

A review on recent advances in the stability study of anti-mycobacterial drugs

Przegląd najnowszych osiągnięć w badaniu trwałości leków przeciwprątkowych

Marta Karaźniewicz-Łada^{A–F}

Department of Physical Pharmacy and Pharmacokinetics, Poznan University of Medical Sciences, Poland

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Address for correspondence

Marta Karaźniewicz-Łada
E-mail: mkaraz@ump.edu.pl

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Abstract

Several factors, including characteristic polymer composition of the cell wall, based on peptidoglycans cross-linked with arabinogalactans, together with the lipid layer contribute to the high resistance of *Mycobacterium tuberculosis* to antibiotics and other anti-tuberculosis drugs, leading to the development of new treatment methods. Implementation of therapeutic drug monitoring for anti-mycobacterial drugs in routine clinical practice requires understanding of the limited stability of these drugs. Rifampicin and isoniazid are the main anti-tuberculosis drugs that generate degradation products during sample handling and storage. Therefore, analytical methods used for analysis of clinical samples collected from tuberculosis patients treated with a combination of different drugs should enable the separation of the studied analytes from their metabolites and degradation products. Moreover, the samples require strictly regulated collection and storage conditions to prevent degradation processes.

The purpose of this review was to present recent data on the stability studies of anti-mycobacterial drugs, specifically used as first-line treatment in patients with tuberculosis. Detailed degradation pathway of rifampicin was described, including conditions influencing the formation of specific rifampicin related substances. Moreover, the results of the stability studies of anti-mycobacterial drugs were presented in various matrices in conditions determined by international guidance such as U.S. Food and Drug Administration (FDA) or International Council for Harmonisation (ICH) guidelines. Particular attention was given to analytical methods designed for analysis of anti-mycobacterial drugs in the presence of their degradation products. Finally, recommendations proposed by different authors for collection, processing and storage of clinical samples to increase stability of anti-mycobacterial drugs were summarized.

Key words: tuberculosis, isoniazid, rifampicin, pyrazinamide, ethambutol

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Streszczenie

Zróznicowane czynniki, w tym charakterystyczny skład polimerowy ściany komórkowej, opartej na peptydoglikanach usieciowanych arabinogalaktanami, wraz z warstwą lipidową, przyczyniają się do wysokiej oporności *Mycobacterium tuberculosis* na antybiotyki i inne leki przeciwgruźlicze, co prowadzi do poszukiwania nowych metod leczenia. Wdrożenie terapeutycznego monitorowania leków przeciwpłatkowych w rutynowej praktyce klinicznej wymaga zrozumienia ograniczonej stabilności tych leków. Ryfampicyna i izoniazyd są głównymi lekami przeciwgruźliczymi, które generują produkty degradacji podczas przygotowywania i przechowywania próbek. Z tego względu metody analityczne stosowane do analizy próbek klinicznych pobranych od pacjentów z gruźlicą leczonych kombinacją różnych leków powinny umożliwiać oddzielenie badanych analitów od ich metabolitów i produktów degradacji. Ponadto próbki wymagają ściśle określonych warunków pobierania i przechowywania, aby zapobiec procesom degradacji.

Celem tego przeglądu jest przedstawienie najnowszych danych na temat badań stabilności leków przeciwpłatkowych, w szczególności tych stosowanych jako leki pierwszego rzutu u pacjentów z gruźlicą. Szczegółowo opisano proces degradacji ryfampicyny z uwzględnieniem warunków wpływających na powstawanie określonych substancji pochodnych ryfampicyny. Ponadto przedstawiono wyniki badań stabilności leków przeciwpłatkowych w różnych matrycach w warunkach określonych przez wytyczne międzynarodowych instytucji takich jak FDA lub ICH. Szczególną uwagę poświęcono metodom analitycznym przeznaczonym do analizy leków przeciwgruźliczych w obecności produktów ich degradacji. Na koniec podsumowano zalecenia zaproponowane przez różnych autorów dotyczące zbierania, przygotowania i przechowywania próbek klinicznych w celu zwiększenia stabilności leków przeciwpłatkowych.

Słowa kluczowe: gruźlica, izoniazyd, rifampicyna, pyrazynamid, etambutol

Introduction

Tuberculosis infections pose a serious threat to the human population. One of the factors influencing the drug resistance of the tuberculosis bacillus is the composition of its cell wall, which contains specific polymers and hydrophobic compounds. The characteristic polymer composition of the cell wall, based on peptidoglycans cross-linked with arabinogalactans, together with the lipid layer protects the bacterium against the influence of xenobiotics.¹ The World Health Organization (WHO) reported that tuberculosis remained the 2nd most frequent cause of death from infection in 2022, just behind coronavirus disease (COVID-19). In addition, this disease caused almost twice as many deaths as HIV/AIDS. Approximately 25% of people in the world are infected with *Mycobacterium tuberculosis* and about 5–10% of them will develop an active form of the disease during their lifetime. Every year, 10 million people suffer from tuberculosis. Most cases of the disease occur in the countries of southern Africa and southeast Asia.² In Poland, 4,314 new cases of tuberculosis were registered in 2022, which means 17.5% of cases more than in the previous year.³ According to the WHO recommendations, first-line treatment should include rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (ETH). These drugs are given in combination to target different enzymes and minimize bacterial resistance.⁴ In cases of treatment resistance, second-line medicines are used, including aminoglycosides, fluoroquinolones (moxifloxacin, levofloxacin), ethionamide, prothionamide, cycloserine, terizidone, and p-aminosalicylic acid.⁵

