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Specific high-performance liquid chromatography assay for determination of rifabutin plasma concentration following Extrelut column extraction

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Abstract

A specific, precise and accurate assay for determination of rifabutin in human plasma using Extrelut column extraction was developed and validated. Rifabutin concentrations were calculated with a standard curve ranging from 5 to 800 ng ml^{-1}, using a split-curve approach. Chromatographic peaks were separated by means of a 5 μm Symmetry Shield RP8 using a KH_2PO_4 (0.05 *M*) buffer–acetonitrile mobile phase. Detection wavelength was set at 275 nm. Chromatography was carried out at room temperature (20–25°C). The limit of quantification was 5 ng ml^{-1}. The recovery was over 71%. The intra-day precision of the assay was 5, 7, and 1% while the inter-day precision was 11.2, 8.1, and 5.8% at concentrations of 30, 150 and 500 ng ml^{-1}, respectively. The accuracy ranged from 99 to 108%. Forty of the drugs most commonly administered to HIV-positive patients were found not to interfere with the assay. The assay has been used in a comparative study of rifabutin pharmacokinetics in HIV-positive patients with or without wasting syndrome.

Keywords: Rifabutin

1. Introduction

Rifabutin, (Fig. 1; 4-deoxo-3,4-[2-*spiro*-(*N*-isobutyl - 4 - piperidyl) - 2, 5 - dihydro - 1H - imidazo] - rifamycin-S), a semisynthetic derivative of rifampicin 5, has been approved for prophylaxis of disseminated *Mycobacterium avium* complex (MAC) in patients infected with human immunodeficiency virus (HIV). Prophylactic treatment with rifabutin was shown to decrease the incidence of MAC by approximately 50% in AIDS patients enrolled in two randomized, placebo-controlled clinical trials [1]. Recently, a three drug combination therapy containing rifabutin has been shown to be more effective in the treatment of MAC bacteremia than a four-drug combination therapy containing rifampin [2]. The clinically significant interactions of the newer antiretroviral agents, belonging to the family of protease inhibitors, with rifampin [3] may foster the usage of rifabutin in some clinical settings. For example, it has been hypothesized that rifabutin could be used for the treatment of tuberculosis in patients concomitantly treated with indinavir [4]. Therefore, rifabutin

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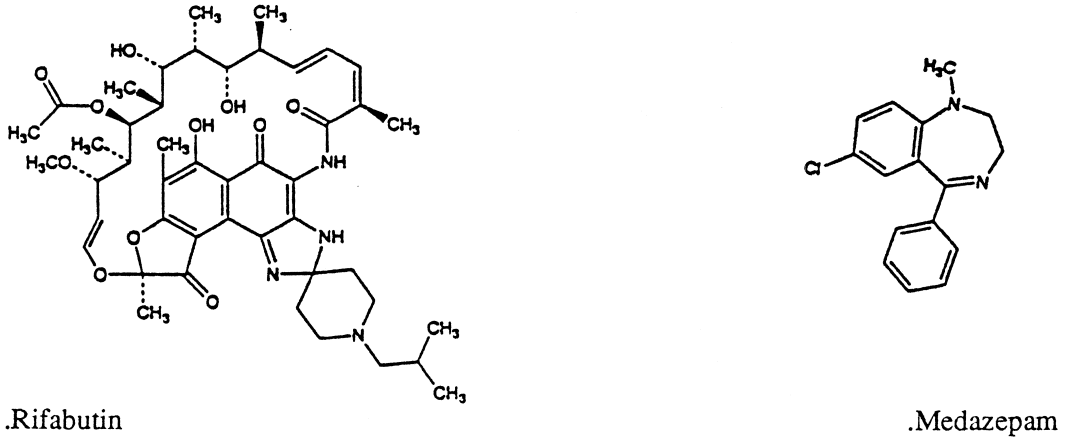

