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Quantitative analysis of RU38486 (mifepristone) by HPLC triple quadrupole mass spectrometry

Natalie Z.M. Homer[a], Rebecca M. Reynolds[a], Cecilia Mattsson[b], Matthew A. Bailey[a], Brian R. Walker[a], Ruth Andrew[a,*]

[a] Mass Spectrometry Core Laboratory and Centre for Cardiovascular Sciences, Wellcome Trust Clinical Research Facility, Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom
[b] Department of Public Health and Clinical Medicine, Umeå University Hospital, S-901 85 Umeå, Sweden

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ABSTRACT

A sensitive liquid chromatography–mass spectrometric method was validated for the quantification of RU38486 (mifepristone) in human and murine plasma. The analyte and internal standard (alfaxolone) were extracted by liquid–liquid extraction with diethyl ether, resolved on a C18 column using gradient elution with methanol and ammonium acetate and detected after positive electrospray ionization (m/z 430 → 372; m/z 333 → 297, respectively). Quantification was linear over the range 0.5–500 ng ($r^2 > 0.997$), precise and accurate (intra-assay RSD ≤ 7.2%, RME ≤ 8.2%; inter-assay RSD ≤ 15.7% RME ≤ 10.2%). The limit of quantification (LOQ) was 50 pg injected on column, permitting reproducible analysis of RU38486 in small volumes of plasma.

1. Introduction

RU38486 (mifepristone) is a progesterone receptor antagonist used clinically as an abortifacient [1,2]. RU38486 has also been used to reduce glucocorticoid receptor activation as a potential therapy in the metabolic syndrome [3] and depression [4], and as an investigational tool to dissect the hypothalamic-pituitary axis which regulates glucocorticoid production [5,6]. The dose used for anti-glucocorticoid therapy is similar or lower than that used for gynaecological purposes, and the circulating concentrations of the drugs must be measured, to account for pharmacokinetic variation between subjects, and also to ensure compliance. Furthermore for preclinical studies in rodent models, plasma volume is limited, necessitating a sensitive and specific assay.

Previous methods of quantitative analysis of RU38486 have included radioimmunoassay [7], however the antibody used exhibited substantial cross-reactivity with the drug's metabolites. More recently the approach of HPLC analysis with UV detection has provided highly accurate and precise measurements, with suitable limits of detection to support its conventional clinical use, following solid phase extraction of plasma [8,9]. Guo et al. attained limits of detection and quantitation of 6 and 10 ng/ml (0.6 and 1 ng on column), respectively, using norethisterone as the internal standard [10], whereas Stith and Hussain developed a method of analysis of RU38486 in coyote (*Canis latrans*) serum [11], achieving a linear range of detection in the range 10 −1000 ng/ml (5–500 ng on column) using a synthetic analog of RU38486, RTI-3021-003, as the internal standard. However when UV detection alone is used there is still potential for interference from co-eluting substances, in particular closely related metabolites which also absorb UV light at 305 nm. Improved sensitivity of detection has been achieved using HPLC-ECD (0.5 ng on column), however run times of 50 min were necessary to ensure separation of the parent drug from its metabolites [12].

The potential risk of interference cannot be easily eliminated without the appropriate analytical standards, which are not readily available. Therefore the aim of this work was to develop an assay by liquid chromatography–tandem mass spectrometry (LC–MS/MS) to enhance the specificity and sensitivity of these analyses and to allow analysis of smaller volumes of plasma and in lower concentrations.

2. Experimental

2.1. Chemicals and reagents

RU38486, alfaxolone and ammonium acetate were purchased from Sigma (Sigma–Aldrich, Poole, UK). HPLC, glass distilled grade diethyl ether, chloroform, dichloromethane, methanol and water were purchased from VWR (VWR, Poole, UK).

* Corresponding author. Tel.: +44 131 242 6763; fax: +44 131 242 6779.
E-mail address: Ruth.Andrew@ed.ac.uk (R. Andrew).

