Bio-evaluation of Poly-Lactate co-Glycolic Acid (PLGA) nanoparticles loaded with radio-labeled rifampicin.

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January 27, 2023

Abstract

The poly lactate co-glycolic acid (PLGA) nanoparticles of tubercular drugs have been demonstrated to have sustained release profile over seven days. There is no information on the location or mode of release of these nanoparticles in living system. Therefore, we have planned the study to explore the pharmacokinetics and biodistribution of PLGA rifampicin nanoparticles in healthy human volunteers. Rifampicin was labeled with 99mTc by indirect method and nanoparticles were prepared by a single emulsion evaporation method. To investigate the pharmacokinetics and biodistribution of nanoparticles, a single dose of 450 mg of rifampicin was administered orally to healthy human volunteers divided into two different groups. Following a single oral dosage of the rifampicin nanoformulation, the PK parameters were significantly different between the nanoparticle and conventional groups AUC (113.8 vs 58.6), MRT (16.2 vs 5.8) and Ke (0.04 vs 0.10). Also SPECT/CT images revealed biodistribution of nanoparticles in the distal portions of the intestine, which is consistent with our dosimetry analysis. Significant difference in PK parameters and bio-distribution of nanoparticles in spleen and lymph nodes with maximum deposition were observed in large intestine. Nanoparticle distribution pattern may be advantageous for the treatment of intestinal or lymph node TB and has the potential to result in a lower dose of rifampicin nanoformulation for the treatment of pulmonary TB.

Title page

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1. Radio-labeling and dosing were done in the Department of Nuclear Medicine before being transferred and admitted to the Clinical Pharmacology Unit (CPU) at the PGIMER Chandigarh India.
2. Principle Investigator: Dr. Samir Malhotra, Head and professor Department of Pharmacology PGIMER Chandigarh India had direct clinical responsibility for healthy volunteers.
3. Key words: Pharmacokinetics, Biodistribution, Nanoparticles, TB

The MS/MS parameters
Q1-823.4, Q3-791.4, CE-25, DP-70, EP-10 and CXP-8.
Assessment of eligibility for enrollment

Inclusion Criteria

1. Normal healthy male volunteers.
2. Age 18 - 45 yrs.
3. Willing to give written, informed consent
4. Willing to comply with protocol requirements
5. Normal physical & systemic examination
6. Biochemistry & hematology (within 15% of laboratory range will be acceptable)
7. No abnormality detected on X-ray chest & ECG
8. No laboratory evidence of hepatic, renal or cardiac dysfunction

Exclusion criteria

1. History of having donated blood in the past 3 months.
2. HIV or HBs Ag positivity
3. History of receiving any enzyme-inducing agent in the past 15 days
4. History of participating in any IND study in last 6 months
5. History of participating in any non-IND study in the past 3 months
6. History of allergy or hypersensitivity to any medication
7. History of peptic disorder
8. Chronic smoker >6 cigarettes/beedies per day
9. Alcoholic (history daily alcohol intake in last 6 months)
10. History of drug abuse
11. History of any chronic disease and taking medication
12. History of taking non-allopathic drug in the past 6 months for any illness
13. History of any major illness in past (viz. TB, hepatic, nephritis)

Abstract

The poly lactate co-glycolic acid (PLGA) nanoparticles of tubercular drugs have been demonstrated to have sustained release profile over seven days. There is no information on the location or mode of release of these nanoparticles in living system. Therefore, we have planned the study to explore the pharmacokinetics and biodistribution of PLGA rifampicin nanoparticles in healthy human volunteers.

Method

Rifampicin was labeled with $^{99m}$Tc by indirect method and nanoparticles were prepared by a single emulsion evaporation method. To investigate the pharmacokinetics and biodistribution of nanoparticles, a single dose of 450 mg of rifampicin was administered orally to healthy human volunteers divided into two different groups.

Results

Following a single oral dosage of the rifampicin nanoformulation, the PK parameters were significantly different between the nanoparticle and conventional groups AUC (113.8 vs 58.6)***, MRT (16.2 vs 5.8)** and $K_e$ (0.04 vs 0.10)*. Also SPECT/CT images revealed bio-distribution of nanoparticles in the distal portions of the intestine, which is consistent with our dosimetry analysis.

Conclusion

Significant difference in PK parameters and bio-distribution of nanoparticles in spleen and lymph nodes with maximum deposition were observed in large intestine. Nanoparticle distribution pattern may be advantageous for the treatment of intestinal or lymph node TB and has the potential to result in a lower dose of rifampicin nanoformulation for the treatment of pulmonary TB.

INTRODUCTION
Tuberculosis (TB), often regarded as a disease of the past, is still one of the leading causes of mortality among adults and children. Each year, globally, around 10.4 million individuals develop TB infection, while 1.8 million people die due to this disease.

