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Determination of rifalazil in dog plasma by liquid–liquid extraction and LC–MS/MS: Quality assessment by incurred sample analysis

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Abstract

Incurred dog plasma samples were utilized for method quality assessment in this study, where a sensitive LC–MS/MS method was used for determination of the antibacterial agent rifalazil (KRM-1648 or ABI-1648). Reproducibility was estimated by repeated analysis of samples from a pharmacokinetic study, where 23 out of 864 study samples were reassayed during the course of the study. Precision for same-day duplicates was %R.S.D. 3.1 (concentration range 0.7–149 ng/ml), and over the whole study %R.S.D. 11.0 (concentration range 0.5–52 ng/ml). Moreover, standard addition experiments with incurred samples (concentration range 0.30–45 ng/ml) are described, where the recovery of spiked rifalazil amount was measured as a surrogate parameter for accuracy. The mean recovery of the added rifalazil amount was 103% (%R.S.D. 26.2). It was concluded that the described method is robust and reproducible for incurred samples.

Liquid–liquid extraction was used for isolation of rifalazil and an isotope labeled internal standard from plasma. A manual procedure, based on an 8 × 12 array format, was used for sample extraction. Extracts were analyzed by reversed-phase liquid chromatography using octylsilica column with gradient elution (1 mM ammonium acetate + 0.01% (v/v) acetic aid in water and methanol). Mass spectrometric detection was made with positive ion electrospray ionization and LC–MS/MS analysis in a triple-quadrupole mass spectrometer. The lower limit of quantification was 50 pg/ml. MS characteristics of rifalazil are presented. In particular, two different sets of ionization and selected reaction monitoring (SRM) conditions, where in-source fragmentation was used for precursor ion formation in one of the sets, were compared. The good correlation found between the two sets of results for authentic sample extracts indicated that either condition could be used for quantification of rifalazil in dog plasma.

Keywords: Dog plasma; Quantification; Incurred samples; Accuracy; Rifalazil; Reproducibility; Stable label isotope internal standard; In-source fragmentation

1. Introduction

Investigations aiming at an assessment of the method reproducibility for study samples, as an extension to the method validation, have recently been reported [1]. It has been shown that a regular method validation using fortified samples made from, e.g., pooled control plasma or urine may not fully reflect the method performance for real-life samples. Therefore, it has been suggested to regularly repeat the analysis of a portion of randomly selected study samples as a mean to better estimate the reproducibility of a method. Justifications for post-validation documentation of methodology through incurred sample analysis were recently presented in a conference report [2].

This communication reports quality assessment data and procedures used for repeated analysis of incurred dog plasma samples during a pharmacokinetic study of rifalazil using a newly established LC–MS/MS method. Rifalazil (also known as KRM-1648 or ABI-1648) is a potent antibacterial agent which is currently evaluated in clinical trials [3–5]. A sensitive bioanalytical method for the determination of rifalazil in human plasma was recently described [6] as an alternative to earlier presented LC–UV methodology [7]. The method utilized liquid–liquid extraction and LC–MS/MS and reached a lower limit of quantification (LLOQ) of 50 pg/ml. In the current

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communication, this method was adapted through modification of the chromatographic conditions in order to facilitate bioanalytical support of preclinical studies undertaken in parallel to the clinical program. This also allowed for further exploration of the analytical conditions for rifalazil and related new chemical entities (NCEs) [4]. In particular, an alternative MS/MS transition was explored for rifalazil quantification. The analytical method described here was applied to a formulation development study, using the dog as a model for rifalazil absorption after oral administration of rifalazil. The variability found for incurred samples by duplicate processing in a run, or by repeated analysis on a second occasion, is reported. Also, standard addition experiments with incurred samples are described, where the recovery of spiked rifalazil amount was measured as a surrogate parameter for accuracy. The results show that the method described has good reproducibility and overall accuracy for dog plasma study samples in the concentration range studied.

2. Experimental

2.1. Materials

Rifalazil (3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin, $C_{51}H_{64}N_4O_{13}$), MW 941.1, and the internal standard $^{13}C_4$-rifalazil (ABI-9901), MW 945.1, were obtained from ActivBiotics, Inc. (Lexington, MA). The structures of rifalazil and ABI-9901 are shown in Fig. 1. The monoisotopic weights of the protonated molecular ions are 941.5 and 945.5, respectively.

Rifalazil has a low solubility in aqueous solution at neutral pH and a tendency to adsorb to surfaces. Adsorptive losses were minimized by preparing the stock solutions in methanol (at 0.1 mg/ml) and the working calibration standards were diluted in blank dog plasma (K_2-EDTA, Valley Biomedical, Winchester, VA, USA). A working solution of 50 ng/ml internal standard (IS) was made in methanol–water (1:1, v/v). The final IS concentration during sample extraction corresponded to 12.5 ng/ml plasma.

All reagents used were of HPLC grade or equivalent. Positive displacement pipettes, Gilson Microman (Rainin, Oakland, CA, USA) were used for pipetting methanol and plasma solutions.