One of the reasons for the limited effectiveness of tuberculosis treatment are subtherapeutic concentrations of the drugs, which lead to drug resistance and death.⁶ Therefore, therapeutic drug monitoring (TDM) is suggested to individualize dosing for a patient. To ensure

the effectiveness of bactericidal treatment, therapeutic concentration ranges for anti-tuberculosis drugs in plasma or serum have been established. Due to the limited time and resources in the clinic, typically, only 2 samples are collected post-dose: at 2 h, which corresponds to the peak concentration for most anti-tubercular drugs, and at 6 h, which allows for distinguishing between delayed absorption and malabsorption.⁷ High-performance liquid chromatography (HPLC) methods are recommended to analyze concentrations for TDM, and in the case of RIF, chemiluminescence and spectrophotometry can be also applied.⁸

Despite recommendations, TDM is not widely used in countries where tuberculosis is common. The main reasons include high costs, limited availability of analytical instruments including mass spectrometry (MS) or HPLC, difficulties in storing and transporting biological material due to high temperatures, and the need for multiple blood collection, which is problematic, particularly in the case of malnourished children. Therefore, a urine sample was proposed as an alternative to blood for TDM. Among anti-tubercular drugs, RIF is monitored using colorimetry due to the red discoloration of urine in people taking this drug.^{8,9} Recently, a colorimetric method using mobile phone application and standardized light box have been proposed to measure RIF in urine samples for personalized treatment of children with tuberculosis based on predicted RIF levels.¹⁰ However, urinary RIF concentrations were poorly correlated with serum C_{max} , which raises doubts regarding the utility of urine as a matrix useful for TDM of RIF.⁹

Difficulties in TDM may be also caused by limited stability of the drugs. Degradation of RIF depends on pH and leads to the formation of rifampicin quinone (RIF-Q), 3-formyl-rifampicin (3-F-RIF) and 25-desacetyl-rifampicin (25-D-RIF).¹¹ Moreover, limited stability of INH at ambient temperature was confirmed for whole blood, serum and

plasma. Hence, it is recommended to process collected specimen at low temperatures.¹² All RIF compounds except RIF-Q have similar UV-VIS absorbance spectra with maximum absorbance at 474 nm, while the maximum absorption of RIF-Q shifts to 540 nm.¹³ Therefore, when using spectrophotometric methods, the observed absorbance values may not correspond to the actual concentrations of RIF because its products of degradation (3-F-RIF and 25-D-RIF) show absorbance at the same wavelength.

To overcome abovementioned problems with stability, Xing et al.¹⁴ suggested adding ascorbic acid to protect RIF against autooxidation in clinical samples. Pršo et al.¹⁵ suspected that the observed inaccuracy in their assay was caused by RIF degradation and therefore RIF was not included in the measurements. Moreover, liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods are recommended for analysis of several anti-mycobacterial agents due to their higher sensitivity and selectivity compared to other analytical methods. As mentioned by Kuhlin et al.,¹⁶ LC–MS/MS offers adequate separation and fast analysis of multi-analyte samples collected from patients with tuberculosis despite using non-selective procedures for preparation of samples. Nevertheless, among articles focusing on the analysis of anti-mycobacterial

agents in various matrices, very few mentioned separation and detection of degradation products.^{14,15,17–19}

This article focuses on a review of recent advances in anti-mycobacterial drug stability studies, including stability-indicating techniques, analysis of degradation products, and recommendations for sample storage and handling to enhance stability.

Degradation pathway of RIF

The RIF is a lipophilic substance with a partition coefficient log p-value of 2.77 and a pKa of 1.7 and 7.9 related to the 4-hydroxy and 3-piperazine nitrogen, respectively.²⁰ The stability of the drug is pH-dependent. In neutral pH, the drug is stable, whereas in acidic and basic pH, its decomposition was observed (Fig. 1). At low pH, RIF undergoes decomposition to 3-F-RIF, which is poorly soluble and contributes to the reduced bioavailability of RIF. The compound possesses high activity against *Mycobacterium tuberculosis* in vitro but not in vivo. At acidic pH of stomach and in the presence of INH, the degradation process of RIF is even more pronounced. Interaction between RIF and INH leads to the formation of the isonicotinyl

