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Determination of rifalazil, a potent antibacterial agent, in human plasma by liquid–liquid extraction and LC–MS/MS

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Abstract

A sensitive assay for determination of rifalazil (also known as ABI-1648 and KRM-1648) in human plasma is described. The analytical method utilizes liquid–liquid extraction of plasma with methyl *tert*-butyl ether, followed by reversed-phase liquid chromatography with a C_{18} column and a mobile phase gradient utilizing 0.1% formic acid in water and acetonitrile, respectively. Electrospray mass spectrometry in the positive ion mode with selected reaction monitoring of rifalazil and an isotope labeled internal standard, $^{13}C_4$-rifalazil (ABI-9901) was used for selective and sensitive detection. The calibration range was 0.050–50 ng/mL plasma using 200 μL plasma sample volume. The absolute extraction recovery of rifalazil from K_2-EDTA plasma, evaluated at three concentration levels, was 88.6–97.3%, and the recovery for the internal standard was 96.8%. A study of plasma matrix effects showed a peak area response at 90–99% compared to neat solutions for both rifalazil and the internal standard. Stability evaluation of rifalazil in plasma, whole blood and methanol showed that the analyte stability was adequate when stored under study conditions. The precision, as evaluated in three validation batches, was consistent for fortified plasma quality control (QC) samples at four concentration levels, with ≤6% R.S.D. except for at the lowest quality control level where it was 10.7% R.S.D. The accuracy for QC samples (difference between found and nominal concentration) ranged from −2.3% to 5.1%. Similar precision and accuracy values were obtained over 6 months of routine application of this method. It was concluded that the performance improved markedly during routine operation by replacing a closely related structural analog internal standard with the stable isotope internal standard.

Keywords: Rifalazil; ABI-1648; KRM-1648; Human plasma; Isotope labeled internal standard; Quantification; Electrospray

1. Introduction

Rifalazil (also known as ABI-1648 and KRM-1648) is an antibacterial agent which has extensive tissue penetration and high intracellular concentrations. These properties may contribute to rifalazil's extraordinary activity against the obligate intracellular pathogens *Chlamydia pneumoniae* and the closely related species, *Chlamydia trachomatis*, in cell culture experiments [1]. A recent phase 2 clinical study demonstrated that a single 25 mg oral dose of rifalazil was sufficient to achieve a long-term microbiological cure in the majority of patients with nongonococcal urethritis who were infected with *Chlamydia* [2]. Rifalazil belongs to the rifamycin family, however, without displaying significant interaction with the cytochrome P450 system [3]. Currently rifalazil is undergoing clinical testing for the treatment of peripheral vascular disease, which has been associated with *Chlamydia* infection of peripheral arteries [4]. A study of the pharmacokinetics of rifalazil in healthy male subjects after a single oral dose at 25 mg was recently reported in a food interaction study [5]. It was found that systemic exposure of rifalazil increased when rifalazil was given with food. In addition, intersubject variability decreased significantly when the dose was given with food compared to after an over-night fast.

Bioanalytical methodology for rifalazil has previously been described by Hosoe et al. [6,7] for determination of rifalazil concentrations in plasma, whole blood, urine and tissue from rats and dogs. Solid phase extraction and liquid chromatography with UV detection was used for rat plasma with a lower limit of quantification (LLOQ) of 10 ng/mL. The pharmacokinetics of rifalazil were studied in rats and dogs, after intravenous and oral administration [7]. Rifalazil distributes readily to tis-

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sues, as reflected in a large volume of distribution in animals [7]. Given the low doses used in human clinical trials with a therapeutic dose at 25 mg, and a long terminal half-life at above 100 h observed in humans [5], it was important to develop a more sensitive assay in order to perform detailed pharmacokinetic studies. This communication describes a sensitive and selective liquid chromatography–mass spectrometry method for human plasma, using liquid–liquid extraction for the isolation of rifalazil from plasma. Method performance during pre-study validation and routine sample analysis is reported. It is shown that the introduction of a stable isotope labeled internal standard resulted in improved performance and robustness.

2. Experimental

2.1. Materials

Rifalazil (ABI-1648 (3′-hydroxy-5′-[4-isobutyl-1-piperazinyl]benzoxazinorifamycin, $C_{51}H_{64}N_4O_{13}$), MW 941.1 (Fig. 1), as well as the stable isotope labeled internal standard (IS) ABI-9901, rifalazil-$^{13}C_4$, MW 945.1, and the isopentyl analog internal standard [6], ABI-2501, MW 955.1, were obtained by ActivBiotics, Inc., Lexington, MA, USA. All solvents were of HPLC grade. Methanol, acetonitrile, acetic acid, formic acid and methyl t-butyl ether were from Fisher Scientific (Fair Lawn, NJ, USA). Positive displacement pipettes from Gilson Microman, Rainin Instrument, LLC (Woburn, MA) were used for organic solvent and plasma solutions.