Fig. 1. Chemical structures of rifabutin and medazepam, the internal standard.

currently remains a major drug for the management of MAC in AIDS patients. Due to the large number of drugs which may be concomitantly administered to patients with HIV infection there is potential for pharmacokinetic interactions. We developed and validated an HPLC assay with liquid–liquid column extraction and we studied the possible chromatographic interference with the most common drugs used to treat HIV infected patients. This assay is faster and easier to execute than the previously published assay using a liquid–liquid extraction method [5]. It does not have the tailing effect observed in another recently published assay [6] and it is more simple than using on line concentration and column switching [7]. This assay has been used in a comparative study of rifabutin pharmacokinetics in HIV infected patients with or without wasting syndrome [8].

2. Experimental

2.1. Materials

Rifabutin was kindly provided by Pharmacia & Upjohn (Milan, Italy). Medazepam, the internal standard, was purchased from Sigma (Milan, Italy). Acetonitrile, methanol, hexane, and ethylacetate were from Merck (Darmstadt, Germany). All reagents were HPLC grade. Phosphoric acid potassium phosphate, monobasic, and potassium hydroxide, were purchased from Analyticals Carlo Erba (Milan, Italy) and 1-heptanesulfonic acid, sodium, was purchased from Sigma–Aldrich (St. Louis, MO, USA). Water deionization and purification was achieved using a Milli-Ro and MilliQ system, Millipore corporation. The columns for liquid–liquid extraction, Extrelut 3, were purchased from Merck.

2.2. Chromatographic system

The HPLC system consisted of a Merck–Hitachi (Darmstadt, Germany) model L-6200 A intelligent pump, a Perkin Elmer (Norwalk, CT, USA) model autosampler ISS-200 automatic injector, a Merck–Hitachi L-4500 diode array detector and a Merck–Hitachi D-6500 chromatography data station software-data system manager installed on a Impex Gavi GAV 484 (Reggio Emilia, Italy) personal computer.

The mobile phase consisted of a mixture of 0.05 *M* potassium phosphate, monobasic, (pH 4.10) and acetonitrile (51.3:48.7, v/v); pH was adjusted with phosphoric acid (85%).

The buffer of the mobile phase was filtered through a Millipore 0.45 μm filter and degassed in an ultrasonic bath.

Peaks were separated with a 5 μm Symmetry Shield RP8 column (25 cm×4.6 mm) (Waters, Milford, MA, USA) using a flow of 1 ml min^{-1} and detected at a wavelength of 275 nm. Chromatography was carried out at room temperature (20–25°C).

The lifetime of the column was greater than 900 injections.

2.3. Preparation of standard solutions

Stock solution of 20 μg ml^{-1} of rifabutin was prepared by dissolving rifabutin in 50 ml of methanol. The purity of the substance was 98.54%.

Using different dilutions in methanol, rifabutin working standard solutions were prepared from the stock solution at the following concentrations: 8, 4, 2, 1, 0.5, 0.2, 0.05 μg ml^{-1}.

A solution of 5 μg ml^{-1} of medazepam in methanol, used as internal standard, was prepared from a stock solution of 1 mg ml^{-1} in methanol.

The working standard solutions were stable at 4°C for approximately two months.

The standard curve ranging from 5 to 800 ng ml^{-1} was prepared on each day of analysis by adding 100 μl of each of the rifabutin working standard solutions to 900 μl of blank plasma.

Rifabutin photodegradation was minimized by preparing the stock solutions in the dark and storing in aluminum foil.

2.4. Quality control samples

Quality control spiked samples were prepared with the same procedure used for standard solution preparations. The final concentrations were 500, 150 and 30 ng ml^{-1}.

2.5. Sample preparation

A 1-ml aliquot of calibration standard or sample was pipetted into a PBI International (Milan, Italy) clean culture polypropylene 17×120 h mm-cap tube.