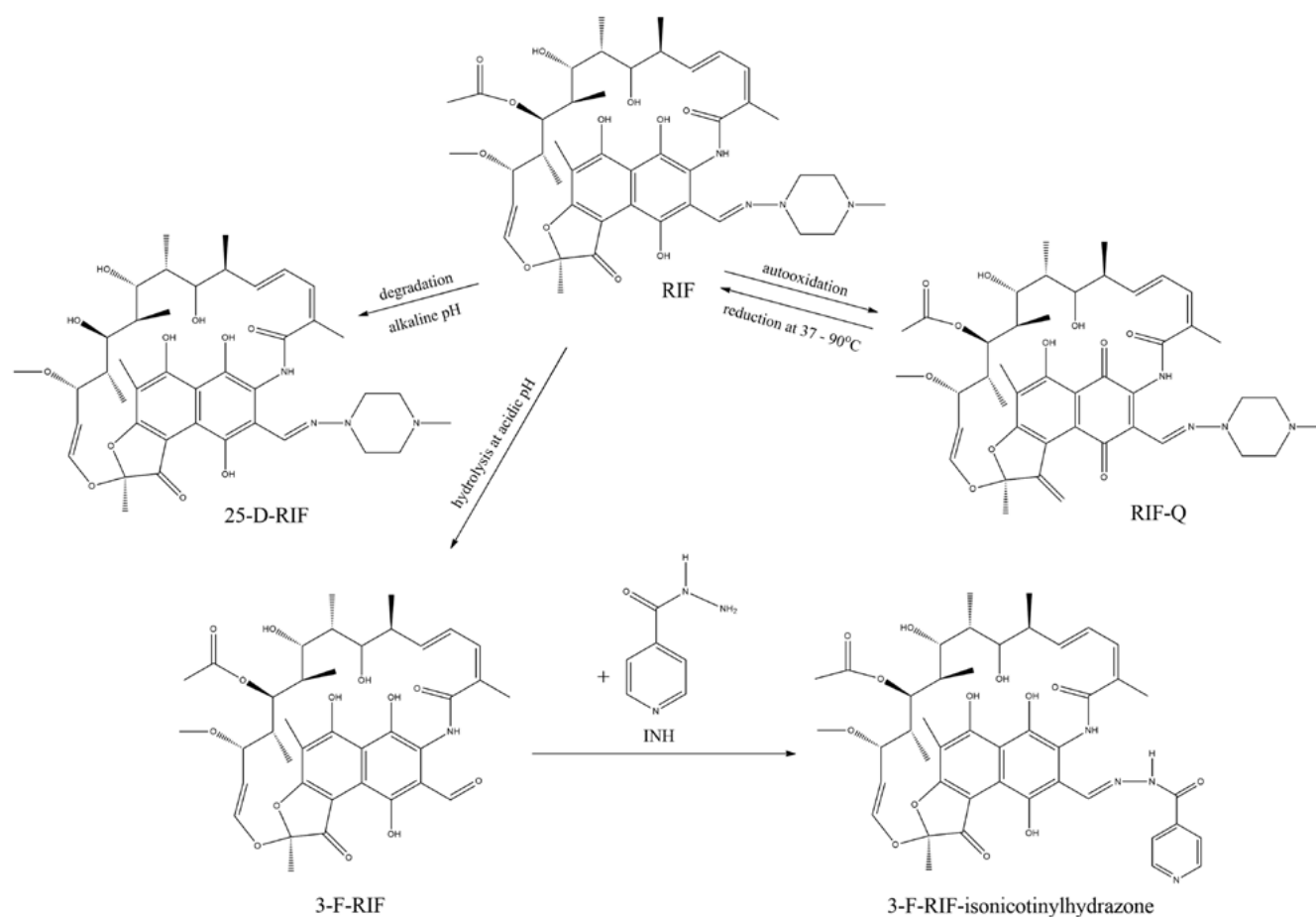


Fig. 1. Degradation pathway of rifampicin (RIF)

RIF-Q – rifampicin quinone; 25-D-RIF – 25-desacetyl-rifampicin; 3-F-RIF – 3-formyl-rifampicin; 3-F-RIF-isonicotinylhydrazone – 3-formyl-rifampicin isonicotinylhydrazone; INH – isoniazid.

hydrazone derivative of 3-F-RIF (3-F-RIF-isonicotinylhydrazone; Fig. 1) and subsequent production of hydrazine and 1-amino-4-methyl-piperazine.¹¹ Sankar et al.²¹ showed that degradation of RIF and INH is caused by interaction between the compounds in fasting pH conditions under which combination of both drugs are administered. At pH 2 (the maximum pH in the fasting condition) and in 50 min, RIF decomposed by approx. 34%, while INH by 10%. The extent of decomposition for RIF and INH ranged between 13–35% and 4–11%, respectively, in several marketed formulations.

At higher pH, deacetylation process of RIF to 25-D-RIF is observed.¹² It is worth mentioning that 25-D-RIF is also the main metabolite of RIF *in vivo*.²² In addition, at the mild alkaline conditions (pH 8.0), RIF is transformed into RIF-Q.¹² The latter is a main compound formed from RIF in a process of nonenzymatic autooxidation.^{13,23,24} The degradation product is regarded as an impurity in RIF samples and an indicator of poor quality of RIF tablets. Sutradhar and Zaman¹³ observed that RIF-Q in solution undergoes chemical conversion to RIF in the presence of microorganisms. This process is temperature-dependent, may result in an increase in antimicrobial activity of RIF, and promotes the development of antimicrobial resistance due to incorrect determination of medicine quality.¹³ Data on potential interconversion between RIF and RIF-Q *in vivo* and the analyte stability in human clinical samples are scarce. Only few articles mentioned the appearance of RIF-Q in plasma and urine of patients treated with RIF.^{17–19} Kivrane et al.¹⁷ noticed the formation of RIF-Q in real samples but did not measure the concentration of the compound. In another report, plasma concentrations of RIF-Q in patients suffering from tuberculosis and being administered the first-line anti-mycobacterial drugs were in the range of 0.114–0.325 mg/L and comprised 2.30–4.50% of the sum of RIF and RIF-Q. Moreover, presence of 3-F-RIF was confirmed in all samples but its concentrations were below the quantification limit of the analytical method.¹⁹ RIF-Q and 3-F-RIF appeared also in urine, suggesting RIF degradation during sample processing and storage. The concentration of RIF-Q was in the range of 0.71–2.62 mg/L, which constituted 3.6–13.2% of the total RIF concentration (RIF plus RIF-Q) and was twice higher than the concentration found in plasma.¹⁸