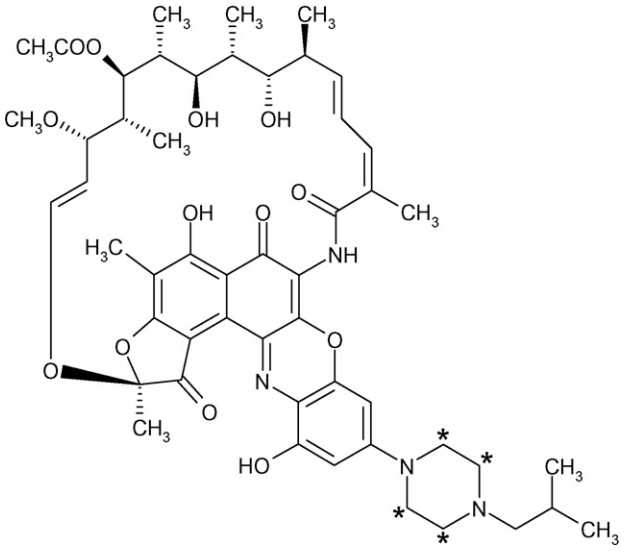

Fig. 1. Structure of rifalazil (ABI-1648). Asterisks (*) denote the position of ^{13}C labels in the internal standard (ABI-9901, $^{13}C_4$-rifalazil).

2.2. LC–MS/MS conditions

An Agilent 1100 series system (Agilent Technologies, Inc., Waldbronn, Germany) consisting of a binary pump, a vacuum degasser, and a column compartment was used. The microplate autosampler with a passive (medium: methanol/water 1:1) and an active (methanol–water–acetic acid at 70:30:1, v/v/v) external needle wash was used. The injection volume was 30 μl and the sample tray temperature was set at 6 °C. The reversed-phase analytical column was an ACE C8, with dimensions 50 mm × 2.1 mm, 5 μm, from MAC-MOD Analytical (Chadds Ford, PA, USA) with a SecurityGuard C8 precolumn filter, 4 mm L × 2.0 mm ID (Phenomenex, Torrance, CA, USA). The mobile phase composition was 0.01% (v/v) acetic acid with 1 mM ammonium acetate in both H_2O (A) and methanol (B), respectively. The flow rate was 0.6 ml/min. The time program was 30% B for 0.75 min, gradient 30–95% in 3.25 min, hold for 2.5 min and then back to 30% B.

A triple-quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a Turbo-V ion source was used with electrospray ionization in the positive ion mode with selected reaction monitoring (SRM) of transition *m/z* 941/909 for rifalazil. Nitrogen (boil-off from liquid nitrogen) was utilized as a nebulizer, curtain and collision gas. Typical mass spectrometer settings are shown in Table 1, where Set 1 was used for study samples, and Set 2 (in-source fragmentation) with the SRM transition at *m/z* 909/517 was explored as an alternative. The dwell time was 150 ms for rifalazil and 100 ms for ABI-9901, and unit resolution was used for quantification. The LC/MS system control and quantification were made in the Analyst 1.4 software (MDS Sciex, Toronto, ON, Canada). For acquisition of mass spectra, a rifalazil solution was continuously infused into a flow of mobile phase via a

Table 1
Mass spectrometry settings for rifalazil analysis with selected reaction monitoring

Parameter	MS conditions Set 1	MS conditions Set 2
SRM transitions (*m/z* in Q1/Q3)		
Rifalazil	941.5/909.4	909.4/517.2
Internal standard	945.5/913.4	913.4/521.2
Ion source temperature (TEM, °C)	450	500–550
Declustering potential (DP, V)	80	135–140
Collision energy (CE, eV)	30	50
CAD gas (collision activated dissociation)	4	7–8
Ion spray voltage (ISV, V)	5200	5200

Set 1: typical MS/MS conditions used for quantitative analysis of study samples. Set 2: precursor ion formed by in-source fragmentation combined with a different product ion.

tee connection (flow rate ratio typically 10:400) located before the electrospray ion source.

2.3. Dog plasma extraction procedure

Liquid–liquid extraction of dog plasma with methyl *t*-butyl ether (MtBE) was made similar to the method for human plasma [6], however, the extraction tube format was replaced by an array of 8×12 in order to simplify sample tube identification during sample processing. Sample extraction was made in 1.2 ml polypropylene cluster tubes, sealed with strip-caps (Costar, Corning, Inc., NY, USA). Dog plasma (200 μl) and internal standard working solution (50 μl) were mixed for 1 min before adding 400 μl of MtBE (EMD, Gibbstown, NJ, USA). An eight-channel pipette from Matrix Technologies (Hudson, NH, USA) was used for IS transfer to the cluster tubes. MtBE was added with a Calibrex 520 dispenser from Wheaton Instruments (Millville, NJ, USA) and samples were extracted by horizontal shaking for 30 min. After centrifugation, and freezing the lower aqueous layer in a dry ice/methanol cryogenic bath, the supernatant was transferred to a 96-well plate (500 μl V-bottom, Costar) with a disposable transfer polyethylene pipette (1.5 ml, VWR International, Bristol, CT, USA). The solvent was removed under nitrogen flow (grade 4.8 or higher) in a Glas-Col Concentrator/Evaporator (Model 9600E, Glas-Col, LLC, Terre Haute, IN, USA) at room temperature. The residue was reconstituted by adding 200 μl H_2O–methanol–acetic acid (60:40:0.5, v/v/v) and vortex mixing. Phase separation was occasionally observed after extract storage at refrigerated conditions. Therefore, the reconstituted extracts were centrifuged, and an aliquot of the supernatant was transferred to a 96-well plate (or to separate vials with polypropylene inserts) for LC–MS/MS analysis.