A 50-μl volume of internal standard was subsequently added and the mixture was vortex-mixed. A 1-ml volume of extraction buffer at pH 7.4 (0.25 *M* potassium phosphate, monobasic, +0.05 *M* 1-heptanesulfonic acid, sodium, adjusted with 1 *M* potassium hydroxide) and a 1-ml volume of deionized water were then added to the tube and the mixture was vortexed for 20 s.

The mixture was then applied to the Extrelut 3 column and the compounds eluted with 6 ml (in two aliquots of 3 ml with a 15 min interval) of a mixture of hexane–ethyl acetate (80:20, v/v). The eluate was collected into a PBI International clean culture polypropylene 17×120 h mm-cap tube. Elution was completed in approximately 20 min.

The eluate was evaporated under a gentle stream of nitrogen.

A 100-μl volume of mobile phase was added and the tube was vortexed for 20 s. A volume of 80 μl was injected into the HPLC system.

2.6. Validation

A standard curve consisting of seven points ranging from 5 ng ml^{-1} to 800 ng ml^{-1} and quality control samples consisting of low (30 ng ml^{-1}), medium (150 ng ml^{-1}) and high (500 ng ml^{-1}) concentration were used to determine the intra- and inter-day precision and accuracy of the assay. Spiked concentrations and peak height ratios of rifabutin standards were fit by unweighted regression to a linear equation ($y=ax+b$) and drug concentrations in control samples as well as in same day standard curve samples were calculated using this equation. A split curve approach was used. Only the concentrations greater than or equal to 100 ng ml^{-1} were calculated with the entire curve ranging from 5 to 800 ng ml^{-1}. Concentrations lower than 100 ng ml^{-1} were calculated using the lower portion of the curve ranging from 5 to 100 ng ml^{-1}. For all curves the correlation coefficients (r^2) were never smaller than 0.998.

3. Results

3.1. Specificity

A chromatogram of extracted plasma (blank control) is shown in Fig. 2a; a chromatogram from a patient plasma sample is shown in Fig. 2b. No peaks interfering with analytes are evident. Specificity of the assay is proven by the absence of interfering peaks in the pre-dose plasma samples obtained from 20 HIV patients who were enrolled in a comparative pharmacokinetic study of rifabutin absorption and disposition in patients with or without wasting syndrome [8]. Also, to verify the specificity of the method, several drugs which could be administered concomitantly with rifabutin were injected using the same chromatographic conditions. A list of the drugs