Stability of anti-mycobacterial drugs

Several guidelines on bioanalytical method validation released by U.S. Food and Drug Administration (FDA), European Medicine Agency (EMA) and International Council for Harmonisation (ICH) provide general principles for the stability testing, including recommendations on the acceptance criteria for stability results and

the duration of stability tests. The chemical stability of the analyzed compound should be proved in stock solutions and biological fluid, and the effects of sample collection, handling and storage of the analyte should be assessed.^{25–27} Hence, most data regarding the stability of anti-mycobacterial drugs were generated during developing and validating methods designed for analysis of these compounds. Several studies confirmed stability of these drugs in stock and working solutions stored for at least 1 month at -80°C ^{28–30} and -20°C .^{18,19} However, significant degradation of INH and RIF was noticed in biological samples. Matrix components were suspected to significantly impact the stability of both compounds. Unsatisfactory benchtop stability was confirmed for INH at room temperature in plasma or serum. Sturkenboom et al.³¹ reported significantly better stability of INH in ultrafiltrate than in plasma, and suggested that plasma proteins might be responsible for INH degradation. The INH stability in samples stored at room temperature for 12 h were reported by some authors.^{15,32} However, other reports indicated that INH undergoes a significant decay at room temperature and is stable for 4 h^{17,33} or as short as for 1 h.^{17,19} There is inconsistency of results obtained in the stability study of RIF in plasma and serum samples. Some studies have shown that RIF remains stable in plasma/serum samples at room temperature for up to 24 h,^{23,34} while other authors have reported lower stability of the compound. Le Guellec et al.³⁵ noticed that RIF undergoes rapid decomposition in plasma at room temperature, and its concentration decreases by 54% within 8 h. Karaźniewicz-Łada et al.¹⁹ reported stability of RIF and 3-F-RIF in plasma samples at room temperature for 4 h, while 25-D-RIF was stable for only 1 h. The same results for 25-D-RIF were obtained by Kivrane et al.¹⁷ However, in the study of Sundell et al.,³⁶ the compound was stable for 4 h of storage. The ambiguous results regarding RIF stability could be partly caused by the fact that RIF is sensitive to light and samples should be protected from light during processing and storage.³⁷ Poor stability of RIF and its derivatives in urine samples was also reported. The compounds were stable for 1 h if urine samples were kept at room temperature.¹⁸ Other anti-bacterial drugs proved to be stable on a benchtop. Most authors noticed that PZA was stable in plasma or serum in room temperature for up to 4 h,^{14,17,19,30,33,36} and in 1 study, the compound was stable for up to 24 h.³¹ In urine samples, stability of INH, PZA and ETH was confirmed during 4 h of storage.¹⁸

There is inconsistency in results of long-term storage of samples containing anti-mycobacterial drugs. In most studies, stability of INH and RIF in plasma or serum samples was confirmed when stored for 1 month^{29,30,38} at -80°C . Longer storage stability of 12 weeks at -80°C was reported by Sundell et al.³⁶ However, Kim et al.³⁸ noticed substantial degradation of the compounds over 12 weeks of storage and confirmed stability for only 4 weeks. Another report indicated stability of RIF in plasma samples kept for

70 weeks at -85°C .³⁹ Such long stability of the compound might be connected to the procedure of sample preparation, which included charcoal-stripping, ultracentrifugation and filtration. As a result, many matrix components of the samples were removed, leading to improved RIF stability. It was proved that higher temperature for long-term storage may cause degradation of RIF and INH within few days; INH was stable for 7-day storage at -20°C ,^{17,19} while RIF stability ranges from <1 week^{19,35} to 3 months.¹⁷ Poor stability of RIF-Q (<1 week) was also confirmed.¹⁹ Other anti-mycobacterial drugs exhibit better stability during long-term storage. Samples with PZA were stable at -20°C when stored for up to 3 months.¹⁷

Freezing and thawing samples is known to affect analyte stability. Both RIF, INH and PZA remained stable after 3 cycles of freezing (at -70°C or -80°C) and thawing at room temperature.^{29,30,36,40} According to Gao et al.,³² INH, PZA and RIF were stable during 3 cycles of freezing at -20°C and thawing at room temperature. In 2 other articles, a significant degradation of INH and RIF was observed under similar conditions.^{17,19}

Abouzid et al.¹⁸ performed extended stability study of anti-mycobacterial drugs in urine considering differences in physiological pH of urine samples ranged from 4 to 8. They proved stability of RIF, RIF-Q, 3-F-RIF, 25-D-RIF, INH, and PZA at pH 6 for 24 h of storage at -20°C , and ETH was stable in all sample pH values. However, after 30 days, significant degradation was observed for RIF-Q and 3-F-RIF in all samples, while other analytes were stable in urine of pH 6–8. Moreover, the stability of RIF, INH, PZA, and ETH were studied in conditions mimicking the urine collection process in medical centers and peripheral clinics at room temperature and at 37.5°C . Abouzid et al. noted that RIF was stable at pH 6–7 for up to 8 h, INH was stable at pH 6–7 for up to 24 h, and both PZA and ETH remained stable at pH 4–8 for up to 24 h. During the stability studies, an increase in the RIF-Q was observed, while RIF concentrations decreased, with this process being significantly accelerated at 37.5°C .¹⁸