2.2. Human blood sample collection

Blood samples were collected in 6 mL Vacutainer Plus Plastic Lavender Hemogard Closure tubes (Fisher Scientific, Pittsburgh, PA, USA) with K_2-EDTA as the anticoagulant. The plasma was separated by centrifugation at 5 °C for 10 min at RCF 1500 within one hour of sample collection. Plasma was subsequently transferred to polypropylene cryo vials with polyethylene closures from Nalgene (Fisher Scientific) before freezing at −20 °C.

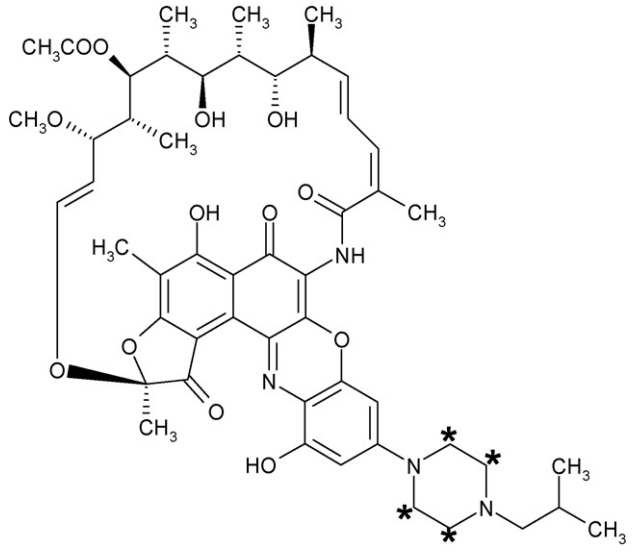

Fig. 1. Structure of rifalazil. Asterisks (*) denote the position of ^{13}C labels in the internal standard (ABI-9901, $^{13}C_4$-rifalazil). The protonated molecular ions were used as precursor ions for selected reaction monitoring of rifalazil and ABI-9901. An isopentyl analog of rifalazil [6], ABI-2501, was initially used as an internal standard.

2.3. Analytical procedure

The rifalazil stock solution at 0.2 mg/mL and a secondary stock solution at 5 μg/mL were made in methanol. Calibration standards (seven concentration levels from 0.050 to 50 ng/mL) were prepared by dilution with human K_2-EDTA plasma. Blank human plasma and whole blood was obtained from Bioreclamation (Hicksville, NY). A separate rifalazil stock solution was made to prepare quality control (QC) samples in human blank plasma. Five QC concentration levels ($N = 6$ per level) were analyzed in each batch during validation experiments and four QC levels ($N = 4$) during sample analysis. The internal standard ABI-9901 stock solution was made in methanol at 0.2 mg/mL and a working solution at 50 ng/mL was made by further dilution with acetonitrile–water (50:50, v/v).

A liquid–liquid extraction procedure was used for rifalazil. Human plasma (200 μL) and the ABI-9901 internal standard solution (50 μL of 50 ng/mL IS working solution) were mixed in a polypropylene tube (13 mm × 100 mm, Fisher Scientific, Pittsburgh, PA, USA) for one minute. The IS concentration corresponds to 12.5 ng/mL plasma. Extraction was made with methyl *t*-butyl ether (3 mL) by shaking for 10 min. Samples were then centrifuged at RCF 1880 for 5 min. The aqueous phase was frozen in a dry ice/methanol bath. The supernatant was then transferred to a clean tube and evaporated to dryness under nitrogen on a water bath set at 40 ± 5 °C in a TurboVap LV evaporator apparatus (Zymark Corp., Hopkinton, MA). The sample was reconstituted with 200 μL of H_2O–methanol–acetic acid (60:40:0.5, v/v/v), vortexed, transferred to autosampler vials and centrifuged before LC–MS/MS analysis.

A procedure for 10-fold dilution with blank human plasma that was used for samples with a concentration above the calibration range was verified, with an acceptance criterion for the result (average, $N = 5$) to be within 85–115% of the nominal concentration.