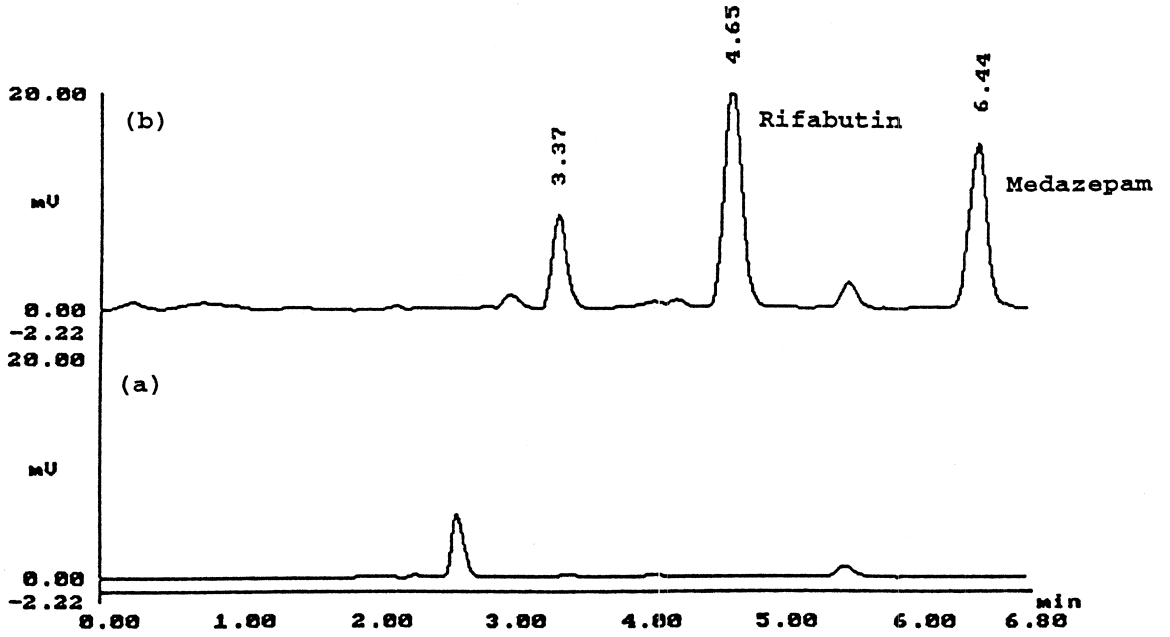

Fig. 2. Chromatograms of (a) blank human plasma, and (b) plasma from a patient taken 5.58 h after dosing with rifabutin (366.4 ng ml^{-1}). Rifabutin RT: 4.6 min medazepam RT: 6.4 min. The peak with a RT of 3.4 min represents rifabutin major metabolite, 25-*O*-desacetyl rifabutin.

which were found to lack interference with the assay is shown in Table 1.

3.2. Recovery

The absolute recovery of rifabutin extracted from plasma was determined by comparing the peak heights of the working standards with the peak height of the respective standards extracted from plasma. The recovery ($n=14$) was 75%, 71% and 78% at the concentrations of 30, 150 and 500 ng ml^{-1}, respectively.

3.3. Precision and accuracy

3.3.1. Intra-day variability

One standard curve and five sets of each of the quality controls were analyzed in the same day. The precision and accuracy for the quality controls are shown in Table 2.

Table 1
List of administered drugs that do not interfere with the rifabutin assay

Calcium Folinate	Loperamide
Ceftriaxone	Lorazepam
Ciprofloxacin	Medrogestone
Clarithromycin	Methadone
Codeine	Metronidazole
Dextrose	Nelfinavir
DDI	Nystatin
D4T	Octreotide
Enalapril	Paracetamol
Ethambutol	Paromomycin
Fluconazole	Pentamidine
Flunitrazepam	Prednisone
Flurazepam	Pyrazinamide
Foscarnet	Rifampicin
Ganciclovir	Ritonavir
Haloperidol	Sulfamethoxazole
Indinavir	Theophylline
Isoniazid	Tranexanic Acid
Ketorolac	Trimethoprim
Lamivudine	Zidovudine

Table 2
Intra-day precision and accuracy of quality control samples ($n=5$)

Spiked concentration (ng ml^{-1})	Mean calculated concentration (ng ml^{-1})	RSD[a] (%)	Accuracy (%)
30	32.6	5	108.7
150	161.5	7	107.7
500	483.9	1	96.8

[a] RSD = relative standard deviation.

3.3.2. Inter-day variability and stability

One standard curve and one set of quality controls were analyzed on twelve different days during the pharmacokinetic study. The precision for the standard curve and quality control samples is shown in Tables 3 and 4, respectively. The RSD of the slopes was 4.2% for the entire curve and 6.1% for the low curve.

This results also indicate that the working solutions were stable for two months when stored at 4°C, this also if standard and controls were prepared fresh or if they were pre-prepared and stored at −20°C for 24 h until the analysis.

Table 3
Precision of the standard curves ($n=12$) utilized for the inter-day experiment

Spiked concentration (ng ml^{-1})	Mean calculated concentration (ng ml^{-1})	RSD[a] (%)
5	5.4	13.8
20	19.8	8.8
50	49.9	4.7
100	99.6	1.1
200	200.9	7.1
400	410.6	5.5
800	792.9	1.7

[a] RSD = relative standard deviation.