Stability-indicating methods

According to the ICH guidance document, a bioanalytical method should be selective and specific enough to detect and differentiate the active substance from other compounds, such as products of degradation found during sample processing.²⁵ Since RIF and INH are the main anti-tuberculosis drugs that generate degradation products during sample handling and storage, the method used in the stability study should enable the separation of all compounds present in clinical samples collected from tuberculosis patients treated with a combination of different drugs. Therefore, the most suitable technique is HPLC because it enables chromatographic resolution

of the analytes from their related substances. This method in combination with MS/MS detection is characterized by high selectivity and specificity, demands low sample volumes and may involve simple preparation techniques such as protein precipitation.¹⁶ There are only few articles on HPLC methods for the determination of RIF and its derivatives in different matrices. Sankar et al.²¹ developed and validated an HPLC with UV detection (HPLC-UV) method for measurements of RIF, INH and their degradation products including 3-F-RIF, RIF-Q and isonicotinyl hydrazone in solutions. After determining validation parameters including linearity, precision and accuracy, this method was applied for the decomposition study of RIF in samples containing INH, in the pH range of 1–3. Sutradhar and Zaman¹³ used HPLC-UV and LC-MS methods to identify RIF as the product of the chemical change of RIF-Q observed upon heating. Both compounds were chromatographically separated and changes in RIF-Q absorbance were measured to track the conversion of the compound for 2 h. However, no validation parameters of the methods were presented. Prasad et al.²⁴ reported an liquid chromatography–diode array detector–mass spectrometry/time-of-flight (LC-DAD-MS/TOF) technique to identify 21 RIF related substances using modern LC-MS tools such as multiple stage MS, high resolution MS and hydrogen/deuterium exchange MS. The method was applied for the detection of the compounds in rat liver microsomes and in rat blood, urine and feces after administration of 50 mg/kg of RIF. The samples were processed prior to the analysis using protein precipitation with acetonitrile, liquid-freeze separation and solid phase extraction. The authors detected 6 known metabolites and degradation products (25-D-RIF, RIF glucuronide, N-demethyl-RIF, 3-F-RIF, RIF-Q, and desacetyl-3-F-RIF) and 15 new RIF related substances. However, their concentrations in biological fluids were not measured. Kivran te al.¹⁷ reported the development and validation of the LC-MS/MS method for simultaneous quantification of first-line antituberculosis drugs (ETH, INH, PZA, and RIF) along with their 6 primary metabolites (25-D-RIF, isonicotinic acid, acetylisoniazid, 5-hydroxypyrazine-2-carboxylic acid, pyrazine-2-carboxylic acid, and 5-hydroxypyrazinamide). They reported 2 RIF peaks as the result of the formation of RIF-Q in plasma samples and used 2 ion-transitions (from 821.4 to 789.3 specific for RIF-Q and from 823.4 to 791.3 specific for RIF) for accurate RIF quantification. Only 2 articles are available on the development and validation of sensitive and selective UPLC-MS/MS methods for the simultaneous analysis of RIF and its metabolite and degradation products – 25-D-RIF, 3-F-RIF and RIF-Q – in the presence of other anti-mycobacterial drugs INH, ETH and PZA in clinical samples.^{18,19} For the measurements, small plasma (20 μL) and urine (10 μL) volumes were required, which were processed using protein precipitation with methanol and

Table 1. Methods for analysis of degradation products of anti-mycobacterial drugs

| Analytical method | Conditions | Analyzed compounds | Application | Reference |
|-------------------|--|---|--|-----------|
| HPLC-UV | column: Supelcosil LC-18-DB (250 × 4.6 mm, 5 µm); mobile phase: 65% methanol and 35% 0.01 M phosphate buffer at pH 7.00; detection: 254 nm | RIF, RIF-Q, 3-F-RIF, INH, isonicotinyl hydrazone | decomposition of RIF in the presence of INH at pH 1–3 | 21 |
| LC-UV, LC-MS | column: C18 (parameters not provided); mobile phase: 80% acetonitrile, 20% water detection: 470 nm | RIF, RIF-Q | evaluation of the effect of temperature on the stability and antimicrobial activity of RIF-Q | 13 |
| LC-UV/DAD-MS/TOF | column: Zorbax C18 (250 × 4.6 mm, 5 µm); mobile phase: acetonitrile, 10 mM ammonium acetate, gradient elution; detection: 254 nm, MS/TOF | RIF, RIF-Q, 3-F-RIF, 25-D-RIF, RIF N-oxide | identification of 21 RIF metabolites and degradation products formed in vitro and in vivo | 24 |
| LC-MS/MS | column: Waters Acquity UPLC BEH C8 (75 mm × 2.1 mm, 1.7 µm); mobile phase: methanol, 0.1% formic acid in water, gradient elution; detection: TQ | RIF, RIF-Q, 25-D-RIF, INH, PZA, ETH and their metabolites | analysis of anti-mycobacterial drugs and their metabolites in plasma | 17 |
| LC-MS/MS | column: Kinetex Polar C18 column (15 × 3 mm, 2.6 µm); mobile phase: 0.1% formic acid in 5 mM ammonium formate and acetonitrile, gradient elution detection: TQ | RIF, RIF-Q, 3-F-RIF, 25-D-RIF, INH, PZA | pharmacokinetic and stability study of RIF, INH, and PZA in plasma samples | 19 |
| LC-MS/MS | column: Kinetex Polar C18 column (15 × 3 mm, 2.6 µm); mobile phase: 0.1% formic acid in 5 mM ammonium formate and acetonitrile, gradient elution detection: TQ | RIF, RIF-Q, 3-F-RIF, 25-D-RIF, INH, PZA, ETH | pharmacokinetic and stability study of RIF, INH, PZA and ETH in urine samples | 18 |
| MLC | column: SPHER-100 C18 (250 × 4.6 mm, 5 µm); mobile phase: 0.15 M SDS-6% 1-pentanol-0.01 M phosphate buffer at pH 7; detection: 337 nm | RIF, RIF-Q | stability study of RIF in solutions and spiked biological fluids | 23 |