Among the arsenal of anti-tubercular drugs, rifampicin is one of the most effective agents. It is the only mycobactericidal drug that kills both dividing as well as non-dividing bacteria, and can eliminate up to 99% of tubercle bacilli within two months of commencement of TB treatment. Rifampicin is also used against meningococcal meningitis, “methicillin-resistant Staphylococcus aureus” (MRSA), Neisseria gonorrhoeae, Haemophilus influenzae, and Legionella pneumophila infections. Unfortunately, mycobacterium responds slowly to rifampicin, resulting in prolonged infectiousness or acquired drug resistance, as well as increasing the cost on public health systems by extending treatment duration.

Sustained therapeutic drug levels can be achieved using controlled-release nanoformulation of rifampicin. A number of biodegradable polymers have been tried for the purpose of drug encapsulation. Of these, poly lactic-co-glycolic acid (PLGA) is the most widely used because of its favorable safety profile, bio-compatibility and degradation characteristics. It is possible to change the overall physical properties of the polymer-drug matrix by controlling the relevant parameters, such as polymer molecular weight, ratio of lactide to glycolide, and drug concentration, to achieve a desired release profile. However, the potential toxicity associated with dose dumping, inconsistent release, and distribution of PLGA nanoformulation is still unclear and requires further evaluation. Radiolabeled pharmaceuticals and mass balance excretion studies are being increasingly conducted to track the bio-disposition of drugs as well the pharmaceutical excipients inside the body.

PK studies carried out on rifampicin and other anti-tubercular drugs by our group have revealed that nano-encapsulation in PLGA provides a sustained release for up to one week after single-dose oral administration in animals. However, the exact pathway of biodisposition of PLGA nanoparticles loaded with rifampicin in human remains unknown. Non-invasive in-vivo imaging (SPECT/CT) techniques are integral part of biodistribution and PK/PD studies. Therefore, we planned to evaluate the PK profile and detailed biodisposition of radio-labeled rifampicin entrapped in PLGA nanoparticles.

MATERIALS AND METHODS

Materials

PLGA (50:50) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rifampicin was purchased from HiMedia, India, and PVA (MW: 30000-70000 Da, 88% hydrolyzed) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (ACN; LCMS grade) and dichloromethane (DCM) were purchased from Merck Ltd. (Mumbai, India). Dialysis bags (cut-off of 12 kDa) were purchased from HiMedia, India.

Preparation and characterization of drug-loaded nanoparticles

Rifampicin-loaded nanoparticles were prepared through single emulsion evaporation method, as previously standardized, with slight modification in preparation of radio-labeled rifampicin nano-particles. DCM was evaporated in rota-evaporator for 15 minutes and drug loading was assessed by using a well counter present in the Nuclear Medicine Department in our institute.

Drug estimation by LCMS-MS

Levels of rifampicin in the formulation and plasma samples were assessed using LCMS/MS (SCIEX triple Quad 35000). Mobile phase consists of ACN and 5mM ammonium formate with 0.1% formic acid for ionization in a ratio of 80:20, flow rate 1ml/minute, coloum C18(Nucleosil-100-3, 150/4.6 mm, 3μm) and injection volume 10μl.

Validation of LCMS-MS method

Accuracy, RSD, intra-batch, and inter-batch variability were calculated using six samples from each lower limit of quantification quality control (LLOQ-QC), lower quality control (LQC), and middle quality control
In-vitro release study

The in-vitro release of the drug-loaded nanoparticles was conducted by the dialysis membrane method. The dialysis membrane with molecular weight cut-off of 12 to 14 kDa was used.

Radiolabeling of rifampicin and optimization of radiolabeling using $^{99m}$Tc tricarbonyls

Rifampicin was labeled by indirect method to study the long-term in-vivo distribution and retention of rifampicin in the body. This method of radio-labeling was carried out in two steps, as described by Alberto et al., (101).

Assessment of quality of radio-labeled rifampicin.

The HPLC method was used to investigate the labeling of rifampicin. Analysis was performed on the HPLC (Shimatzu, SPD 2A LC-20AD), using a reverse phase ultrabase-C18 column (250mm4.6mm, 5m particle size). The mobile phase ratio was 70:30 v/v of 0.05M disodium phosphate and ACN at 1 ml/min flow rate, 25°C oven temperature, and 335 nm wave length.

Radiochemical purity testing

The radio-labeling efficiency or radiochemical purity of the labeled product were determined using paper chromatography. Paper chromatography was performed on Whatman paper #3 using acetone and saline as mobile phase. Labeling efficiency was calculated using a ITLC scanner by selecting area under the single/more than one peak.

Stability

Stability of rifampicin-$^{99m}$Tc was assessed in PBS for up to 12 h. Rifampicin-$^{99m}$Tc (0.5ml) was incubated in PBS (0.5ml). ITLC was performed in both normal saline and acetone at 1 h, 2 h, 3 h, 4h, 6h and 12 h.