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2.2. Biological samples

The clinical study was approved by the local research ethics committee and subjects gave written informed consent. Thirty one healthy male subjects (aged 41 ± 2.5 (SEM) years and BMI 26 ± 0.9 (SEM) kg/m^2), took either 400 mg RU38486 (Mifegyne®, Nordic Drugs AB, Limhamn, Sweden) at 1100 and 1600 h or placebo at least 7 days apart, in a randomized double-blind cross-over design, and had blood collected at 1800 h. Plasma was obtained following centrifugation (3000 × *g*, 10 min, 4 °C) and stored at −80 °C prior to analysis. Mice (C57BL/6 male, age 12 weeks) were implanted with elastomer pellets (Silastic, Dow Corning, USA) under isofluorane anaesthesia. Each pellet contained 10 mg RU38486. After 10–14 days arterial blood was drawn into lithium heparin tubes via a cannula placed in the carotid artery and processed/stored similarly. All animal procedures were performed under the Home Office guidelines, UK.

2.3. Instrumentation

Analyses were performed using a Thermo Electron Surveyor 1.3 SP1 pump, Surveyor 1.3 SP1 HPLC Autosampler (maintained at 10 °C) and Thermo Electron TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Electron, Hemel Hempstead, UK). Data were acquired and processed with Xcalibur 1.2 and LC Quan 2.0 SP1 software. Compounds were separated at 35 °C on a Hypersil C18 BDS column (2.1 mm × 50 mm, 5 μm) protected by a Hypersil C18 guard cartridge (Thermo Electron, Hemel Hempstead, UK).

Two sets of chromatographic conditions were developed. Rapid elution may be achieved using a 5 min linear gradient from 30:70 (ammonium acetate (5 mM): methanol) to 40:60 and a flow rate of 0.5 ml/min. Conditions were maintained for 1 min and the column equilibrated to initial conditions (total run-time 12 min). A slower gradient was developed (and utilised here for full validation) to allow concomitant analysis of RU38486 with spironolactone and canrenone, since these may be co-administered in the research setting. This necessitated using slower gradient elution using the same components; initial conditions (50:50) were maintained for 1 min and then the proportion of methanol increased to 90% over an 8 min linear gradient. Elution conditions were maintained until 14 min and then returned to initial conditions. The mass spectrometer was operated in positive electrospray ionization mode, with a source temperature of 300 °C, a spray voltage of 3 kV and a collision gas (argon) pressure of 0.3 Pa. The ionisation conditions for the analytes were optimised using the software auto-tune facility for selective reaction monitoring of transitions as follows; transitions *m*/*z* 430 → 372 and *m*/*z* 333 → 297, tube lens (V) 162 and 136 and collision energy (eV) 23 and 18 for RU38486 and alfaxolone, respectively.

2.4. Preparation of stock and working solutions of standards

Stock solutions (1 mg/ml) of RU38486 and alfaxolone (internal standard) were prepared by dissolution in methanol and stored at −20 °C. Standards were prepared on the day of analysis by serial dilution of the stock solutions.

2.5. Extraction of RU38486 from plasma and method optimisation

Plasma (100 μl) was dispensed into a glass centrifuge tube and alfaxolone (100 ng, as 10 μl of a 10 μg/ml solution) added. The steroids were extracted by addition of solvent in specific proportions described below to the aqueous phase. The mixture was vortexed and, after centrifugation (500 × *g*, 10 min, 4 °C), the supernatant was transferred to a glass vial. Where stated, the sample was re-extracted a second and third time with the same volume of solvent as for the first extract and the further supernatants combined with the first. The combined supernatants were reduced to dryness under a stream of oxygen free nitrogen (OFN, 60 °C) and reconstituted in methanol/ammonium acetate (5 mM, pH6) (100 μl, 70:30 v/v). The injection volume was 10 μl.

Three systems of liquid–liquid extraction were compared to assess recovery of RU38486 (500 ng) and alfaxolone (100 ng) from plasma. Three solvents (chloroform, diethyl ether and dichloromethane) were compared at different ratios (5:1, 10:1 and 20:1) of solvent:aqueous. Proportions and repetitions were compared to assess optimal extraction efficiency.

