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RAPID SIMULTANEOUS LC/MS^2 DETERMINATION OF RIFAMPICIN AND 25-DESACETYL RIFAMPICIN IN HUMAN PLASMA FOR THERAPEUTIC DRUG MONITORING

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ABSTRACT. A rapid and sensitive liquid chromatography coupled with tandem mass spectrometry (LC/MS^2) method for the simultaneous quantification of rifampicin and its main active metabolite 25-desacetyl rifampicin in human plasma was developed and validated. The separation was performed on a Gemini NX C18 column under isocratic conditions using a mobile phase of 40:60 (V/V) methanol and 2mM ammonium formate in water, at 40 ºC, with a flow rate of 0.6 mL/min. The detection of rifampicin and its metabolite was performed in multiple reaction monitoring mode using an ion trap mass spectrometer with positive electrospray ionization. The human plasma samples (0.1 mL) were deproteinized with methanol and aliquots of 0.3 µL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity ($r > 0.993$), precision (CV < 8.2%) and accuracy (bias < 6.3%) over the range of 411 - 19737 ng/mL for rifampicin and good linearity ($r > 0.992$), precision (CV < 10.1%) and accuracy (bias < 8.2%) over the range of 70 - 3379 ng/mL for 25-desacetyl rifampicin. The lower limit of quantification (LLOQ) was 411 ng/mL and recovery was between 90.3-108.2% for rifampicin, whereas for 25-desacetyl rifampicin the LLOQ was 70 ng/mL and recovery between 93.1-107.5%, respectively. The developed and validated method is simple, rapid

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and specific for the high throughput simultaneous determination of rifampicin and 25-desacetyl rifampicin in human plasma and was successfully applied in therapeutic drug monitoring of rifampicin in patients with tuberculosis.

Keywords: *rifampicin, 25-desacetyl rifampicin, LC/MS^2, human plasma*

INTRODUCTION

Rifampicin, a semisynthetic antibiotic produced from *Streptomyces mediterranei* (Fig. 1) is used as first-line therapy for the treatment of tuberculosis in combination with at least one other anti-tuberculosis drug. As the incidence of infections is increasing, rifampicin is also preferred in combination therapy in the treatment of infections associated with prosthetic devices (e.g., implantable cardiac electronic devices) or in antimicrobial-resistant infections, especially methicillin-resistant Staphylococcus aureus (MRSA) [1,2].

Rifampicin is characterized by wide inter- and intra-individual variability in healthy volunteers, but also in tuberculosis patients [3]. Although the majority of patients respond to a standardized therapy, low drug concentrations are commonly associated with treatment failure or bacteria resistance, resulting in poor clinical outcomes [4,5]. 25-desacetyl-rifampicin is the main active metabolite of rifampicin, thus it is also responsible on the clinical efficiency of the treatment [3]. Rifampicin stimulates the metabolism of isoniazid and of other concomitant administered drugs and also induces its own metabolism over the first few weeks of treatment. In most patients with reduced renal or hepatic function, the doses should be reduced to prevent toxicity, but for rifampicin doses might need to be increased to avoid under-dosing due to enzyme induction. The determination of rifampicin and its metabolite in biological samples is useful for the evaluation of rifampin pharmacokinetics in combination therapy, facilitating early screening of therapeutic failure and preventing adverse effects [6,7]. Pharmacokinetic/ pharmacodynamic studies are needed to evaluate the interrelation between the free plasma drug concentration and microbiological and clinical outcomes of the treatment with rifampicin [5].

Several chromatographic analytical methods have been reported for the determination of rifampicin and 25-desacetyl rifampicin involving various techniques such as high performance thin layer chromatography (HTPLC) [7], high performance liquid chromatography (HPLC) [11], liquid chromatography coupled with mass spectrometry (LC-MS/MS) [10,12,13].