Extraction recovery was measured during method development for rifalazil using 200 μl plasma and 400 μl MtBE and ranged 73–85%. Matrix effect observations (signal suppression and/or enhancement), comparing the response for a rifalazil solution added to blank dog plasma extract with the neat rifalazil solution, showed 96–108% response (plasma matrix vs. neat) in different LC systems. In addition, matrix effects were studied by post-column infusion of a neat rifalazil solution during LC elution of a blank plasma extract, in order to ensure that the rifalazil MS signal was unperturbed by potentially co-eluting endogenous components present in the plasma extract.

Data reported here for quality assessment in incurred samples is based on results from a formulation development study performed in the dog, in which rifalazil was determined in 864 dog plasma samples during 4 months. While quality assessment experiments provided information on the overall method performance during the study, no action was taken based on results from reassay or standard addition. During routine sample analysis, the target performance of spiked calibrants and quality control samples was 85–115% of nominal concentration (20% at the LLOQ). Study samples were selected randomly over treatment groups for intra-day ($N = 26$) and inter-day ($N = 23$) variability assessment. Moreover, standard addition of rifalazil to incurred samples ($N = 34$) was used for accuracy evaluation. In this procedure, 20 μl rifalazil solution (5 or 50 ng/ml methanol) was spiked into the selected study plasma sample (200 μl) before processing. The spiking concentration corresponded to 0.5 or 5 ng/ml plasma, respectively. The spiked sample was analyzed in the same run as the unmanipulated study sample.

2.4. Quantification

Quality control (QC) samples and calibration standards were made from separate rifalazil stock solutions. A calibration curve was prepared in blank beagle dog plasma by fortification with rifalazil at eight concentrations in the range 50 pg/ml (LLOQ) to 50 ng/ml (upper limit of quantification, ULOQ). Quality control samples were prepared in blank plasma at three concentration levels: 0.15 ng/ml, 1.5 ng/ml and 50 ng/ml. Duplicate calibration and QC samples were processed in each analytical batch. The validity of the dilution procedure used for samples with plasma concentrations above ULOQ was verified.

Quantification was performed in Analyst 1.4 using peak area measurements of rifalazil and the internal standard ABI-9901, and a quadratic regression model with $1/x^2$ weighting.

3. Results and discussion

3.1. Rifalazil detection by mass spectrometry

A triple-quadrupole mass spectrometer was utilized for selected reaction monitoring (SRM) of rifalazil (structure, see Fig. 1). A mass spectrum of rifalazil typically displays three major ions: the protonated molecular ion, MH^+, the sodiated molecular ion MNa^+, and the rifalazil fragment $(M-32)H^+$. Fig. 2 shows mass spectra for rifalazil under analytical conditions (trace A) and under ion source conditions that promotes in-source fragmentation of *m/z* 941 → 909 (trace B), respectively. Rifalazil fragmentation of MH^+ from *m/z* 941 → 909 was enhanced at elevated temperature and at an increased declustering potential, while MNa^+ appears more stable at extremer conditions. Thus, excessive in-source fragmentation would affect the abundance of the precursor ion used for SRM, and thereby the assay sensitivity. Typical MS settings are listed in Table 1.

MS/MS experiments were performed on the protonated molecular ions of rifalazil and the isotope labeled internal standard ABI-9901 at *m/z* 941 and 945, respectively. Product ion spectra after collision-activated dissociation (CAD) are shown in Figs. 3A and 4A. The major fragments were found at *m/z* 909 and 913, respectively, which were used for rifalazil quantification in plasma by SRM. In addition, product ion spectra of *m/z* 909 and 913 are presented in Figs. 3B and 4B, along with a suggested fragmentation. In this case, the precursor ion formation was enhanced by more extreme ion source conditions (Table 1). The main CAD fragments formed from these alternative precursor ions for rifalazil and the internal standard were *m/z* 517 and 521, respectively.