MLC – micellar liquid chromatography; RIF – rifampicin, RIF-Q – rifampicin quinone; 3-F-RIF – 3-formyl-rifampicin; 25-D-RIF – 25-desacetyl-rifampicin; INH – isoniazid; PZA – pyrazinamide; ETH – ethambutol; TQ – triple quadrupole tandem mass spectrometer; HPLC-UV – high-performance liquid chromatography with UV detection; LC-MS – liquid chromatography–mass spectrometry; LC-MS/MS – liquid chromatography–tandem mass spectrometry; LC-UV/DAD-MS/TOF – liquid chromatography with UV detection/diode array detector–mass spectrometry/time-of-flight.

further diluted with acetonitrile. For optimal separation of the compounds significantly different in polarity (logP from –0.7 for INH to 2.7 for RIF), Kinetex Polar C18 column and gradient elution were applied, allowing for the total analysis time of 12 min. Both methods were successfully validated in terms of selectivity, linearity and lower limit of quantification, precision and accuracy, matrix effect, carry-over, and stability. The applicability of the methods for analysis of INH, PZA, ETH, and RIF was verified in plasma and urine samples collected from patients with tuberculosis and in the extensive stability study of RIF under various conditions of sample collection and storage.^{18,19}

Another useful analytical technique for studying the conversion of RIF to RIF-Q in plasma and urine is micellar liquid chromatography (MLC), as proved by Mishra et al.²³ The advantage of the method was simple sample preparation consisting of dilution in a micellar sodium dodecyl sulfate (SDS) solution. The method validation was carried out according to the EMA recommendations and tested employing the analysis of RIF in samples collected from tuberculosis patients. Moreover, the authors applied the method for the degradation study of RIF to RIF-Q in solutions and biological fluids spiked with RIF.

The summary of the methods for analysis of degradation products of anti-mycobacterial drugs are presented in Table 1.

Recommendations to increase stability

Limited stability of anti-mycobacterial drugs may impact TDM results. Therefore, results of numerous stability studies of the analytes at various storage conditions were utilized to prepare recommendations for handling the clinical samples. Some articles suggested that antioxidants, e.g., ascorbic acid, should be added after sample collection to protect RIF from autooxidation occurring when the samples are processed or stored.¹⁴ Le Guellec et al.³⁵ indicated better stability of RIF in solutions when stored in higher concentrations and when ascorbic acid was added as a protecting agent. However, Peloquin³⁹ reported no benefits from such stabilization. The author suggested using a specific sample preparation procedure before adding RIF, including charcoal stripping, ultracentrifugation and filtration to remove matrix components that may increase RIF degradation. Mishra et al.²³ indicated that oxidation of RIF to RIF-Q is accelerated

in higher temperatures; thus, storage conditions for samples containing RIF should be strictly controlled. Similarly, INH and ethionamide are not stable in human serum and whole blood at room temperature. Therefore, Peloquin⁴¹ suggested that the blood samples should be promptly centrifuged, the serum harvested and frozen immediately after collection. According to the latest recommendations,¹⁹ centrifugation should be performed at low temperatures (preferably 4°C); samples can be left on the bench at ambient temperature for no longer than 1 h; protection from light is recommended by using amber and non-transparent glass tubes for sample storage and processing. In addition, storage at –80°C for no longer than 1 month, shipment of frozen samples with dry ice and limited sample thawing-freezing cycle number were suggested. Abouzid et al.¹⁸ presented recommendations specifically for urine samples based on the observation that pH of urine and temperature affect the analyte stability. The authors suggested that stability of RIF during TDM can be prolonged to 8 h if the pH of collected urine is maintained in the range of 6–7.

Conclusions

Implementation of TDM for anti-tuberculosis drugs to individualize dosing for a patient requires determination of stability of these drugs in different conditions mimicking the sample collection, processing and storage in clinics. Numerous studies have confirmed the limited stability of RIF and INH in biological fluids, which is probably due to the presence of proteins and other matrix components. Factors that accelerate the decomposition process include higher temperatures during long-term storage, multiple freeze-thaw cycles or acidic and alkaline pH of the sample. Therefore, to increase analyte stability, strictly regulated handling conditions for samples containing anti-mycobacterial drugs have been established. It is increasingly apparent that methods used for determination of anti-tuberculosis agents in real samples should separate the analytes from their degradation products. These conditions are met by LC–MS/MS, which is the most suitable technique for stability studies and pharmacokinetic applications due to its selectivity and specificity.