Table 4
Inter-day precision and accuracy of the quality control samples ($n=12$)

Spiked concentration (ng ml^{-1})	Mean calculated concentration (ng ml^{-1})	RSD[a] (%)	Accuracy (%)
30	31.9	11.2	106
150	152.3	8.1	102
500	512.4	5.8	102

[a] RSD = relative standard deviation.

3.3.3. Accuracy

The assay accuracy was calculated using the following formula:

% accuracy =

(mean calculated concentration/spiked concentration)

×100.

Accuracy was always less than 8.7% as it is shown in Table 4.

3.4. Limit of quantification

The lower limit of quantification was 5 ng ml^{-1}. At such concentration the inter-day precision was 13.8 and the accuracy was 108%.

3.5. Ruggedness

This assay showed a good ruggedness; in fact, little changes either in the mobile phase pH value or in the normal laboratory conditions of humidity, light, air exposure and temperature did not influence the retention time of rifabutin and medazepam.

4. Discussion and conclusion

The assay published by Lewis and coworkers [5] has been used for determining rifabutin concentrations in most of the pharmacokinetic studies regarding this drug. Such assay has been reported to be very reliable, precise and accurate. However, it involves large amounts of extraction solvents and it requires an intensive sample preparation, since it involves a back-extraction procedure. Our assay was developed on the basis of the one published by Lewis and coworkers [5] in an attempt to simplify the extraction procedures without compromising its performance. A few characteristics of our assay, such as mobile phase and extraction conditions resemble the ones used by Lewis. However, a number of modifications were made which resulted in less preliminary sample work-out.

One of the innovative aspects of our assay is the use of a column (Symmetry Shield RP_8 5 μm, Waters) capable of reducing tailing-effects by means of a polar carbammate bound to the silica bonded-phase packing. Therefore we did not need to use any additive to correct for peak tailing [5–7].

Extraction was made with a liquid–liquid partition method, using Extrelut 3 columns (Merck) which consist of a specially processed wide-pore diatomaceus earth of high pore volume. Such columns decrease emulsion formation and are solvent saving. The usage of such extraction procedure represents a key advantage of our methodology. In fact, it allowed us to obtain a reproducible peak for medazepam, the internal standard, without the need of a back extraction of the ethyl acetate–hexane residue, as in the method published by Lewis [5], in which the recoveries of medazepam were variable and low, unless all of the organic phase was removed from the acidic aqueous extract. Also, Extrelut extraction resulted in a cleaner extract, as it usually does, compared to liquid–liquid extraction. This is evident by comparing our chromatograms with the one reported in previous assays [5–7]. Of note, we had only minor peaks in the HPLC void volume, while previous assays had large peaks eluting at 0–2 min. This was unexpected. It may be that, at the particular conditions used in our assay, most of the molecules eluting in the void volume were extracted.

It has been noted that the amount of 100 μl of rifabutin methanolic solution used for quality controls and standards preparation may be excessive and potentially harmful, since it may result in a higher recovery of rifabutin in quality controls and standards compared to patient samples. In order to address this issue the peak high ratios obtained in a set of quality controls prepared by spiking 980 μl of plasma with 100 μl of rifabutin methanolic solution were compared with a set of quality controls prepared by spiking 980 μl of plasma with 20 μl of solution. The final rifabutin concentrations were 30, 150, 500 ng ml^{-1} ($n=5$ for each quality control in each set). We did not find a statistically significant difference (Mann Whitney *U*-test, unpaired *t*-test). However, quality controls prepared by spiking 20 μl showed a trend for lower peak high ratios. Mean values were 2, 3, and 5% lower than the ones spiked with 100 μl at the QC concentrations of 30, 150, 500