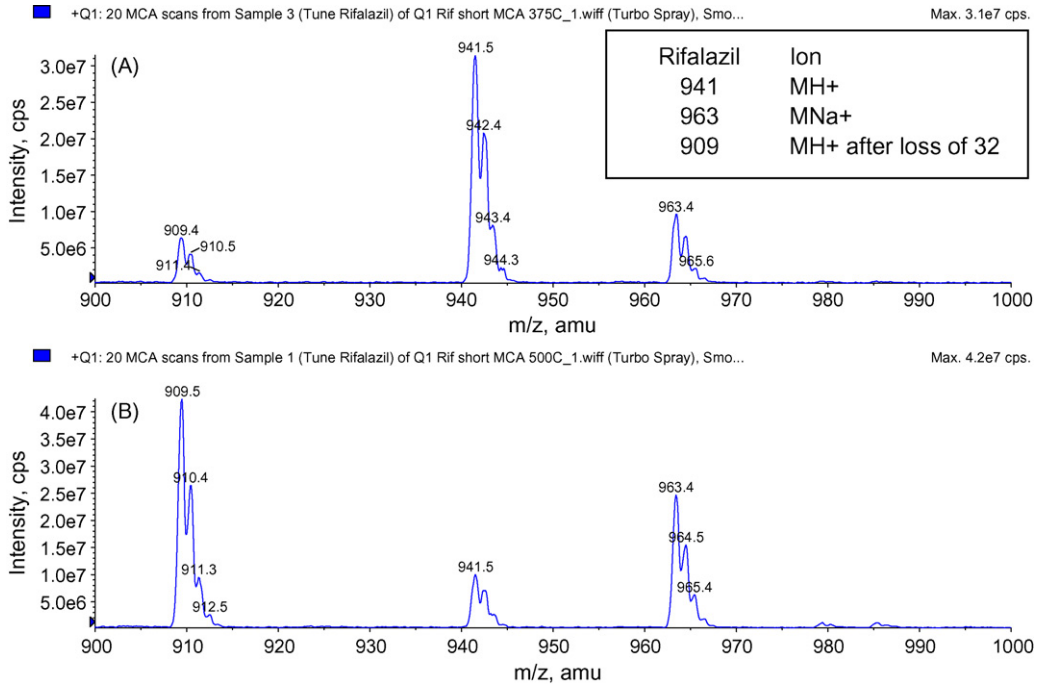

Fig. 2. Mass spectra (Q1) of rifalazil at two ion source conditions, illustrating increased in-source fragmentation at elevated temperature (TEM) and declustering potential (DP): (A) TEM 375 °C, DP 80 V; (B) TEM 500 °C, DP 140 V.

3.2. Evaluation of alternative SRM transition with plasma extracts

The identification of two alternative MS/MS transitions for rifalazil quantification presented an opportunity to evaluate the robustness of the LC/MS analysis of incurred samples. Plasma sample extracts were analyzed by the two different SRM transitions. If a bias were to be found for one of the two transitions in authentic and/or calibration samples, this could indicate interference in the ionization process. Moreover, based on the selection of a larger fragment for SRM of *m/z* 909/517, this transition could potentially be more selective than the loss of 32 amu, and might be useful if any interference would appear in future studies.

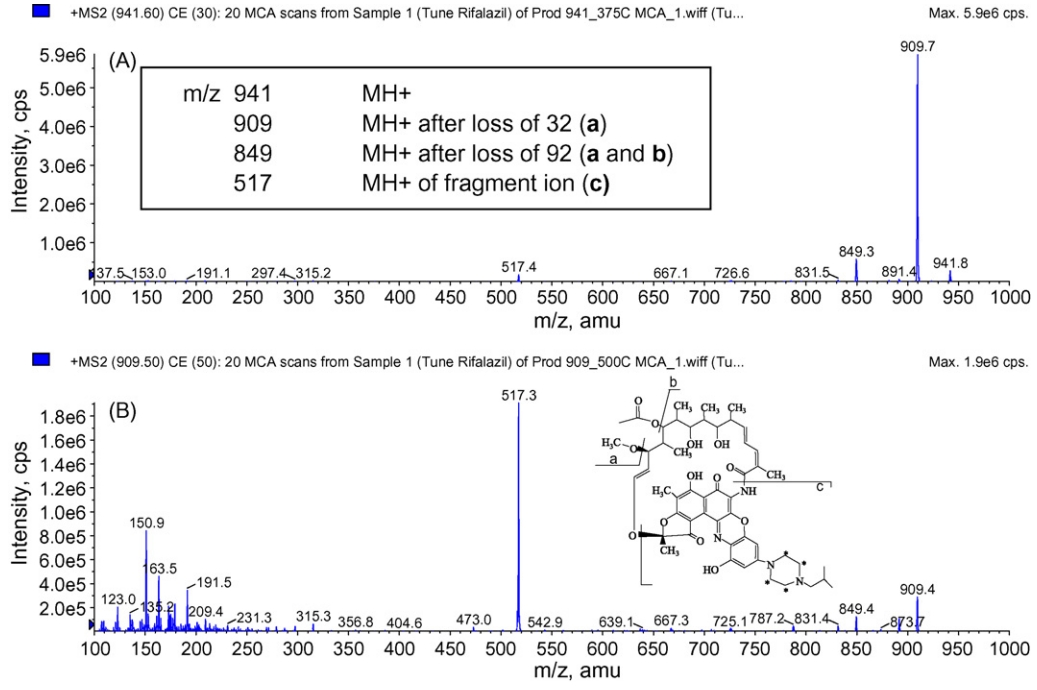

Fig. 3. Product ion scans of rifalazil for two precursor ions and suggested CAD fragmentation (insert). See Table 1 for parameter information. (A) Precursor ion *m/z* 941.6: TEM = 375 °C, DP = 80 V, CAD = 4 and CE = 30 eV; (B) precursor ion *m/z* 909.5: TEM = 500 °C, DP = 140 V, CAD = 7 and CE = 50 eV.