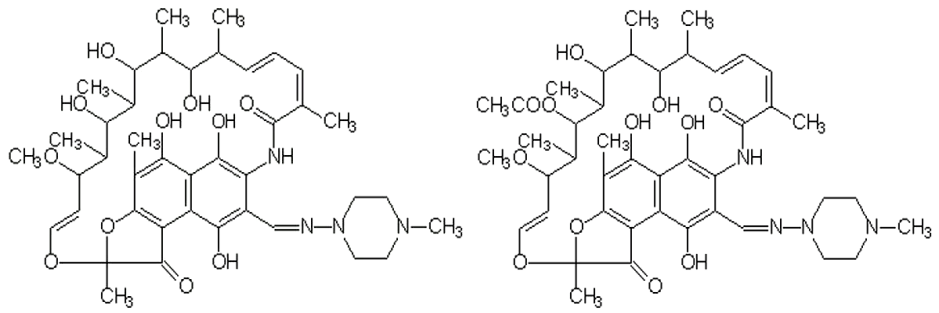

Figure 1. Chemical structures of rifampicin (left) and 25-desacetyl rifampicin (right)

Rifampicin can be determined using HPTLC [7], which is a quick and cheap method, however LC/MS methods are much more sensitive and more selective. HPLC methods have also been used for the determination of rifampicin [11], but we decided to develop a new LC/MS method due to its better selectivity in comparison to HPTLC and HPLC methods.

For analysis of biological samples using chromatographic methods, the samples must first undergo an extraction / purification process. A purification process often used is solid phase extraction (SPE) [9]. Although it is a very effective purification method, it is time consuming and raises the cost of analysis significantly. A fast, simple and inexpensive method for biological sample purification is protein precipitation (PP) method, widely used especially when LC/MS analysis is employed [10,12,13].

RESULTS AND DISCUSSION

Sample preparation

Although the MS detection type and parameters greatly influence the sensitivity of LC/MS assay methods, the sample preparation method can also have an influence on the chromatographic background noise level and can create suppression effects. Generally the analyte is extracted from the matrix prior to analysis using SPE or LLE for reasons such as sample purification and sample pre-concentration. The extraction processes (both SPE and LLE) are tedious, time and resource consuming, and will usually require the use of internal standards in order to reduce extraction variability. For these reasons PP as a sample processing technique is recommended whenever high-throughput and low extraction variability are required. The

disadvantage of PP is the fact that the samples are not highly purified as in the case of SPE or LLE, samples get diluted in the process, thus reducing sensitivity. The sample preparation technique must be chosen in a way to assure the best performance needed for a given analysis, whether reduced time and costs are needed, or a high sensitivity is needed.

The range of the calibration curves was adapted to the expected concentration levels of biological samples to be analyzed, 411.2 - 19737.6 ng/mL for rifampicin and 70.4 - 3379.2 ng/mL for 25-desacetyl rifampicin, respectively. As the developed analytical method shows good sensitivity for both analytes, the PP extraction method (which implies sample dilution) was considered a good alternative to SPE or LLE providing shorter processing times and high reproducibility.

The method developed by us requires samples of only 0.1 mL plasma, processed by PP with methanol (0.3 mL), the supernatant being injected into the HPLC system directly after centrifugation. The sensitivity obtained meets the requirements in order for the method to be usable for the given drug monitoring application and absolute recoveries were close to 100%.

LC-MS assay

The detection parameters were optimized in order to achieve best sensitivity and specificity for both rifampicin and 25-desacetyl rifampicin. Both compounds were ionized in ESI source by proton addition, giving pseudomolecular ions with m/z 824 for rifampicin and m/z 750 for 25-desacetyl rifampicin. By fragmentation, these ions are broken down to ions with m/z 792 for rifampicin and m/z 732 for 25-desacetyl rifampicin respectively (Fig. 2 and Fig 3). The extracted ion chromatograms (EIC) of m/z 792 from m/z 824 for rifampicin and m/z 732 from m/z 750 for 25-desacetyl rifampicin were analyzed for quanfication purposes. In the chromatographic conditions of the method the retention times were 1.1 minutes for rifampicin and 0.6 minutes for 25-desacetyl rifampicin, with a run-time per sample of 1.4 minutes. Typical chromatograms of human plasma samples spiked with rifampicin and 25-desacetyl rifampicin at LLOQ are shown in Fig. 3. No interfering peaks attributed to plasma components were observed at the retention times of rifampicin and 25-desacetyl rifampicin (Fig.3).