2.6. Quantification of RU38486

A standard curve was generated by adding alfaxolone (100 ng) to blank plasma and increasing amounts of RU38486 (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 150, 200, 300 and 500 ng). The peak areas of RU38486 and alfaxolone were integrated and a calibration curve constructed (peak area of RU38486/peak area internal standard vs. amount of RU38486) using a linear regression analysis with equal weighting. Further quality control (QC) samples were prepared containing 0.5 ng (low), 50 ng (medium) and 500 ng (high) of RU38486 and their calculated amounts determined from the regression equation.

The limits of detection (LOD) and quantification (LOQ) were determined by extracting steroids from plasma (100 μl) from six subjects spiked with RU38486 (0.5 ng), injecting one tenth of the final volume. Signal-to-noise (SNR) ratios were calculated and LOD assigned as >3 × SNR. LOQ values were subsequently confirmed using six replicates, as being within an acceptable variance of <20%.

2.7. Determination of recovery, accuracy and precision

The absolute recovery of RU38486 and alfaxolone were determined by comparison of the integrated peak area from the non-extracted and extracted samples of a high QC (500 ng) in triplicate. The intra-assay accuracy and precision were determined in six replicate QC samples at the three different amounts of RU38486. The inter-assay accuracy and precision were determined at the three concentrations from six replicate QC samples prepared on three independent occasions. The intra-assay precision of analysis of concentrations of RU38486 determined in plasma obtained from subjects receiving this drug was assessed by analysing six duplicate extracts. The inter-injection variability was assessed by injecting the same sample (medium QC) six times.

The precision was calculated as the relative standard deviation of the mean (RSD) with RSD (%) = (standard deviation of the mean/mean) × 100. The accuracy was calculated as the relative mean error (RME) with RME (%) = [(mean measured concentration − theoretical concentration)/theoretical concentration] × 100.

2.8. Ion suppression by biological matrix

Blank plasma (human and rodent) was extracted as above, in the absence of internal standard, the extracts reduced to dryness under OFN and the dried residue reconstituted in mobile phase (100 μl). Samples were injected (20 μl) into the HPLC and eluted under chromatographic conditions as above and fractions collected from 1 min before until 1 min after the retention times of the anticipated peaks. The fractions were reduced to dryness under OFN and reconstituted in mobile phase (100 μl). The column was detached from the system, and solutions (10 μg/ml) of RU38486 and alfaxolone infused separately into the LC flow entering the electrospray source, to increase the signal to 1000× background noise (typical of peak height in clinical samples). Fractions were injected (3×) into the flow in the absence of the column and the baseline response

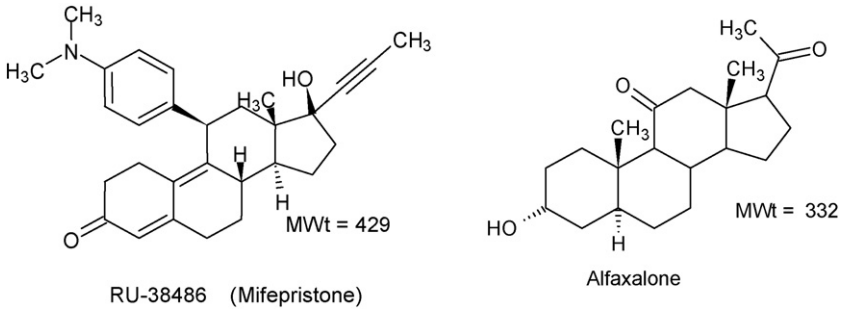

Fig. 1. Chemical structures and molecular weights of RU38486 (mifepristone) and alfaxolone. MWt, molecular weight.

monitored and assessed for ion suppression after the injection lag time of 0.8 min. The experiment could not be performed by infusing the analytes post-column since the infusion pump could not operate under the high back pressures achieved.