ORCID iDs

Marta Karażniewicz-Łada  <https://orcid.org/0000-0003-4091-7035>

References

- Brennan PJ. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis*. 2003;83(1–3):91–97. doi:10.1016/S1472-9792(02)00089-6
- World Health Organization (WHO). *Global Tuberculosis Report 2023*. Geneva, Switzerland: World Health Organization (WHO); 2023. <https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2023>. Accessed November 13, 2024.
- European Centre for Disease Prevention and Control (ECDC). Tuberculosis surveillance and monitoring in Europe: 2024–2022 data. Stockholm, Sweden: European Centre for Disease Prevention and Control (ECDC); 2024. <https://www.ecdc.europa.eu/en/publications-data/tuberculosis-surveillance-and-monitoring-europe-2024-2022-data>. Accessed November 13, 2024.
- Sachan RSK, Mistry V, Dholaria M, et al. Overcoming *Mycobacterium tuberculosis* drug resistance: Novel medications and repositioning strategies. *ACS Omega*. 2023;8(36):32244–32257. doi:10.1021/acsomega.3c02563
- Patil K, Bagade S, Bonde S, Sharma S, Saraogi G. Recent therapeutic approaches for the management of tuberculosis: Challenges and opportunities. *Biomed Pharmacother*. 2018;99:735–745. doi:10.1016/j.biopha.2018.01.115
- Hiruy H, Rogers Z, Mbowane C, et al. Subtherapeutic concentrations of first-line anti-TB drugs in South African children treated according to current guidelines: The PHATISA study. *J Antimicrob Chemother*. 2015;70(4):1115–1123. doi:10.1093/jac/dku478
- Peloquin C. The role of therapeutic drug monitoring in mycobacterial infections. *Microbiol Spectr*. 2017;5(1):5.1.03. doi:10.1128/microbiol.spec.TNMI7-0029-2016
- Xie YL, Modi N, Handler D, et al. Simplified urine-based method to detect rifampin underexposure in adults with tuberculosis: A prospective diagnostic accuracy study. *Antimicrob Agents Chemother*. 2023;67(11):e00932-23. doi:10.1128/aac.00932-23
- Zentner I, Schlecht HP, Khensouvan L, et al. Urine colorimetry to detect low rifampin exposure during tuberculosis therapy: A proof-of-concept study. *BMC Infect Dis*. 2016;16(1):242. doi:10.1186/s12879-016-1576-1
- Szipszky C, Van Aartsen D, Criddle S, et al. Determination of rifampin concentrations by urine colorimetry and mobile phone readout for personalized dosing in tuberculosis treatment. *J Pediatric Infect Dis Soc*. 2021;10(2):104–111. doi:10.1093/jpids/piaa024
- Shishoo CJ, Shah SA, Rathod IS, Savale SS, Kotecha JS, Shah PB. Stability of rifampicin in dissolution medium in presence of isoniazid. *Int J Pharm*. 1999;190(1):109–123. doi:10.1016/S0378-5173(99)00286-0
- Singh S, Mariappan TT, Shankar R, Sarda N, Singh B. A critical review of the probable reasons for the poor variable bioavailability of rifampicin from anti-tubercular fixed-dose combination (FDC) products, and the likely solutions to the problem. *Int J Pharm*. 2001;228(1–2):5–17. doi:10.1016/S0378-5173(01)00754-2
- Sutradhar I, Zaman MH. Evaluation of the effect of temperature on the stability and antimicrobial activity of rifampicin quinone. *J Pharm Biomed Anal*. 2021;197:113941. doi:10.1016/j.jpba.2021.113941
- Xing Y, Yin L, Le X, et al. Simultaneous determination of first-line anti-tuberculosis drugs and one metabolite of isoniazid by liquid chromatography/tandem mass spectrometry in patients with human immunodeficiency virus-tuberculosis coinfection. *Heliyon*. 2021;7(7):e07532. doi:10.1016/j.heliyon.2021.e07532
- Pršo K, Žideková N, Porvazník I, Solovič I, Mokry J, Kertys M. A high-throughput LC–MS/MS method for simultaneous determination of isoniazid, ethambutol and pyrazinamide in human plasma. *Rapid Commun Mass Spectrom*. 2023;37(2):e9425. doi:10.1002/rcm.9425
- Kuhlin J, Sturkenboom MGG, Ghimire S, et al. Mass spectrometry for therapeutic drug monitoring of anti-tuberculosis drugs. *Clin Mass Spectrom*. 2019;14:34–45. doi:10.1016/j.clinms.2018.10.002
- Kivrane A, Grinberga S, Sevostjanovs E, et al. LC–MS/MS method for simultaneous quantification of the first-line anti-tuberculosis drugs and six primary metabolites in patient plasma: Implications for therapeutic drug monitoring. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2021;1185:122986. doi:10.1016/j.jchromb.2021.122986
- Abouzid M, Kosicka-Noworzyń K, Karażniewicz-Łada M, et al. Development and validation of a UPLC–MS/MS method for therapeutic drug monitoring, pharmacokinetic and stability studies of first-line antituberculosis drugs in urine. *Molecules*. 2024;29(2):337. doi:10.3390/molecules29020337
- Karażniewicz-Łada M, Kosicka-Noworzyń K, Rao P, et al. New approach to rifampicin stability and first-line anti-tubercular drug pharmacokinetics by UPLC–MS/MS. *J Pharm Biomed Anal*. 2023;235:115650. doi:10.1016/j.jpba.2023.115650