Assay validation

Validation of the method was carried out in accordance with international regulations [14]. Calibration curves prepared in human blank plasma were linear for both rifampicin and 25-desacetyl rifampicin over their concentration ranges of 411.2 - 19737.6 ng/mL, and 70.4 - 3379.2 ng/mL respectively.

Correlation coefficients were greater than 0.993 for rifampicin and greater than 0.992 for 25-desacetyl rifampicin. The lower limit of quantification (LLOQ) was established at 411.2 ng/mL for rifampicin and 70.4 ng/mL for 25-desacetyl rifampicin. Results for within-day and between-day precision and accuracy determined during the validation are shown in Tables 1 and 2 for rifampicin, and Tables 3 and 4 for 25-desacetyl rifampicin, respectively. All values for accuracy and precision were within acceptance criteria (< ±15%) [14]. Recovery values were between 90.3-108.2% for rifampicin and 93.1-107.5% for 25-desacetyl rifampicin.

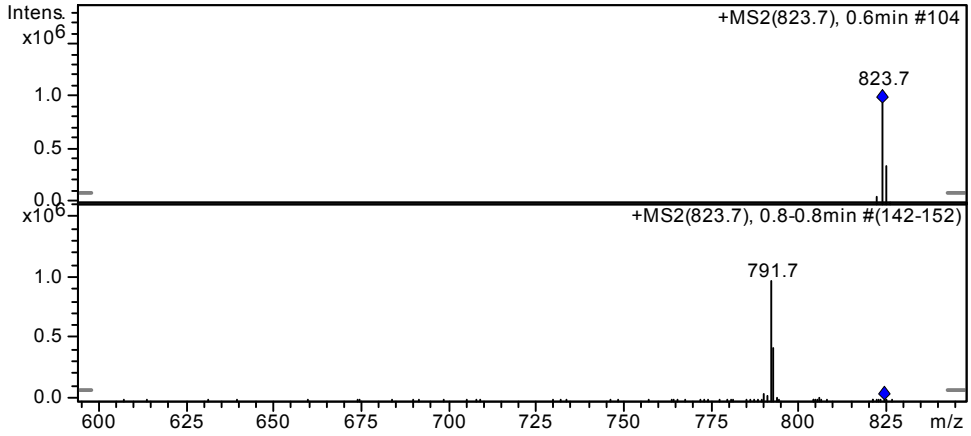

Figure 2. MS spectra of rifampicin – MS spectra (upper image), MS/MS spectra (lower image)