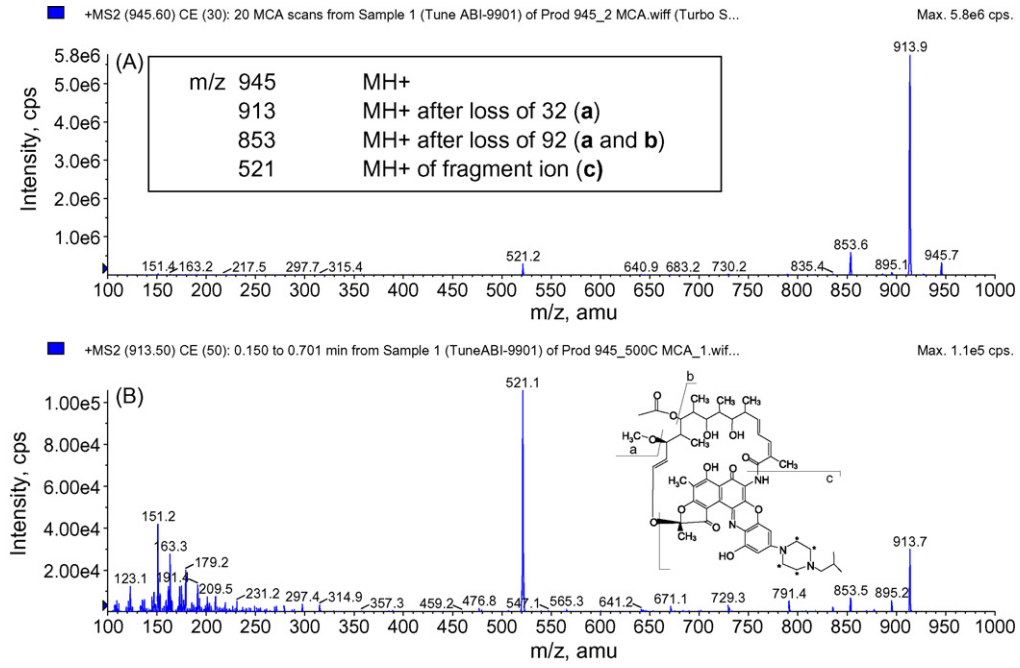

Fig. 4. Product ion scans of the internal standard, ABI-9901, for two precursor ions, and suggested CAD fragmentation (insert). See Table 1 for parameter information. (A) Precursor ion *m/z* 945.6: TEM = 375 °C, DP = 80 V, CAD = 4 and CE = 30 eV; (B) precursor ion *m/z* 913.5: TEM = 500 °C, DP = 140 V, CAD = 7 and CE = 50 eV.

In this experiment, one batch of extracts of incurred dog plasma samples, fortified calibration samples and QC samples were analyzed by LC/MS using two different SRM transitions, *m/z* 941/909 (the regular transition) and *m/z* 909/517 (after in-source fragmentation). The rifalazil response was about a threefold lower with the *m/z* 909/517 transition and the lowest calibration level was therefore elevated from 0.050 ng/ml to 0.10 ng/ml. Fig. 5 shows the relation between found rifalazil concentrations in the authentic sample extracts from the two analytical runs. The concentration range of study samples was from 0.7 ng/ml to 96 ng/ml in this batch. The results correlate well, with a regression line slope of 1.012 and the correlation factor R^2 of 0.9994. Fig. 6 presents the relation of concentrations found for the experimental transition *m/z* 909/517 versus *m/z* 941/909 (percent of initial result). The results from *m/z* 909/517 conditions were within 95–106% (mean 100.4, %R.S.D. 2.3) of the initial value. The corresponding concentration found in fortified plasma standard and QC samples in the same analytical batch was 90–111% (mean 100.5, %R.S.D. 5.1; $N = 20$) of the initial value. The excellent agreement between found mean concentrations in study samples indicates that no bias was found for study samples relative to calibration samples under any of the two different MS/MS conditions. Also, the high reproducibility (low %R.S.D.) of concentrations obtained for extracts from both fortified and authentic plasma samples in two consecutive LC/MS runs and under different ionization conditions further showed that the mass spectrometric detection was robust. Moreover, the results imply absence of ionization interferences in incurred samples that could potentially affect the rifalazil quantification. It can be concluded that either SRM transition can be used for sample analysis, however, with the limitation of an elevated LLOQ from 0.050 ng/ml to 0.10 ng/ml for the *m/z* 909/517 transition.

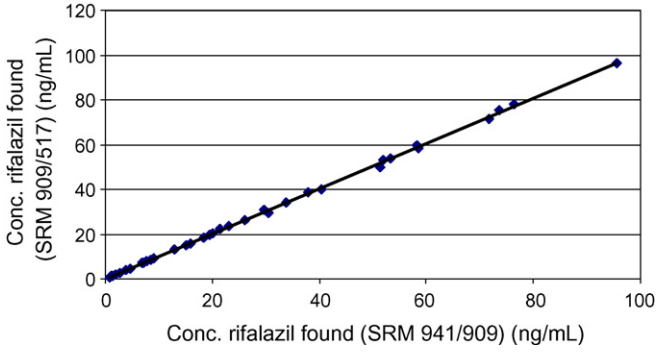

Fig. 5. Correlation of rifalazil concentrations found in incurred dog plasma samples from a formulation study using two different SRM transitions: *m/z* 941/909 and 909/517 (IS: *m/z* 945/913 and 913/521). The equation for the regression line is $y = 1.012x - 0.1171$.

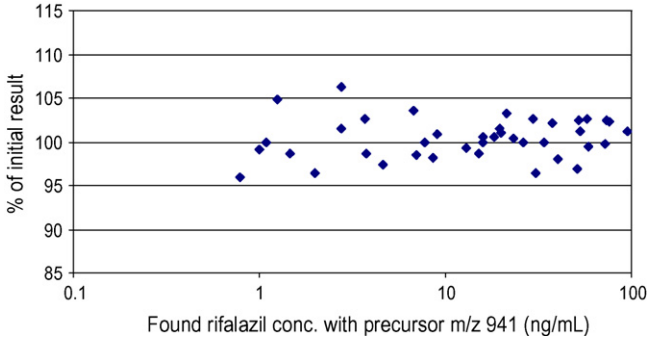

Fig. 6. Analysis of incurred dog plasma sample extracts with two different SRM transitions. Data is presented as % found rifalazil concentration using the explorative transition *m/z* 909/517 vs. the result from the regular *m/z* 941/909 SRM transition (initial) (same sample data as in Fig. 5).