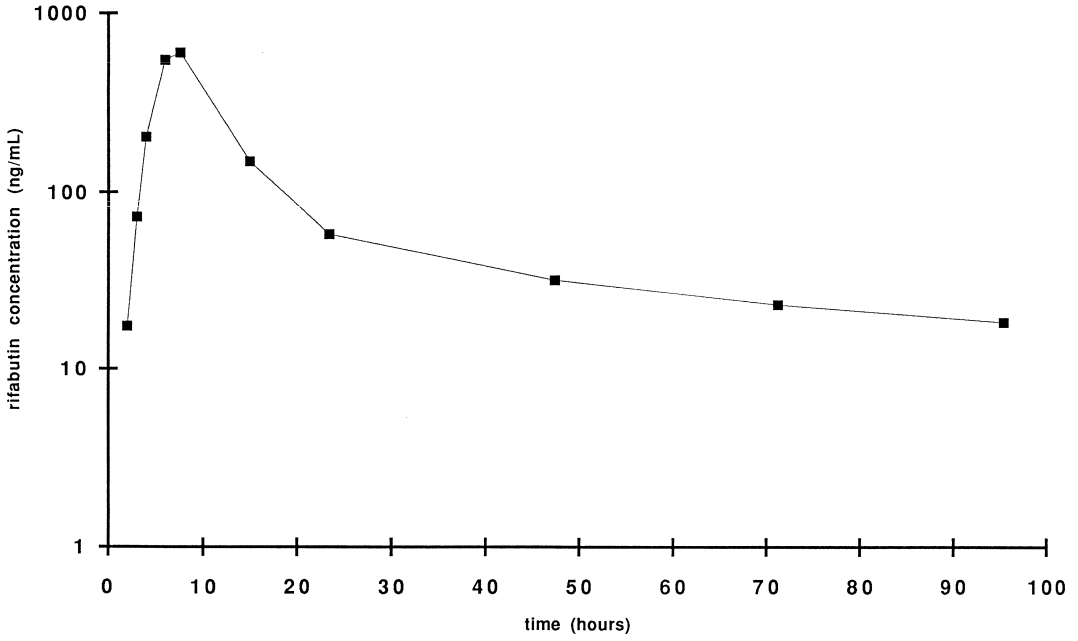

Fig. 3. Pharmacokinetic profile of rifabutin obtained in a patient with wasting syndrome following administration of a single 300 mg dose.

ng ml^{-1}, respectively. Therefore, in order to avoid potential bias in pharmacokinetic studies it is advisable to prepare quality controls and standards spiking 20 μl of rifabutin methanolic solution.

We did not validate our assay for rifabutin major metabolite, 25-*O*-desacetyl rifabutin, since analysis of the metabolite was not included in the objectives of the pharmacokinetic study [8]. However, the assay may be suitable for determination of metabolite concentrations without modification. In fact, at the present conditions the metabolite had a retention time of 3.4 min, as it can be seen in Fig. 2.

The assay has been used in a comparative study of rifabutin absorption and disposition in HIV infected patients with or without wasting syndrome (submitted for publication) [8]. A representative pharmacokinetic profile of rifabutin obtained in a patient with wasting syndrome enrolled in the study mentioned above is shown in Fig. 3. Although none of the patients enrolled in the pharmacokinetic study was receiving protease inhibitors, the assay may be used without modifications for rifabutin studies in patients receiving indinavir, nelfinavir or ritonavir. In fact, the retention time for such drugs was 3.8, 8.8 and 13.9 min, respectively. For patients receiving saquinavir, however, the assay should be modified because this drug retention time was too close to that of rifabutin. The concomitant administration of rifabutin and saquinavir is extremely unlikely, however, since rifabutin decreases saquinavir concentrations to a clinically significant extent [9].

The assay appeared to be reproducible and reliable. The short retention times (4.6 min for rifabutin and 6.4 min for the internal standard) and ease of sample preparation make this assay suitable for future studies of rifabutin pharmacokinetic in the HIV infected patients.

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