The absolute extraction recovery and the matrix effects for rifalazil and ABI-9901 were measured at 0.15, 1.5 and 15 ng/mL rifalazil in plasma ($N = 5$) and at one concentration (50 ng/mL, $N = 15$) for the internal standard. The peak areas of the reference, or unextracted, samples were determined by spiking an equivalent amount of analyte into an extract of blank plasma and injecting onto the LC–MS/MS. Recovery of drug and internal standard were determined from the ratio of the mean peak area of extracted samples to the mean peak area of reference samples.

The matrix effect was evaluated by measuring the Matrix factor, as defined by the ratio (analyte peak area in presence of plasma matrix)/(analyte peak area in absence of plasma matrix), and was expressed as percent response relative to the neat solution. In addition, six lots of human blank plasma, fortified with rifalazil at 50 pg/mL (LLOQ) were analyzed as a specificity and

matrix effect evaluation. Blank extracts of these six lots were evaluated for interfering peaks.

Stability of rifalazil in several matrices was established by using freshly prepared standards for quantification. The rifalazil concentration in each fortified matrix was assessed at the start of the experiment (day 0 or time 0). The acceptance criterion for stability assessments was a maximum allowed change in concentration of 15%. Long-term frozen stability of rifalazil in human plasma at two concentrations (0.15 and 45 ng/mL, $N = 5$) was determined after storage at −20 °C and −70 °C for 126 and 212 days. Whole blood stability was evaluated at room temperature at two concentrations of rifalazil (0.15 and 45 ng/mL, $N = 3$) after 0, 0.25, 0.5, 1, 2, and 3 h of storage. Whole blood samples were centrifuged, the plasma was harvested and analyzed. Refrigerated stability of rifalazil in human plasma was determined after storage at 4–8 °C. Stability was determined at two concentrations (0.15 and 45 ng/mL, $N = 3$) after 0, 0.083, 1, 4, 28, 52, 76, and 100 h of storage prior to analysis. Freeze/thaw stability was performed to evaluate the stability of rifalazil in human plasma after three freeze/thawcycles. QC samples at two concentrations (0.15 and 45 ng/mL, $N = 5$) were frozen at −70 °C (for a minimum of 24 h for cycle 1 and a minimum of 12 h for cycles 2 and 3) and thawed at room temperature. This freeze/thaw cycle was repeated three times. After the completion of the third cycle, the samples were analyzed.

2.4. Liquid chromatography–mass spectrometry

The separation column was Luna C18(2), 50 mm × 2.0 mm, 5 μm from Phenomenex (Torrance, CA, USA). The mobile phase flow rate was 0.4 mL/min and the composition was (A) 0.1% (v/v) formic acid in H_2O and (B) 0.1% (v/v) formic acid in acetonitrile, with a gradient program 40–100% B in 1.5 min, then back to 40% B in 0.1 min and hold for 3 min. The eluent was directed to waste after the LC column at 0–0.75 min via a diversion valve. The time between injections was about 5 min. The injection volume was 8 μL. The LC mobile phase was delivered by an Agilent 1100 series binary pump, equipped with a vacuum degasser and a column compartment (Agilent Technologies, Waldbronn, Germany). The autosampler was typically a HTS PAL from CTC Analytics (LEAP Technologies, Carrboro, NC). An injector rinse solvent consisting of methanol–water–acetic acid (70:30:1, v/v/v) was used.

Mass spectrometric detection was made with a triple quadrupole mass spectrometer API 4000 (Applied Biosystems, Inc., Foster City, CA, USA), with electrospray ionization in the positive ion mode. Typical settings were: Ion source temperature 450 °C, collision gas 8, collision energy 29 V, declustering potential 56 V, unit resolution (Q1 and Q3). Selected reaction monitoring (SRM) transitions (as optimized) for rifalazil was typically m/z 941.4 → 909.4 and for ABI-9901 (IS) m/z 945.6 → 913.5, with a dwell time of 150 ms. Nitrogen gas (boil-off from liquid nitrogen) was used as the mass spectrometer gas supply. Quantification was based on peak area measurements and made with a linear regression model with $1/x^2$ weighting. The mass spectrum (Fig. 3) and the product ion scan (Fig. 4) were obtained by infusion of a rifalazil solution at 10 μL/min into a mobile phase flow at 0.6 mL/min without an LC column, via a tee connection. The mass spectrometer acquisition was made in the MCA mode (Multiple channel acquisition).