2.9. Stability

Short-term stability was assessed in three aliquots of standards at each QC concentration allowed to stand at room temperature (18–22 °C) for 24 h before analysis. Long-term stability was assessed in three aliquots of standards, at each of the low and high concentrations stored at −70 °C for 6 months. In each case, concentrations were analysed and compared to those of freshly prepared solutions.

2.10. Data analysis and statistics

All data relating to clinical and murine studies are presented as mean ± SEM. % Recoveries were compared by repeated measure two-way ANOVA, testing for effect of solvent, proportion and repetition, followed by Fisher's post hoc testing.

3. Results and discussion

The method was validated in terms of extraction efficiency from plasma, limits of quantification recovery, specificity, sensitivity, precision and accuracy and stability. Alfaxolone (Fig. 1), a related progestogen, was used as an internal standard.

3.1. Liquid chromatographic–mass spectrometric analysis

Although steroids are hydrophobic, RU38486 and the related steroid alfaxolone were ionized under electrospray conditions, yielding the protonated molecular ions, *m*/*z* 430 and *m*/*z* 333, respectively. The product ion of RU38486 with *m*/*z* 372 corresponded to loss of the propargyl and hydroxyl groups from C17. The product ion of alfaxolone with *m*/*z* 297 corresponds to loss of two water molecules. The analytes and internal standard were resolved under the conditions used, permitting acceptable analytical throughput (Fig. 2).

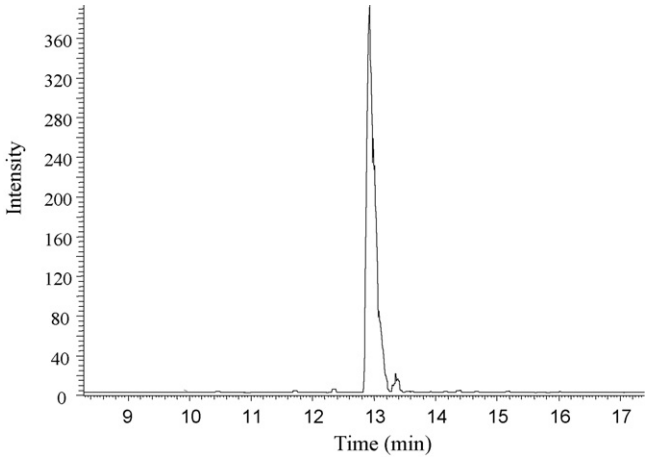

Fig. 2. Mass chromatogram of RU38486 (0.5 ng) extracted from plasma.

The chromatographic conditions presented were selected to allow analysis of RU38486 and alfaxolone in the presence of other drugs which may be administered concomitantly in a research setting, e.g. spironolactone and canrenone [13]. This necessitated a longer chromatographic run than would be necessary for study of RU38486 alone, when analysis may be executed more rapidly (~12 min).

3.2. Extraction efficiency

Comparison of recovery of standards from plasma between extracted and unextracted standards was assessed using three different solvents as a single, double or triple extraction and the results are shown in Table 1. RU38486 and alfaxolone contain more polar residues than endogenous progestogens and therefore a more polar solvent than is commonly used to extract progesterone, diethylether, was tested. More efficient recovery for RU38486 was obtained using diethyl ether as opposed to the less polar solvents (chloroform and dichloromethane) ($p < 0.001$ vs. other solvents). Repeating the extraction and increasing the proportion of diethyl ether to plasma further increased the recovery ($p < 0.001$) and maximal extraction efficiencies were achieved using either triple extractions with ratios of 5:1 or 10:1 or double extraction with diethyl ether at a ratio 20:1, the last being selected. These recoveries were similar to those reported using solid-phase extraction [8,9,11]. Under these conditions the recovery of alfaxolone was 92% which was therefore suitable for further validation as an internal standard. Of note, initial studies attempted recovery of RU38486 from water and were unsuccessful, indicating that this method of extraction may not be extended to assays using aqueous buffers for example.

The presence of biological matrix did not induce ion suppression for either the analyte or internal standard, inferred both by comparable peak areas of unextracted and extracted samples and also following injection of matrix into a steady infusion of RU38486 or alfaxolone; the intensity of response remained within ±10% of its initial value.

