugation step of the separated supernatant at 14 000×*g* for 10 minutes at 4 °C. The tissue homogenates were then dried at room temperature under nitrogen and reconstituted in 100 μl diethylether. MIF and MET extractions from tissue homogenates were performed as stated above.

Chromatographic and mass spectrometric conditions. Reversed-phase LC separation was carried out on an ExionLC system (Sciex, Darmstadt, Germany) using a Phenomenex Synergi Fusion-RP column (50 × 2 mm, 4 μm particle size, 80 Å pore size) with a flow rate of 0.4 ml $minute^{-1}$ maintained at 35 °C.

Water containing 0.1% FA and 95/5 (vol/vol) acetonitrile/water containing 0.1% FA were used as eluents A and B, respectively. Gradient elution was carried out as follows: 20.0% to 80.0% B in 4.5 minutes, followed by a flushing step with 95.0% B for 1.0 minutes, followed by a re-equilibration step with 20.0% B for 1.5 minutes. The total time for a single chromatographic run was 8.0 minutes. For plasma and tissue extracts, injection volumes of 15 μl were used.

Selected Reaction Monitoring (SRM) for MIF, MET, and the internal standard LNG in the samples were performed on a TripleQuad5500+ (Sciex, Darmstadt, Germany) in positive ion mode. Quantifier and qualifier ions for MIF, MET, and LNG were recorded with the settings shown in Table 3. Additional mass spectrometric parameters were set as follows: source temperature of 500 °C, collision gas of 9 (AU), curtain gas of 40 (AU), ion source gas 1 of 40 (AU), ion source gas 2 of 60 (AU), ion spray voltage of 5500 V, and entrance potential of 10 V. Analyst software 1.7.1 was used for the acquisition of data. SciexOS (version 1.7.0.36606) was used for data analysis and quantification.

Method validation. The method validation for plasma was generally performed according to the Eurachem guideline.[32] In summary, performance characteristics such as selectivity, carry-over, linearity, intra-day precision and trueness, time-dependent intermediate precision (*i.e.*, inter-day precision) and trueness, and robustness were evaluated in plasma. Linearity and time-dependent intermediate precision were additionally assessed for calibrants in neat standard solutions. Furthermore, stability experiments were carried out, and the ion ratio of quantifiers and qualifiers of calibrators and QC samples in plasma and neat solvent solutions obtained during the validation process were evaluated.

Six double blank human plasma samples without internal standard and six blank human plasma samples with internal standard were injected to address selectivity. The SRM traces of MIF, MET, and the ISTD for all samples were inspected for any interfering substances.

Carry-over effects were evaluated by solvent injections after the injection of the highest calibrant. The SRM traces of MIF, MET, and ISTD in solvent injections were checked for any carry-over peaks.

To assess the linearity of calibration in the range of 0.039–40 ng ml^{-1}, six independent calibration curves were analyzed on six different days. Calibration curves were derived from ratios of the peak areas of MIF or MET to the ISTD using $1/\chi$-weighted linear least-squares regression of the area ratio *versus* the concentration ratio. Analytical limits such as limit of detection (LOD) and limit of quantification (LOQ) were theoretically calculated based on and defined as three-fold and nine-fold signal-to-noise ratios (S/N), respectively.

Intra-day, time-dependent intermediate trueness and precision were evaluated by analyzing at least five replicates of low and high QC samples during short-term intervals or on at least six independent days of measurements. Absolute peak areas were compared to peak areas of the analytical run on day 0, where the first and initial analytical run took place.

The robustness of the analytical method was tested by small changes in the parameters of the method like increased column temperature (45 °C instead of 35 °C) and replacement of mobile phase additive from water or 5/95 (vol/vol) acetonitrile containing 0.1% FA to 1 mM ammonium formate in water or 5/95 (vol/vol) acetonitrile.

Moreover, evaluation of the short- and long-term stability of MIF and MET was performed for processed (*i.e.*, post-preparation) plasma QC samples spiked with low and high concentrations of the analytes. Processed sample stability was analyzed after 28 and 42 days of storage at −20 °C and −80 °C. Additionally, the evaluation of processed samples after three freeze/thaw cycles within 28 days as well as the concentrations of samples retained in the autosampler rack for 24 hours were determined. Ratios of absolute peak areas of the analytes to absolute peak areas of the ISTD (area/area ISTD) were compared to ratios of the analytical run on day 0, where stability samples were prepared.

Furthermore, we assessed the greenness and the applicability of the analytical method with the freely available online software tools Analytical GREEnness calculator (AGREE) and blue applicability grade index (BAGI), respectively[33,34].

Plasma and tissue collection for the application of the validated method to murine plasma and tissue samples. Animal experiments were performed at the animal facility of the Paracelsus Medical University Salzburg in accordance with the Austrian federal ministry of education, science, and research (BMBWF), study approval No. 2021-0.236.530. After one week of adaptation to the environment, 7 weeks old female BALB/c mice (Charles River, Sulzfeld, Germany) received 3 mg of MIF (#M8046, Sigma-Aldrich) in the form of slow-release (90 days), custom-made pellets (#NX-999, Innovative Research of America, Sarasota, FL, USA) by subcutaneous implantation. Throughout the study, mice had free access to food and water. All samples

Table 3 Precursor and product ions of MIF, MET and LNG with MS parameters in positive electrospray ionization mode

Analyte		Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Dwell time (ms)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential
Mifepristone (MIF)	Quantifier	430.2	372.3	80	161	31	20
	Qualifier	430.2	134.2	80	161	37	16
Metapristone (MET)	Quantifier	416.3	358.3	80	131	27	18
	Qualifier	416.3	120.2	80	131	31	14
Levonorgestrel (LNG)	Quantifier	313.1	245.2	80	136	27	10
	Qualifier	313.1	109.0	80	136	29	16

were collected at a mouse age of 22–23 weeks. Blood was collected by cardiac puncture and stored in heparin-coated tubes on ice until centrifugation at 1550×*g* for 10 minutes at 4 °C to collect plasma. Plasma samples were frozen in liquid nitrogen and stored at −80 °C until measurements. Approximately 50 mg of mammary glands, liver, uterus, and spleen tissue were frozen in liquid nitrogen and then stored at −80 °C until tissue extraction and subsequent HPLC-MS/MS analysis of MIF and MET.

Conclusions

We developed, validated and applied a rapid and robust method to quantify the amount of MIF and its metabolite MET in plasma and tissue samples from BALB/c mice that had a MIF pellet implanted subcutaneously. Sample preparation consisted of a LLE with diethylether and required 100 µl of plasma or homogenates of approximately 50 mg of tissue, resulting in sufficient intensities for subsequent quantification of the analytes. The presented HPLC-MS/MS method showed high sensitivity with baseline separation within a run time of 8.0 min resulting in low LOQs for plasma and tissue extracts. The presented method has been validated for murine plasma and tissue samples but can be easily applied to human samples.

Data availability

The data supporting this article have been included as part of the main article and the ESI.†

Author contributions

Conceptualization, BK, TKF; methodology, JT, SAG; formal analysis, JT; resources, TKF; investigation, JT, SAG, DDW, LC, VES; data curation, JT; software, JT; validation, JT; visualization, JT; supervision, RL, TKF, BK; writing – original draft, JT; writing – review & editing, SAG, DDW, LC, VES, ER, CH, RL, MW, TKF, BK; funding acquisition, MW, BK; project administration, BK.

Conflicts of interest

There are no conflicts to declare.

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