3. Results and discussion

The quantitative method for rifalazil in human plasma was designed to reach a low limit of quantification, 0.050 ng/mL. This was achieved by liquid–liquid extraction with methyl t-butyl ether, which in combination with mass spectrometry and selected reaction monitoring gave sufficient selectivity and sensitivity. A less selective sample preparation method, such as protein precipitation, would not provide sufficient sensitivity. The absolute extraction recovery of rifalazil and matrix effects were evaluated at three rifalazil concentration levels, 0.15, 1.5 and 15 ng/mL ($N = 5$). The extraction recovery was 88.6–97.3% with a similar result, 96.8% (average, $N = 15$) for the stable label isotope internal standard, ABI-9901. Potential matrix effects on the mass spectrometric peak area response were evaluated for rifalazil by comparing a spiked reconstituted blank plasma matrix with the direct injection of the corresponding neat solutions. Plasma matrix effects on the electrospray MS detector response for rifalazil were found to be moderate, with a response at 90.0% (0.15 ng/mL), 96.0% (1.5 ng/mL) and 98.7% (15 ng/mL) compared to neat solutions. The stable isotope internal standard showed similar effects (89.8%, 98.6% and 98.7%, respectively), thus compensating well for response variation between samples.

Method specificity was evaluated for six lots of human blank plasma after fortification with rifalazil at 50 pg/mL (LLOQ). The average found concentration of rifalazil was 108% of nominal (%R.S.D. 7.4). No interfering peaks were detected for either rifalazil or the internal standard, ABI-9901. A dilution test made for rifalazil in plasma at 100 ng/mL ($N = 5$) showed that a 10-fold dilution provided accurate results.

3.1. Stability of rifalazil in various matrices

The stability of rifalazil under various study conditions was examined. Calibrants and solutions used as a reference at each time point were made from freshly prepared solutions. The rifalazil concentration in each fortified matrix was assessed at the start of the experiment (day 0 or time 0). A standard solution of rifalazil at 0.1 μg/mL in methanol was stable for at least 9 months at −20 °C and at room temperature for at least 6 h. No degradation was observed in human blood fortified with rifalazil (0.15 and 45 ng/mL) after 3 h at room temperature or on ice bath. The result (mean, $N = 3$ at each time point) at five time points was all within 90–110% of the time zero concentration. Fortified human plasma samples were stable for at least 15 h at room temperature (4.5 and 45 ng/mL). Long-term freeze stability was demonstrated at −20 °C and −70 °C for both concentration levels (0.15 and 45 ng/mL) for at least for 7 months. The concentration change, relative to day 0, after 126 days storage ranged from −7.1% to −13.7% over both temperatures and concentration levels studied, while the results after 212 days storage showed a range from −4.3% to −7.1%. Stability

of rifalazil in human plasma after three freeze-thaw cycles of plasma samples (both experiments at 0.15 and 45 ng/mL) was also confirmed (<10% change). In addition, rifalazil was found stable in human plasma for at least 4 days (100 h) when stored at +4 °C to 8 °C. The concentration difference found between the last time point and Time zero was −7.9% at 0.15 ng/mL and +3.1% at 45 ng/mL. Reconstituted extract stability was also confirmed for fortified human plasma samples (4.5 and 45 ng/mL) after storage in the autosampler for 140 h at room temperature, allowing for reinjection of sample extracts in an analytical batch.