3.3. Specificity

The specificity was tested to ensure that the method could differentiate and quantitate the analyte in the presence of other endogenous constituents in the biological sample and to detect potential interferences. A mass chromatogram of RU38486 recov-

Table 1
Recovery of RU38486 from plasma % recovery of RU38486 from plasma (n = 6 replicates); comparison of solvents, solvent:aqueous ratios and repetitions of extraction. CV, coefficient of variation.

	5:1		10:1		20:1	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Diethylether						
Single	88.6	3.6	80.2	4.5	84.9	11.5
Double	93.5	4.8	89.7	10.0	102.3	7.3
Triple	98.2	11.1	99.4	3.5	96.2	1.3
Chloroform						
Single	72.9	0.2	27.1	1.9	55.9	4.0
Double	74.1	3.3	82.0	1.0	73.9	2.0
Triple	91.7	1.3	92.3	0.8	73.7	1.0
Dichloromethane						
Single	52.6	6.7	61.0	8.1	59.4	2.4
Double	57.8	0.8	76.9	0.9	86.2	3.5
Triple	72.5	4.4	82.6	2.9	86.1	5.8

ered from plasma at the LOQ is presented in Fig. 2. Interfering peaks were not observed close to or at the retention times of RU38486. Analytical standards were not commercially available to test whether there were potential interferences generated by the metabolites of RU38486 (e.g. demethylated and hydroxylated [12]), however it would be anticipated that these would generate different precursor ions and exhibit mass transitions distinct from the parent drug.

3.4. Linearity and sensitivity

Standard curves were performed in triplicate and were linear over a concentration range of 5–5000 ng/ml of RU38486 in plasma and were prepared in the range 0.5–200 ng extracted from plasma (100 μl). A typical equation of the regression line is $y = -0.034 + 0.0114x$. In all cases the regression coefficient (r) was >0.99 (mean (95% confidence intervals), 0.997 (0.995–0.999), n = 10). Equal weighting was selected when the concentrations of analytes exceeded or were equal to 10 ng, with inter-assay accuracy (RME) remaining <15%. If the lower range of concentrations on the curve was being utilised (0.5–5 ng/ml), then a weighting of $1/x$ was necessary. The calculated LOQ was 50 pg injected on column for RU38486, with a RSD of 7.2%, whereas the LOD was 30 pg. This LOQ was lower than those reported previously (0.5 ng [12], 300 pg to 1 ng on column [10,11]). Accordingly the analysis could be performed using only 100 μl of plasma, in contrast to volumes of approximately 0.5–1 ml in previous reports [9–11]. In development, it was found that an alternative steroidal drug, spironolactone could also be used as suitable internal standards, yielding linear responses. However this was not selected since, in some experiments investigating glucocorticoid action, this drug may be given concomitantly. A deuterated isotopomer of RU38486 was not commercially available but may merit investigation in the future.

3.5. Intra- and inter-assay accuracy and precision

The accuracy and precision were calculated from six replicate QC samples prepared at three concentrations, summarised in Table 2. The intra-assay accuracy, as determined by the relative mean error, had a minimum of −2.9% and a maximum of 8.2% of the nominal concentration in plasma. Inter-assay accuracy ranged from −10.2 to 10.2%. While these data relating to accuracy are similar to those of Stith and Hussain [11], and Guo et al. [8] the new assay demonstrated improved precision. The precision, evaluated by the RSD for concentrations as low as 0.5 ng/ml, ranged from 4.3% to 7.2% intra-assay increasing to 15.7% inter-assay (in contrast to 19.8% [11] and 20.2% [8] RSD at 10 ng/ml). These data confirm that this is a more reproducible assay for these measurements and is an improvement on that of previous approaches at low concentrations. In samples obtained from subjects who had received RU38486, as opposed to blank samples spiked with standard, the RSD between duplicates was similar, 7.9%. Variability induced through injection and integration was 2.7% and therefore contributed in part to the RSD.