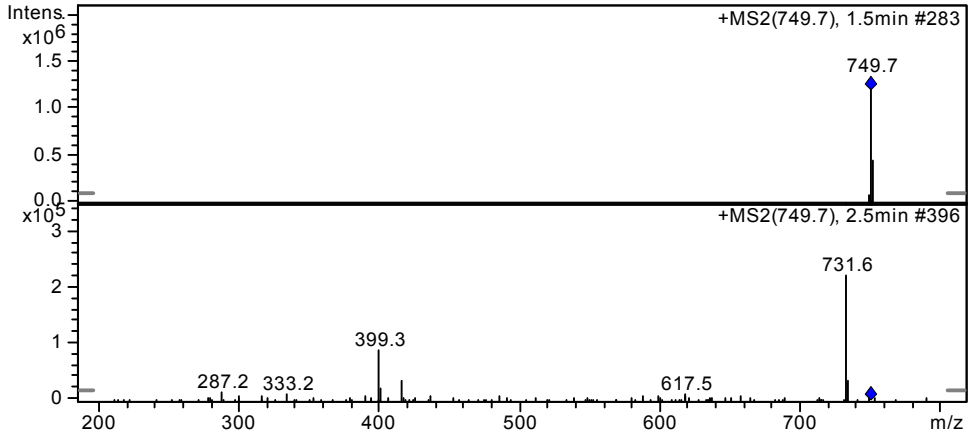

Figure 3. MS spectra of 25-desacetyl rifampicin – MS spectra (upper image), MS/MS spectra (lower image)

Method application

The validated method for simultaneous determination of rifampicin and 25-desacetyl rifampicin in human plasma was successfully applied in therapeutic drug monitoring and pharmacokinetic assessment of rifampicin and 25-desacetyl rifampicin in tuberculosis patients. Typical chromatograms of rifampicin and 25-desacetyl rifampicin in a tuberculosis patient plasma sample is shown in Fig. 3 and the overlapped plasma profiles obtained in the whole study (spaghetti plot chart) are presented in Fig. 4.

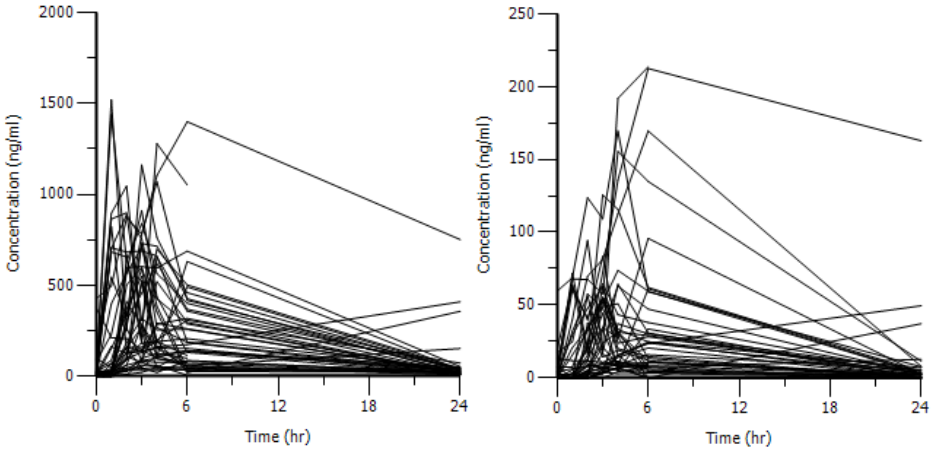

Figure 4. Overlapped plasma profiles of rifampicin (left) and 25-desacetyl rifampicin (right) administrated to patients with tuberculosis (600 mg rifampicin/day)

CONCLUSION

Antimicrobial therapeutic drug monitoring could be an important tool in clinical practice if the administered drug shows large inter-subject variability in pharmacokinetics or when the patient's compliance is poor. Therefore, quantification of drug in plasma is an important issue in clinical practice to enhance efficacy and to reduce toxicity.

The LC/MS^2 method developed by us for the quantification of rifampicin and 25-desacetyl rifampicin is specific, accurate, fast and inexpensive, as long as the analytical technology used (LC/MS) is available. It is one of the fastest analytical methods for simultaneous determination of rifampicin and 25-desacetyl rifampicin in biological matrices published, also having the advantage of using a simple, high throughput sample preparation technique. The method has a runtime of 1.4 minutes. Compared to other methods described in literature with runtimes of 2.1 minutes [10], 3.5 minutes [12] and 6 minutes [13]. This allows for shorter analysis times and higher throughput,