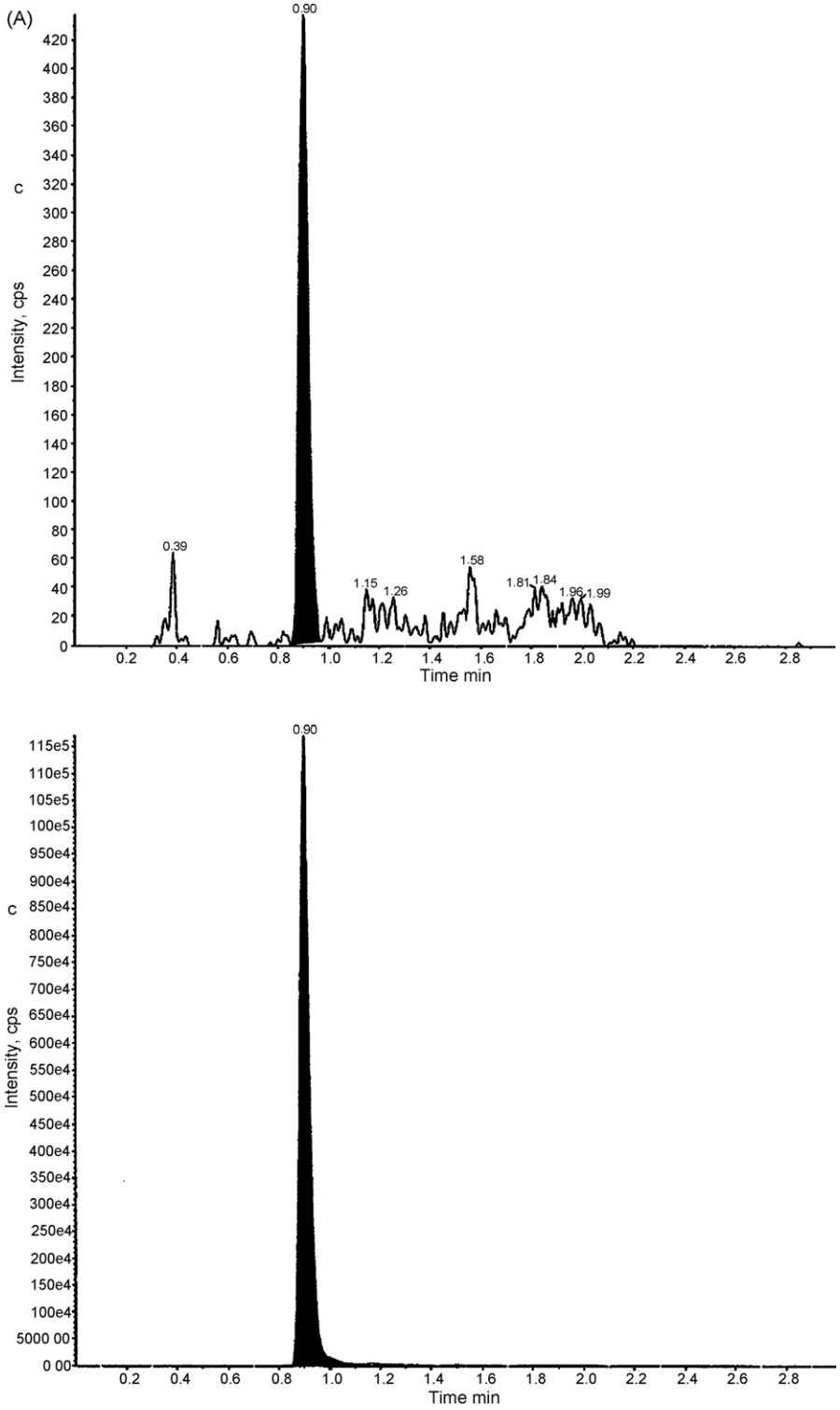

Fig. 2. LC–SRM chromatograms from extracted human plasma samples generated during determination of rifalazil. Mobile phase gradient: 0.1% formic acid in water–acetonitrile. Upper trace: rifalazil, *m/z* 941.4/909.4. Lower trace: internal standard ABI-9901, *m/z* 945.6/913.5. (A) Plasma calibration standard at 0.050 ng/mL rifalazil (LLOQ) with internal standard; (B) plasma sample blank without internal standard.