3.6. Sample stability

Stability in a biological fluid is a function of the storage conditions, the chemical properties of the analyte, the matrix and the container system. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling, and as such stability was evaluated in the short-term where samples were left in the autosampler overnight and in the longer term when samples were stored at −70 °C for 6 months (Table 3). RU38486 was stable in the conditions simulating overnight storage in the autosampler, with recoveries tested in plasma at three concentrations ranging from 99.5% to 112.8% in agreement with previous reports [9]. These comparisons were made following storage of solutions at room temperature, however as a further precaution the autosampler carousel was chilled to 10 °C during routine analysis.

In the longer term, RU38486 degraded more rapidly in more dilute solutions, and was undetectable in solutions of concentrations of 10 ng/ml after 6 months; length of storage of samples prior to analysis should therefore be minimised. However in more concentrated solutions, recovery was satisfactory and reliable. Previous reports have highlighted the necessity of freezing plasma at −20 °C

Table 2
Intra- and inter-assay precision (RSD) and accuracy (RME) for RU38486 extracted from plasma, calculated from six replicates analysed on three occasions.

QC (ng)	Intra-assay		Inter-assay	
	RSD (%)	RME (%)	RSD (%)	RME (%)
0.5	7.2	−2.9	15.7	−10.2
50	4.3	8.2	3.9	10.2
500	6.2	−0.1	5.4	5.5

Table 3
Stability of RU38486 in plasma following short-term (24 h) storage at room temperature and longer term storage (6 months) at −70 °C.

	Short-term stability		Long-term stability	
	Recovery (%)	SD	Recovery (%)	SD
10	112.8	3.2	n.d.	n.d.
50	103.3	5.0	111.1	2.4
500	99.5	3.9	96.8	5.5

rather than storing at 4 °C to maintain stability of RU38486 [8,9], although have not studied these parameters after as long a period as 6 m.

3.7. Application of the method

In plasma from subjects who had received treatment, the concentrations of RU38486 achieved were 3670 ± 288 ng/ml. These were easily detectable within the limits of the assay. RU38486 was not detected in plasma obtained following placebo but could still be detected at concentrations of 288 ± 90 ng/ml 1 week after dosing of the active drug, indicating that a washout period of greater than 1 week is required to totally eliminate the drug from the body. This was consistent with a long half-life, e.g. 33.1 h [12], probably as a result of extensive protein binding, and necessitating ~7 days (~5 half-lifes) to eliminate it fully from the body. The assay was also performed using murine plasma and detected at concentrations of 28–77 ng/ml; the low volume of plasma required for the assay permits analyses in individual animals.

4. Conclusion

Several methods have been developed in the last few years for the quantification of RU38486 by HPLC-UV, utilising solid phase extraction. The method reported here has the advantage of an improved LOQ, 20-fold lower than these previous methods, with improved precision and retaining high accuracy. In contrast to previous methods, it utilises more economical liquid–liquid extraction and also benefits from analysis by a liquid chromatographic method with triple quadrupole mass spectrometric detection, gaining from the inherent specificity and noise reduction of this technique. Use of LC–MS/MS reduces the chance of interference from drug metabolites.

Furthermore, by this approach, small quantities of biological material (100 μl of plasma) can be used, allowing minimum invasive sampling and investigation of concentrations in models where plasma volume is more limited, e.g. rodent models; the maximum blood volume obtained from an experimental mouse at cull is ~1 ml, yielding at most ~400 μl of plasma. RU38486 is highly protein bound [12] and only the unbound proportion (~5%) may pass across membrane barriers, e.g. into aqueous humour, cerebrospinal, sinovial, amniotic and follicular fluids. Therefore the increased sensitivity of this method may open up the possibility of investigating drug concentrations in extracellular fluid nearer the target tissue. Lastly by extension this method would also be suitable for analysis of alfaxolone, although full parameters for quality control have not been determined. Therefore, this versatile method may be used for both preclinical and clinical studies with RU38486 and will be widely applicable with the increasing availability of tandem mass spectrometry.

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