especially when large batches of samples need to be analysed. The faster runtimes were achieved without loss of accuracy or precision of the method. For rifampicin the within-day precision ranged from 1.0% to 7.7%, with an accuracy (bias, %) of -6.3% to 5.3%, while between-day precision ranged from 3.0% to 8.2%, with an accuracy (bias, %) of -3.2% to 4.7%. For 25-desacetyl rifampicin the within-day precision ranged from 1.8% to 4.2%, with an accuracy (bias, %) of 0.1% to 8.2%, while between-day precision ranged from 5.3% to 10.1%, with an accuracy (bias, %) of -2.5% to 1.0%. These values are similar to those of other methods described in literature [10,12,13]. The sample preparation using protein precipitation is simple and fast, ideal for high throughput LC-MS methods, and is also used in other methods described in literature [10, 12, 13]. The lower limit of quantification was 411 ng/mL for rifampicin and 70.4 ng/mL for 25-desacetyl rifampicin, higher than other methods described for the quantification of rifampicin [10, 12, 13] and 25-desacetyl rifampicin [10]. However, the purpose of our analytical method was its suitability for therapeutic drug monitoring, when steady-state plasma concentrations are much higher than after single-dose administration and a shorter analysis time is more desirable than a higher sensitivity.

The method was successfully applied in therapeutic drug monitoring of rifampicin and 25-desacetyl rifampicin in patients with tuberculosis.

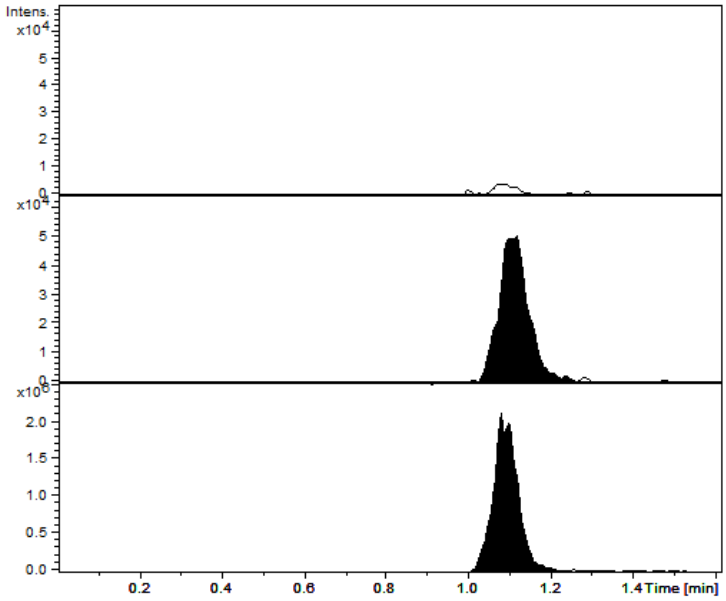

Figure 5. Representative chromatograms of blank sample (upper image); plasma spiked with rifampicin at lower limit of quantification (411 ng/mL, middle image); and a plasma sample from a patient under treatment with 600 mg rifampicin/day, sample taken after 2h from administration, concentration found 13672 ng/mL (lower image).