3.3. Liquid chromatography

The LC method used for human plasma samples [6] at the time of method set-up was modified because parallel studies of new chemical entities with chemical structures related to rifalazil called for a general separation system. The original reversed-phase octadecyl silica column was replaced by an octyl silica material with the intent of facilitating removal of late eluting endogenous components. The addition of ammonium acetate to the low-ionic strength acetic acid (0.01% v/v) modifier in a water–methanol gradient improved the peak shape of related compounds. A low (1 mM)-ammonium acetate concentration was found to be a good compromise between MS response and general analyte peak shape. An intermediate LC/MS run time (8–9 min between injections) was found robust and was considered acceptable. A typical chromatogram of rifalazil at 0.050 ng/ml (LLOQ) is shown in Fig. 7.

The selectivity between rifalazil and known metabolites in the dog [8] was explored through selected reaction monitoring at *m/z* 899/867 for ABI-1671 (desacetyl-rifalazil) and at *m/z* 957/925 for 30-hydroxy-rifalazil. Both metabolites were observed in incurred dog plasma samples. They separated well

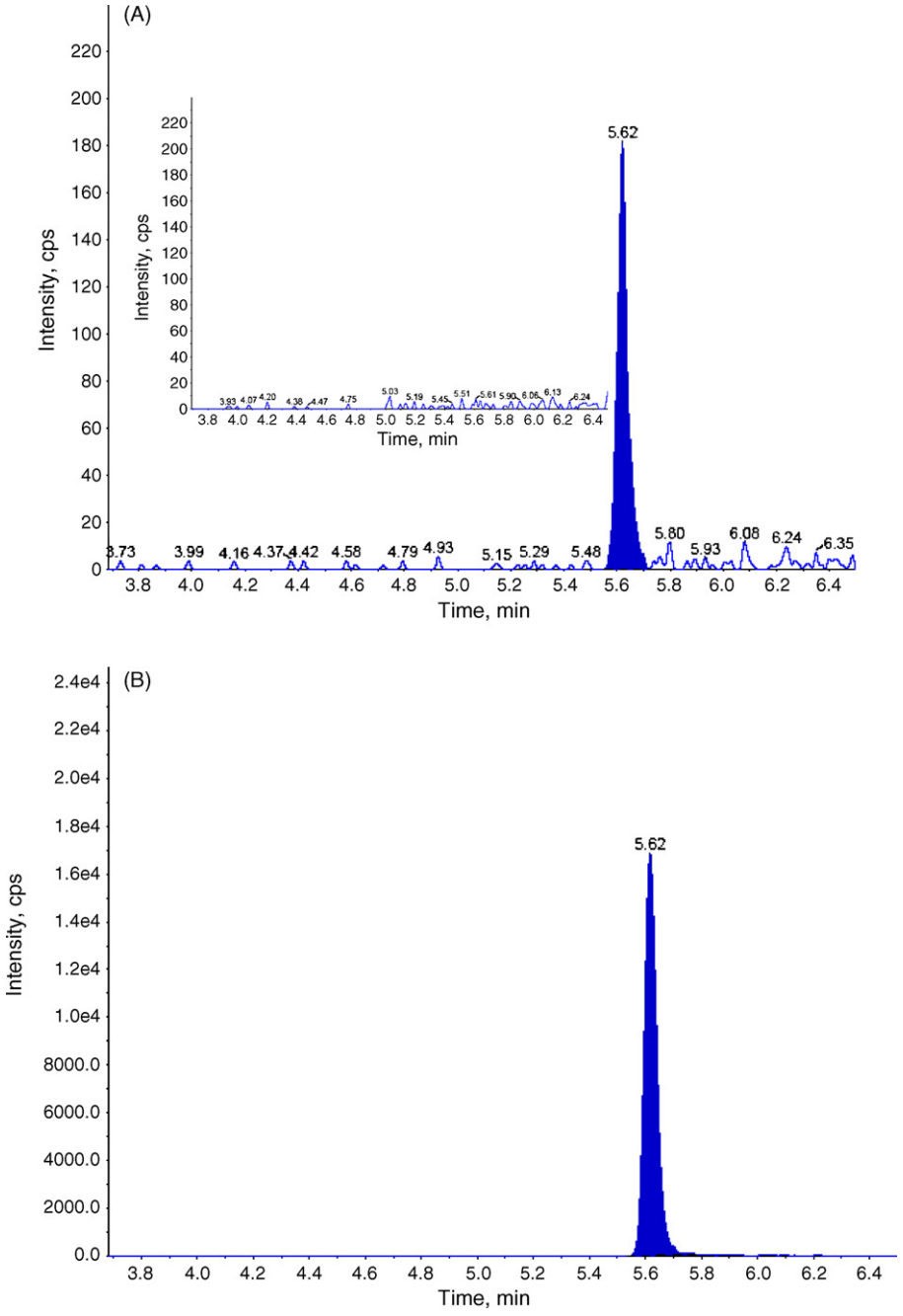

Fig. 7. LC–SRM chromatogram from dog plasma calibration sample extract of rifalazil at 0.050 ng/ml (LLOQ), retention time window 3.7–6.5 min. See Section 2 for LC conditions. (A) Rifalazil, *m/z* 941.6/909.4 with insert of blank plasma sample chromatogram; (B) internal standard ABI-9901, *m/z* 945.6/913.4.