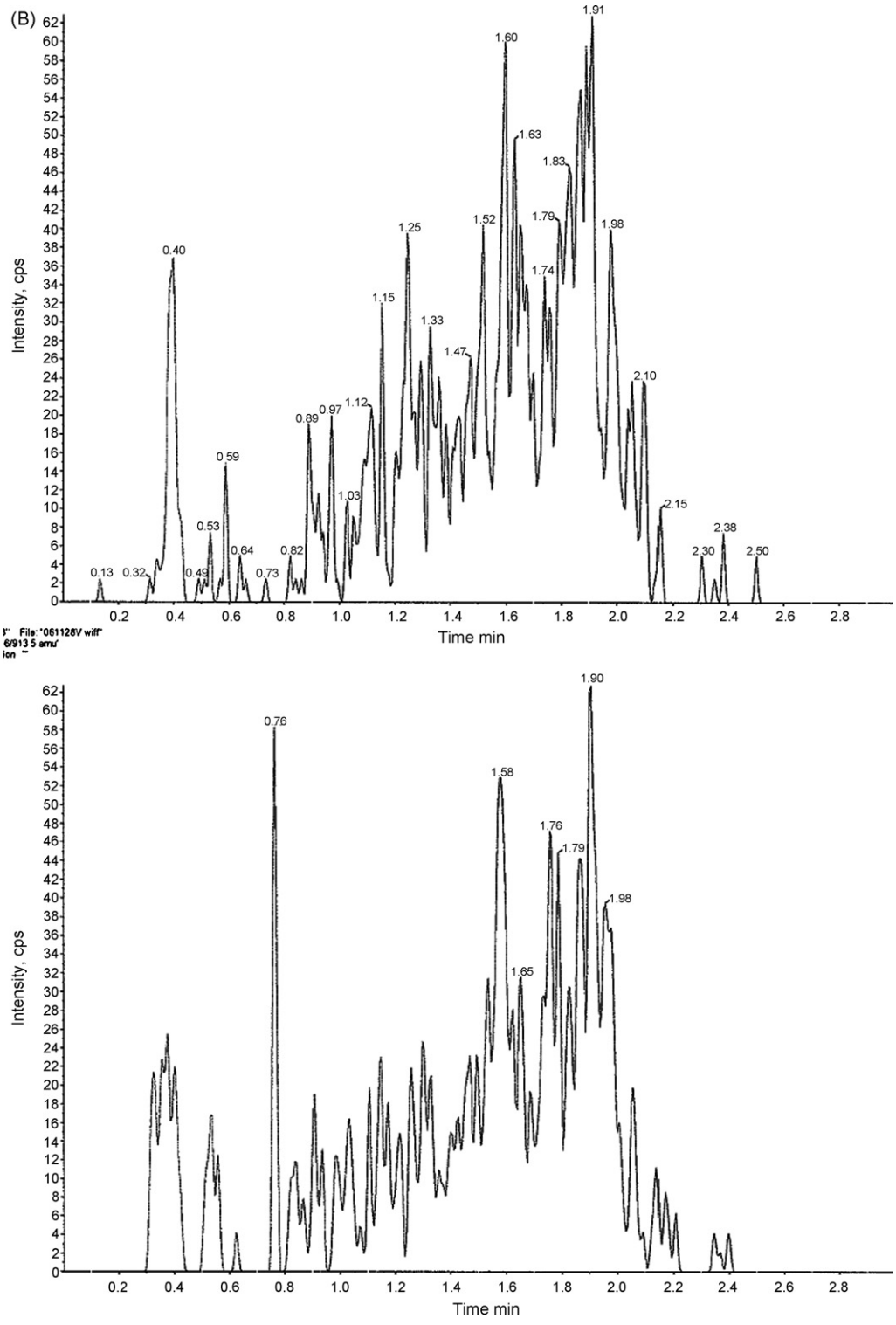

Fig. 2. (*Continued*).

3.2. Liquid chromatography–mass spectrometry

Reversed-phase liquid chromatography with an octadecyl silica column was used for sample analysis. Selected ion monitoring chromatograms of rifalazil and the internal standard MS/MS transitions at the lower limit of quantification, (LLOQ), 0.050 ng/mL, and from a blank plasma sample extract are shown in Fig. 2A and B, respectively. Rifalazil has a low solubility in aqueous media at neutral pH. The use of an injector wash step with a low-pH solution (methanol–water–acetic acid 70:30:1, v/v/v), in which rifalazil has a higher solubility than at neutral pH, reduced injector carry-over.

Rifalazil ionizes readily by positive ion electrospray and a Q1 mass spectrum is shown in Fig. 3. For quantitative analysis,