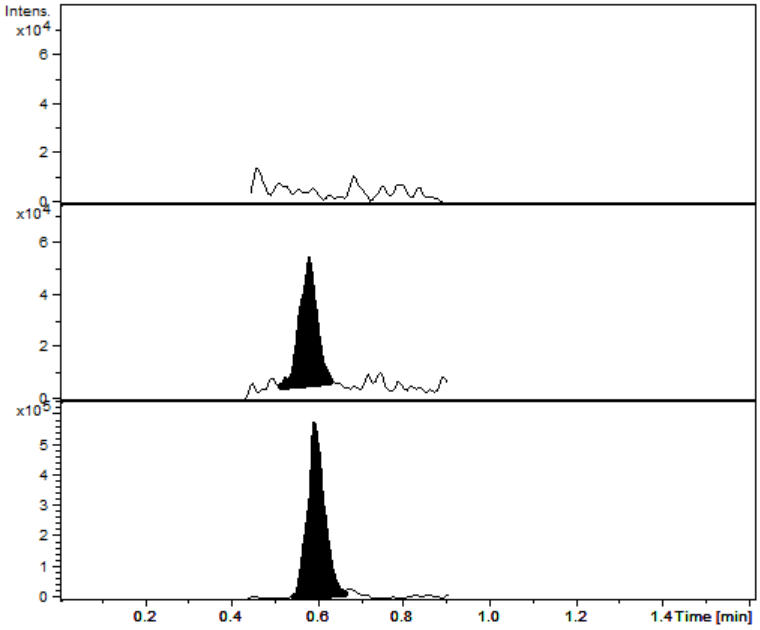

Figure 6. Representative chromatograms of blank sample (upper image); plasma spiked with 25-desacetyl rifampicin at lower limit of quantification (70 ng/mL, middle image); and a plasma sample from a patient under treatment with 600 mg rifampicin/day, sample taken after 2h from administration, concentration found 645 ng/mL (lower image).

Table 1. Within-day precision, accuracy and recovery (n = 5) for rifampicin

$c_{nominal}$ ng/mL	Mean c_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
411.2	436.5±33.6	7.7	6.2	94.8±8.6
822.4	770.4±13.5	1.8	-6.3	100.8±10.2
3289.6	3241.0±67.8	2.1	-1.5	108.2±11.6
9868.8	10390.5±101.6	1.0	5.3	96.6±9.6

Table 2. Between-day precision, accuracy and recovery (n = 5) for rifampicin

$c_{nominal}$ ng/mL	Mean c_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
411.2	430.3±35.4	8.2	4.7	96.2±12.8
822.4	804.6±44.2	5.5	-2.2	100.1±12.9
3289.6	3182.8±203.3	6.4	-3.2	90.3±4.7
9868.8	10191.6±304.1	3.0	3.3	102.2±12.7

Table 3. Within-day precision, accuracy and recovery (n = 5) for 25-desacetyl rifampicin

$c_{nominal}$ ng/mL	Mean c_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
70.4	74.0±2.7	3.7	5.1	99.4±6.6
140.8	152.3±2.7	1.8	8.2	102.1±4.9
563.2	577.8±24.5	4.2	2.6	97.3±6.3
1689.6	1691.8±61.3	3.6	0.1	99.4±7.0

Table 4. Between-day precision, accuracy and recovery (n = 5) for 25-desacetyl rifampicin

$c_{nominal}$ ng/mL	Mean c_{found} ng/mL (± S.D.)	CV %	Bias %	Recovery % (± S.D.)
70.4	71.13±4.9	7.0	1.0	99.6±9.2
140.8	140.33±14.1	10.1	-0.3	96.4±2.3
563.2	549.35±29.0	5.3	-2.5	93.1±0.9
1689.6	1659.9±126.2	7.6	-1.8	107.5±3.2

EXPERIMENTAL SECTION

Reagents

Rifampicin and 25-desacetyl rifampicin reference standards were from USP (Rockville, MD, USA). Gradient grade methanol for liquid chromatography and ammonium formate of analytical-reagent grade were supplied by Merck KGaA (Darmstadt, Germany). Bidistilled deionised water for injections was supplied by the Infusion Solution Laboratory of the University of Medicine and Pharmacy Cluj-Napoca (Cluj-Napoca, Romania). Human blank plasma was obtained from healthy volunteers, both male and female.

Apparatus

The apparatus and equipment used were: Sigma (Osterode am Harz, Germany) 204 series Centrifuge; Mettler-Toledo (Greifensee, Switzerland) Analytical Plus Balance and Mettler-Toledo (Greifensee, Switzerland) Precision Standard Balance; Scientific Industries (New York, USA) Vortex Genie 2 mixer; Elma (Singen, Germany) Transsonic 700/H Ultrasonic bath. An Agilent Technologies (Darmstadt, Germany) 1100 series LC/MS system was used consisting of: G1312A binary pump, G1379A in-line degasser, G1329A autosampler, G1316A column thermostat and an 1100 SL Ion Trap Detector.