20. Khan MF, Rita SA, Kayser MdS, et al. Theoretically guided analytical method development and validation for the estimation of rifampicin in a mixture of isoniazid and pyrazinamide by UV spectrophotometer. *Front Chem*. 2017;5:27. doi:10.3389/fchem.2017.00027
21. Sankar R, Sharda N, Singh S. Behavior of decomposition of rifampicin in the presence of isoniazid in the pH range 1–3. *Drug Dev Ind Pharm*. 2003;29(7):733–738. doi:10.1081/DDC-120021772
22. Seng KY, Hee KH, Soon GH, Chew N, Khoo SH, Lee LSU. Population pharmacokinetics of rifampicin and 25-deacetyl-rifampicin in healthy Asian adults. *J Antimicrob Chemother*. 2015;70(12):3298–3306. doi:10.1093/jac/dkv268
23. Mishra P, Pawar RP, Bose D, et al. Stability studies of rifampicin in plasma and urine of tuberculosis patients according to the European Medicines Agency guidelines. *Bioanalysis*. 2019;11(8):713–726. doi:10.4155/bio-2018-0174
24. Prasad B, Singh S. In vitro and in vivo investigation of metabolic fate of rifampicin using an optimized sample preparation approach and modern tools of liquid chromatography–mass spectrometry. *J Pharm Biomed Anal*. 2009;50(3):475–490. doi:10.1016/j.jpba.2009.05.009
25. European Medicines Agency (EMA). ICH M10 on bioanalytical method validation: Scientific guideline. Amsterdam, the Netherlands: European Medicines Agency (EMA); 2023. <https://www.ema.europa.eu/en/ich-m10-bioanalytical-method-validation-scientific-guideline>. Accessed November 17, 2024.
26. European Medicines Agency (EMA). Bioanalytical method validation: Scientific guideline. Amsterdam, the Netherlands: European Medicines Agency (EMA); 2012. <https://www.ema.europa.eu/en/bioanalytical-method-validation-scientific-guideline>. Accessed November 17, 2024.
27. U.S. Food and Drug Administration (FDA). Bioanalytical method validation Guidance for industry. Rockville, USA: U.S. Food and Drug Administration (FDA); 2018. <https://www.fda.gov/files/drugs/public/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf>. Accessed November 17, 2024.
28. Notari S, Mancone C, Sergi M, et al. Determination of antituberculosis drug concentration in human plasma by MALDI-TOF/TOF. *IUBMB Life*. 2010;62(5):387–393. doi:10.1002/iub.321
29. Baietto L, Calcagno A, Motta I, et al. A UPLC–MS–MS method for the simultaneous quantification of first-line antituberculars in plasma and in PBMCs. *J Antimicrob Chemother*. 2015;70(9):2572–2575. doi:10.1093/jac/dkv148
30. Zhou Z, Wu X, Wei Q, et al. Development and validation of a hydrophilic interaction liquid chromatography–tandem mass spectrometry method for the simultaneous determination of five first-line antituberculosis drugs in plasma. *Anal Bioanal Chem*. 2013;405(19):6323–6335. doi:10.1007/s00216-013-7049-0
31. Sturkenboom MGG, Van Der Lijke H, Jongedijk EM, et al. Quantification of isoniazid, pyrazinamide and ethambutol in serum using liquid chromatography–tandem mass spectrometry. *J Appl Bioanal*. 2015;1(3):89–98. doi:10.17145/jab.15.015
32. Gao S, Wang Z, Xie X, et al. Rapid and sensitive method for simultaneous determination of first-line anti-tuberculosis drugs in human plasma by HPLC–MS/MS: Application to therapeutic drug monitoring. *Tuberculosis*. 2018;109:28–34. doi:10.1016/j.tube.2017.11.012
33. Luyen LT, Hung TM, Huyen LT, et al. Simultaneous determination of pyrazinamide, rifampicin, ethambutol, isoniazid and acetyl isoniazid in human plasma by LC–MS/MS method. *J Appl Pharm Sci*. 2018;8(9):61–73. doi:10.7324/JAPS.2018.8910
34. De Velde F, Alffenaar JWC, Wessels AMA, Greijdanus B, Uges DRA. Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography–tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877(18–19):1771–1777. doi:10.1016/j.jchromb.2009.04.038
35. Le Guellec C, Gaudet ML, Lamanetre S, Breteau M. Stability of rifampin in plasma: Consequences for therapeutic monitoring and pharmacokinetic studies. *Ther Drug Monit*. 1997;19(6):669–674. doi:10.1097/00007691-199712000-00011
36. Sundell J, Bienvenu E, Birgersson S, Äbelö A, Ashton M, Hoffmann KJ. Simultaneous quantification of four first line antitubercular drugs and metabolites in human plasma by hydrophilic interaction chromatography and tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2019;1105:129–135. doi:10.1016/j.jchromb.2018.10.027
37. Hartkoorn R, Khoo S, Back D, et al. A rapid and sensitive HPLC–MS method for the detection of plasma and cellular rifampicin. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007;857(1):76–82. doi:10.1016/j.jchromb.2007.07.005
38. Kim HJ, Seo KA, Kim HM, et al. Simple and accurate quantitative analysis of 20 anti-tuberculosis drugs in human plasma using liquid chromatography–electrospray ionization–tandem mass spectrometry. *J Pharm Biomed Anal*. 2015;102:9–16. doi:10.1016/j.jpba.2014.08.026
39. Peloquin CA. Rifampin stability. *Ther Drug Monit*. 1998;20(4):450–451. doi:10.1097/00007691-199808000-00017
40. Wu L, Ye Z, Liu H, et al. Rapid and highly sensitive quantification of the anti-tuberculosis agents isoniazid, ethambutol, pyrazinamide, rifampicin and rifabutin in human plasma by UPLC–MS/MS. *J Pharm Biomed Anal*. 2020;180:113076. doi:10.1016/j.jpba.2019.113076
41. Peloquin CA. Therapeutic drug monitoring in the treatment of tuberculosis. *Drugs*. 2002;62(15):2169–2183. doi:10.2165/00003495-200262150-00001