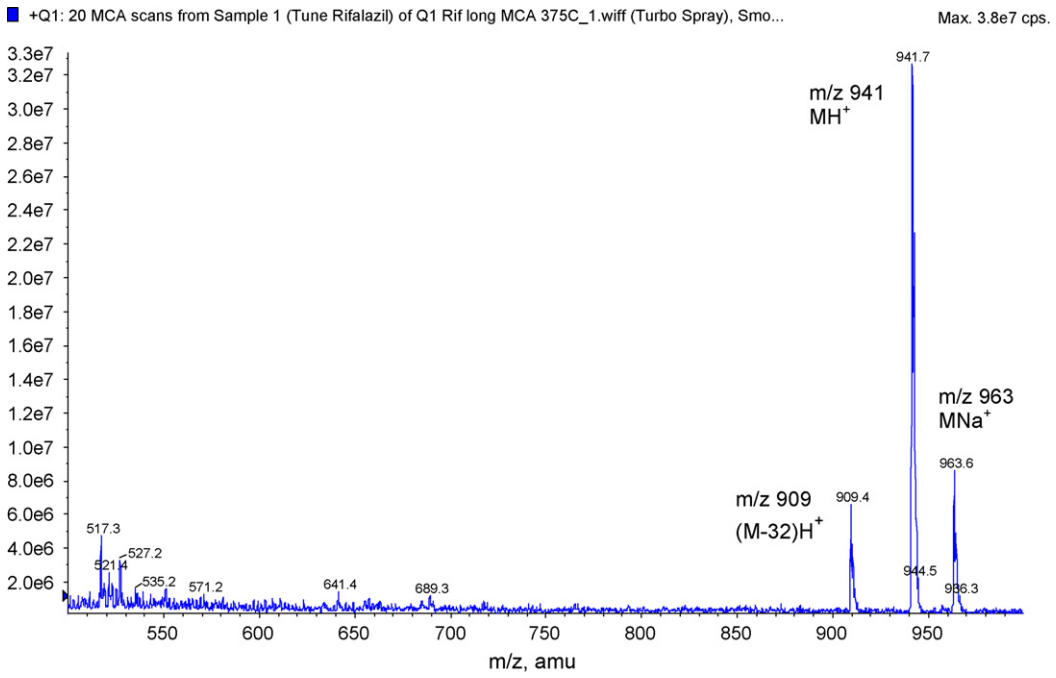

Fig. 3. Q1 mass spectrum of rifalazil in positive ion electrospray MS. The spectrum was recorded with MCA during compound infusion at 10 μL/min into a flow of mobile phase (50% organic, isocratic) at 0.6 mL/min.

rifalazil detection was made by selected reaction monitoring (SRM) after fragmentation of the protonated molecular ion, MH^+, (*m/z* 941.4) to $(M-32)H^+$ (*m/z* 909.4). A product ion mass spectrum of *m/z* 941 is shown in Fig. 4. The fragment ion *m/z* 909 can also be observed in the Q1 mass spectrum (Fig. 3). This ion is formed by in-source fragmentation which can be further promoted at increased temperature and declustering potential [9]. The use of *m/z* 909 → 517 SRM transition was investigated as an alternative to the transition of *m/z* 941 → 909 for quantification of rifalazil in dog plasma with liquid–liquid extraction and LC–MS/MS [9]. It was found that results correlate well between the two sets of data for spiked quality control samples as well

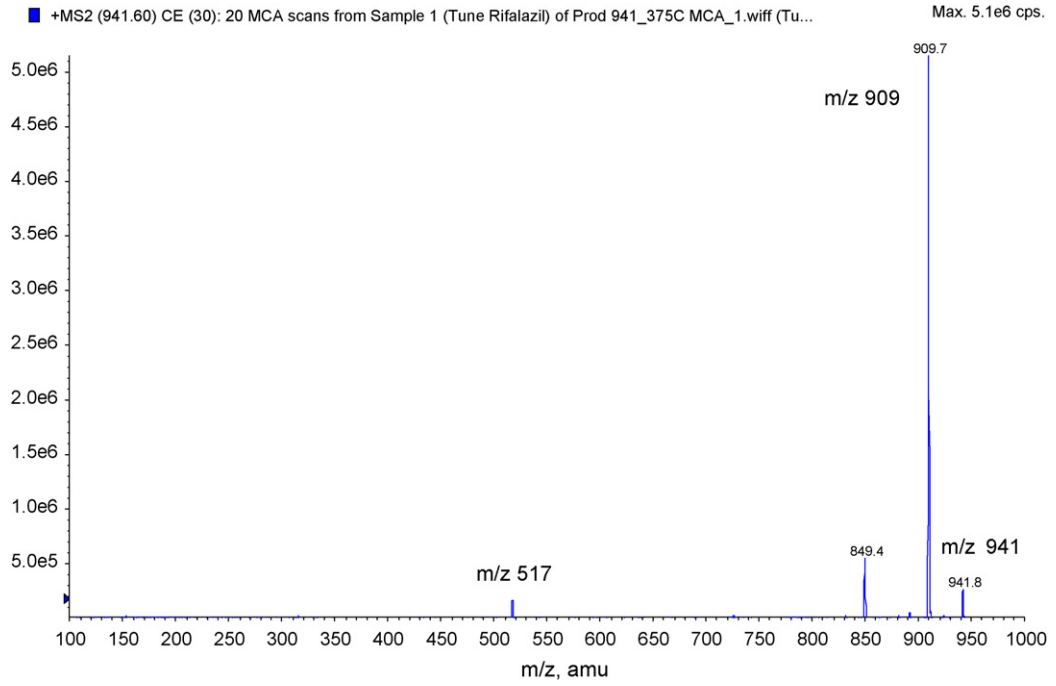

Fig. 4. Product ion mass spectrum of rifalazil, precursor ion *m/z* 941. For quantification, selected reaction monitoring was performed with the transitions *m/z* 941 → 909 for rifalazil and *m/z* 945 → 913 for the internal standard ABI-9901 ($^{13}C_4$-rifalazil). Conditions as in Fig. 3. See Ref. [9] for suggested MS/MS fragmentation of rifalazil.