Chromatographic and spectrometric conditions

A Phenomenex (Torrance, California, USA) Gemini NX C18 (50 x 2.0 mm i.d., 3.0 μm) chromatographic column was used for separation, using a mobile phase of a 40:60 (V/V) mixture of methanol and 2mM ammonium formate in water under isocratic conditions, kept at 40 ºC with a flow rate of 0.6 mL/min. Detection of rifampicin and 25-desacetyl rifampicin was carried out using multiple reaction monitoring (MRM) mode of the ion trap mass spectrometer with electrospray ionization (ESI) source, positive ionization (capillary exit 175 V, nebulizer 60 psi (nitrogen), dry gas (nitrogen) at 11 L/min, dry gas temperature 350ºC). The column effluent was diverted to waste for the first 0.4 minutes in order to keep the ESI source clean of contamination. The extracted ion chromatograms (EIC) of m/z 792 from m/z 824 for rifampicin and m/z 732 from m/z 750 for 25-desacetyl rifampicin were analyzed (MS^2 mode).

Standard solutions

Stock solutions of 1.028 mg/mL rifampicin and 0.440 mg/mL 25-desacetyl rifampicin were prepared by dissolving the appropriate quantity of substance in methanol. Working solutions of rifampicin (41.12 μg/mL) and 25-desacetyl rifampicin (7.04 μg/mL) were prepared by appropriate dilution of stock solutions in human blank plasma. The working solutions were then used to prepare seven plasma calibration standards and three quality control standards for both rifampicin and 25-desacetyl rifampicin. Concentrations of calibration standards for rifampicin were between 411.2 ng/mL and 19737.6 ng/mL, and quality control standards had concentrations of 822.4 ng/mL (low), 3289.6 ng/mL (medium) and 9868.8 ng/mL (high). For 25-desacetyl rifampicin the calibration standards were between 70.4 ng/mL and 3379.2 ng/mL, and quality control standards had concentrations of 140.8 ng/mL (low), 563.2 ng/mL (medium) and 1689.6 ng/mL (high). All calibration standards and quality control standards were prepared by diluting the appropriate volumes of the working solutions with blank plasma.

Sample preparation

0.1 mL of standard and plasma sample respectively were added to a 1.5 mL plastic tube and precipitated with 0.3 mL methanol. The samples were then vortex-mixed (10 seconds) and centrifuged (3 minutes at 12000 rpm), and 0.15 mL of the supernatants were transferred to autosampler vials and 0.3 μL were injected into the HPLC system.

Method validation

Rifampicin and 25-desacetyl rifampicin concentrations were determined automatically by the instrument's QuantAnalysis data system using the peak areas, using the external standard method. The calibration curve model was determined to be y = b + ax, weighted ($1/y^2$) linear regression, where y is the peak area and x is the concentration of the analyte (μg/mL).

Within-day precision and accuracy were determined analyzing five different samples (n = 5) of each of the QC standards (low, medium, high) in the same run. The between-day precision and accuracy were determined analyzing five different samples (n = 5) of each of the QC standards (lower, medium, higher), each on one of five different days. Precision was expressed as the coefficient of variation (CV %) of five samples, while accuracy was expressed as relative difference between mean of measured and the calculated concentrations (bias %). The lowest calibration standard with an accuracy and precision within the 20% acceptance was established as the lower limit of quantification (LLOQ). Relative recovery values (at LLOQ and low, medium, high QC levels) were determined by comparing chromatographic peak areas of spiked plasma samples with peak areas of samples in pure solvent, both having the same concentration of rifampicin and 25-desacetyl rifampicin respectively [15-16].

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