Table 2
Variability for incurred dog plasma samples and quality control samples analyzed in the same study

	Incurred samples, same run	Incurred samples, between runs	QC: 0.15 ng/ml	QC: 1.5 ng/ml	QC: 50 ng/ml
Average (%)	99.6	100.8	99.0	100.4	97.6
%R.S.D.	3.1	11.0	19.2	10.7	7.0
N	26	23	29[a]	34	34

Incurred samples were processed in duplicates on a single day (same run) or a single plasma sample was reassayed on a second occasion (between runs). Average (%) for incurred samples is given as the ratio: second/initial result × 100. Average% for QC samples: compared with nominal concentration. Statistics are based on data from the entire study.

[a] QC: 0.15 ng/ml: excluded for runs where the calibration curve was truncated at 0.50 ng/ml. In addition, one outlier (300% of target) was excluded in the statistical calculations.

from rifalazil, eluting at a shorter retention time (data not shown).

The autosampler carry-over was minimized by utilizing an active external needle rinse with a low-pH solution, in which the solubility of rifalazil is higher than at neutral pH. A mixture of methanol–water–acetic acid (70:30:1, v/v/v) was found effective for wash of the injector needle.

3.4. Reproducibility of incurred sample analysis

Due to the inherent complexity of quantitative determination of drug-related compounds at low-concentration levels in plasma samples, it can be essential during method development and implementation to study the reproducibility of the method not only for fortified samples, prepared from pooled blank plasma from commercial sources, but also for study samples. Analysis of real-life samples may be influenced by many variables, such as sampling procedures, feeding conditions, individual variations of endogenous substance levels, etc. Early observations of deviations are important, as method modifications might be warranted to improve robustness.

A formulation development study, comprising of 864 study plasma samples which were analyzed over 4 months, was used to explore the method reproducibility after implementation. This study was generally suited, as the available plasma sample volume sufficed for several analyses, using 200 μl sample volume for the assay. Another prerequisite when repeating the analysis of study samples for quality assurance purposes is that the analyte is sufficiently stable in the sample matrix. Rifalazil stability in authentic dog plasma samples was therefore established in a pilot study ($N=4$) over three to five analytical occasions (including repeated thaw/freeze).

Incurred dog plasma samples were processed in duplicates on a single day, or a single plasma sample was reassayed on a second occasion. Within-day repeatability for incurred samples analyzed in duplicates is presented in Fig. 8 as the second duplicate result (% of the initial/first duplicate). The concentration range for the selected study samples was from 0.7 ng/ml to 149 ng/ml. The precision was %R.S.D. 3.1 (Table 2) and the variability appeared random over the concentration range studied. Method reproducibility over the study is shown in Fig. 9, where 23 out of 864, i.e., 2.7% of the study samples were subjected to repeated assay. Sample concentrations in the selected samples ranged from 0.5 ng/ml to 52 ng/ml. The precision for incurred samples between runs was %R.S.D. 11.0 and the second result versus the initial value was 100.8% (Table 2). The good precision for incurred samples indicates that the method provided consistent data over the study course. Additional evaluation of incurred sample reproducibility at lower concentration levels (up to 10× LLOQ) could be suggested.

Results for quality control (QC) samples from the same study are presented in Table 2 for comparison. The overall precision for QC samples at 1.5 ng/ml and 50 ng/ml in the study was similar to the between run precision for incurred samples. QC level 0.15 ng/ml (3× LLOQ) showed a higher variability with a %R.S.D. of 19.2. On a similar note, the calibration curve was truncated to a LLOQ of 0.50 ng/ml due to elevation of individual

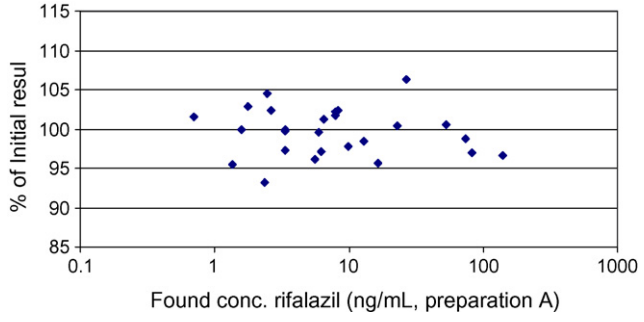

Fig. 8. Incurred dog plasma sample analysis. Each sample was processed in duplicates in the same analytical run. Data is presented as second replicate result (% of the initial). See Table 2 for summary statistics.

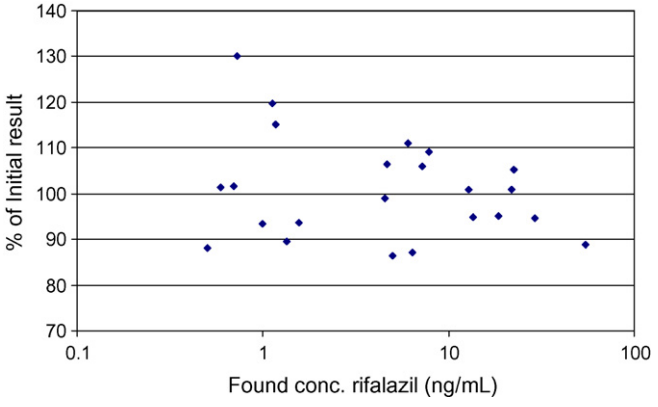

Fig. 9. Incurred dog plasma sample analysis. Each sample was processed in two different analytical batches. Data is presented as second occasion result (% of the initial result). See Table 2 for summary statistics.

low-level calibration standards on two occasions. Overall, quality control sample performance seemed to reflect the variability of authentic samples in the concentration range studied.