Table 1A
Quality control sample performance during pre-study method validation for rifalazil in human plasma

Concentration (ng/mL)	0.15	4.5	12	45
N	18	18	18	18
Mean concentration (ng/mL)	0.150	4.73	11.7	44.0
%R.S.D.	10.7	3.0	6.0	4.3
%Diff.	0.0	5.1	−2.3	−2.2

Internal standard: ABI-9901 (stable isotope label of rifalazil). %Diff. denotes difference between mean found concentration and the nominal concentration.

Table 1B
Quality control sample performance during routine sample analysis over 6 months for rifalazil in human plasma

Concentration (ng/mL)	0.15	4.5	12	45
N	76	77	77	77
Mean concentration (ng/mL)	0.150	4.33	12.1	44.9
%R.S.D.	11.3	4.8	5.3	4.8
%Diff.	0.0	−3.8	0.9	−0.3

Internal standard: ABI-9901 (stable isotope label of rifalazil).

as for incurred samples, which indicates that MS detection of rifalazil is robust in either mode [9].

3.3. Method performance

The precision and accuracy for fortified quality control samples were evaluated during a pre-study method validation (Table 1A), and also during routine sample analysis over several months (Table 1B). Excellent performance with low variability and consistent accuracy was observed over 6 months for rifalazil using the stable label isotope ABI-9901 as the internal standard. The benefit of using such internal standards for LC–MS analysis, compared to analog internal standards, was thoroughly discussed by Matuszewski, especially with respect to biological sample matrix effects [10]. The closely related isopentyl analog to rifalazil [6], ABI-2501, was initially used by the current authors as the internal standard for determination of rifalazil in human plasma with LC–MS/MS. For comparison, the performance of the analog as an internal standard is presented below. As shown in Table 2A, the method readily met acceptance criteria for imprecision (<15%R.S.D.; at LLOQ < 20%) and inaccuracy (deviation from nominal <15%; at LLOQ < 20%) during the pre-study validation. During routine operation over 6 months, however, a larger variability was observed (Table 2B). Moreover, calibrants at the highest concentration level failed on several occasions to meet the acceptance criteria. This resulted in a truncated calibration range, associated also with more frequent repeated sample analysis and accompanied by a lower sample through-put. As shown above, the introduction of a stable isotope label internal standard improved robustness and productivity considerably for the rifalazil assay.

Table 2A
Quality control sample performance during pre-study method validation for rifalazil in human plasma. Internal standard: ABI-2501 (structural analog of rifalazil)

Concentration (ng/mL)	0.15	4.5	45
N	18	18	18
Mean concentration (ng/mL)	0.149	4.73	45.2
%R.S.D.	4.7	5.8	4.6
%Diff.	−0.7	5.0	0.5

Table 2B
Quality control sample performance during routine sample analysis over 6 months for rifalazil in human plasma

Concentration (ng/mL)	0.15	4.5	12	45
N	43	43	43	16[a]
Mean concentration (ng/mL)	0.153	4.74	12.6	50.7
%R.S.D.	18.3	14.8	14.5	10.0
%Diff.	2.0	5.4	5.2	12.7

Internal standard: ABI-2501 (structural analog of rifalazil).
[a] QC samples at 45 ng/mL were not included for batches where the high calibration standard level (50 ng/mL) failed to meet acceptance criteria. In such cases, the calibration range was truncated resulting in an upper limit of quantification at 15 ng/mL.

While acceptance criteria for routine sample analysis generally are based on QC and calibration sample performance, such parameters may not necessarily reflect all quality aspects of a bioanalytical method. However, repeated analysis of study samples from dosed subjects can provide additional evidence for method reproducibility [8]. Reproducibility of rifalazil determination in dog plasma with LC–MS/MS was recently reported for routine sample analysis by repeating the analysis of study samples ($N = 23$) in a second analytical run [9]. Sample concentrations in the selected study samples ranged from 0.5–52 ng/mL. The precision for incurred samples between-runs was %R.S.D. 11.0 and the ratio of the second result versus the initial value was 100.8%, indicating that the method is reproducible not only for fortified blank plasma quality control samples but also for study samples [9].

4. Conclusions

A robust, sensitive and selective method for rifalazil determination in human plasma was developed. The introduction of a stable isotope labeled internal standard markedly improved method performance during routine operation. LC–MS/MS methodology has been used for the analysis of thousands of human plasma samples during the clinical development of rifalazil.

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