3.5. Accuracy assessment for incurred sample analysis

An attempt was made to evaluate the accuracy of rifalazil determinations in incurred dog plasma samples by a standard addition procedure. Such a procedure could possibly detect extraction recovery anomalies for authentic samples and also matrix effects on the LC/MS response. On the other hand, it cannot be considered a universal tool for accuracy evaluation for plasma analysis, as it would not account for, e.g., metabolite to analyte interconversion or analyte degradation during the analytical procedure, which however was not a concern for rifalazil which shows moderate metabolism and overall high stability.

A rifalazil standard solution (20 μl) corresponding to 0.5 ng/ml or 5 ng/ml in plasma was added to randomly selected study samples (200 μl) with unknown concentrations before processing by liquid–liquid extraction. Sample analysis was performed in 18 analytical batches. The recovery of spiked amount of rifalazil was calculated from the concentration difference between the spiked study sample and the original study sample: (result for spiked sample − result for sample)/nominal spiking concentration. An alternative approach is to derive the recovery from the ratio (result for spiked sample)/(result for spiked sample + result for sample), which would give a lower relative deviation from target. The first, more stringent, approach of recovery calculation was selected here. It can also be noted that the effect of analytical errors for two measurements will reduce the precision in the recovery measurement. However, the goal of this experiment was to estimate the average recovery (method accuracy) which would be feasible with a sufficient number of observations.

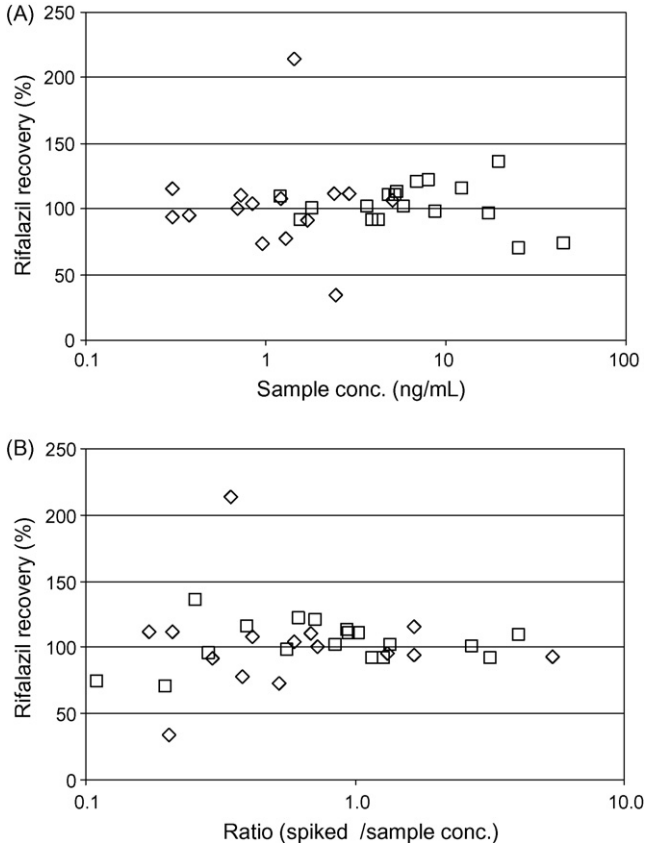

Fig. 10. Accuracy of rifalazil determination in incurred dog plasma samples. Routine sample analysis was performed in 18 analytical batches. Standard addition procedure: (A) rifalazil standard solution (20 μl) corresponding to 0.5 ng/ml or 5 ng/ml in plasma was added to randomly selected study samples (200 μl) with unknown concentrations before processing by liquid–liquid extraction. The recovery of spiked amount of rifalazil was calculated from the concentration difference between the measured spiked study sample and the original study sample. See text for discussion on accuracy calculation. (A) Recovery of spiked amount of rifalazil, arranged by incurred sample concentration (spiked rifalazil concentration: diamond, 0.5 ng/ml; square, 5 ng/ml); (B) recovery of spiked amount of rifalazil, arranged by ratio of spiked vs. actual sample concentration (spiked rifalazil concentration: diamond, 0.5 ng/ml; square, 5 ng/ml).

In order to reasonably match the spiking concentration to the original sample concentration, the analyst selected sample time points (time elapsed after an oral dose of rifalazil) based on previous experience from a similar study. The ratio (spiked/original concentration) ranged from 0.1 to 5.4. The recovery of spiked rifalazil in incurred dog plasma samples is presented in Fig. 10A and B shows the recovery in relation to the ratio of spiking concentration versus the sample concentration. The average recovery was 103% (%R.S.D. 26.2) over the study and the variation seemed randomly distributed over the plasma concentration range studied (0.30–45 ng/ml).

4. Conclusions

The presented results from repeated analysis of incurred dog plasma samples imply that the described LC–MS/MS method generates reproducible results during routine operation. Incurred sample data variability for within-run analysis of duplicates was %R.S.D. 3.1 and between run for repeat analysis %R.S.D. 11.0. Spiked QC samples in the same concentration range, run throughout the study, were found to reflect the performance of authentic samples. In addition, accuracy evaluation by standard addition of rifalazil to authentic dog plasma samples showed an average recovery of the spiked amount of 103%. It was further shown that in-source fragmentation of rifalazil followed by selected reaction monitoring is a viable alternative for rifalazil quantification.

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