Biodistribution of labeled rifampicin in animals

The Institutional Animal Ethical Committee approved the study, and the treatment of the animals was carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals’ recommendations (CPCSEA). Wistar rats (n=12) weighing 150-250 g were orally administered 4mg/kg rifampicin dissolved in 1ml of vehicle containing 100-200 μCi of activity of $^{99m}$Tc. “Tissues and organs were excised from the rats following sacrifice by cervical dislocation at” pre-defined time points of 0.5h, 4h, and 8h post-oral administration of radiolabeled rifampicin. “Three rats were used for each time point. The radioactivity in each organ/tissue was measured” using a flat-type solid scintillation counter (15-cm diameter) with a NaI(Tl) detector. The percentage of administrated activity (%AD) in each organ as well as per g of the tissue” was calculated. “Blood, bone, and muscle weight were taken as 7%, 6.5%, and 40% of the body weight, respectively”.

Pharmacokinetics and bio-distribution of PLGA nano-partials of rifampicin in healthy human volunteers.

A prospective, open-label, non-randomized, single-dose study, approved by our Institutional Ethics Committee (IEC) and Institutional Bio-Safety Committee (IBSC). Registered in the Clinical Trials Registry-India (CTRI/2022/02/040372). This study was conducted to characterize the bio-distribution and pharmacokinetics of a single oral dose of radiolabeled PLGA nanoparticles loaded with rifampicin, containing approximately 5-10 mCi of radioactive material, administered to normal healthy volunteers. Urine and blood were collected, and images were acquired to study pharmacokinetics (PK) and biodistribution till the end of the study.

The following parameters were assessed

1. Total and relative excretion of radioactivity in urine and blood following a single 450-mg oral solution dose of PLGA loaded with rifampicin
2. Pharmacokinetic parameters, such as AUC (0-t), C_{max}, half-life, t_{max}, of orally administered radiolabeled rifampicin

3. Distribution pattern of radiolabeled PLGA loaded with rifampicin by means of gamma camera scan at various time intervals

**Image acquisition**

Images were acquired periodically after oral administration of radiolabeled rifampicin and radiolabeled rifampicin nanoparticles under gamma camera at various time intervals (0h, 1h, 2h, 7h, 12h, and 24h post-administration) to study the distribution of radiolabeled rifampicin nanoparticles inside the body. Images were acquired on a SPECT/CT gamma camera equipped with high energy collimator. A standard of known activity (~200 μCi in 50 ml syringe) was placed at the lowest uptake area and was imaged with the patient.

**Sample collection for pharmacokinetics of rifampicin and bio-distribution of radio-labeled rifampicin**

Participants remained at the clinical site from 10h prior to the administration of the study drug on day 1 till “the completion of all post-dose sample collections on day 8. Blood was collected at 0 (pre-dose), 0.5-1, 2", 6, 24, 48, 72, 96, 120, 144, and 168h after dose from an indwelling IV catheter. Time and date of dose administration, exact volume of the sample were noted. The blood samples were divided into two aliquots of volume 2 ml, one for radio-active counts another for the analysis of rifampicin from whole blood. “Participants were asked to void their bladder in the morning prior to dosing, from which the pre-dose urine sample was obtained. After dose administration, participants were asked to note the total volume of urine whenever they voided the bladder. One ml of urine and 1 ml of blood samples were stored for radioactivity measurement. The time-activity curve was plotted using the OLINDA software.

**STATISTICAL ANALYSIS**

Continuous normal data was expressed as mean and SD/median and range. Categorical data was expressed as percentage. Comparison of the continuous data was made by un-paired student’s t-test for normal date and Mann-Whitney U test for non normal data. P < 0.05 was considered as significant. Analysis was done by using GraphPad Prism-6.

**RESULTS**

**Drug encapsulation efficiency**

Nanoparticles were developed by single emulsion evaporation method with Polydispersity and zeta-potential. The majority of the nanoparticles produced under optimal conditions were in the 475–136 nm size range (Table.1 and 2). Characteristics of radio-labeled nanoparticles were in agreement with un-labeled nanoparticles (Table.3).

**Surface morphology**

The surface morphology of empty and rifampicin-entrapped PLGA nanoparticles was studied using SEM, and both nanoparticles were found to be the same (Fig. 1A and 1B)

**LCMS/MS estimation of rifampicin and validation used for in vivo and in vitro analyses**

The assay was robust, accurate, and repeatable concerning international recommendations ICH. The deviation between and within the run did not exceed ±20% of the mean concentrations in the LCMS-MS (Table.4).

**In-vitro drug release studies of rifampicin nanoparticles**

An initial burst release 20% in 4 hours due to the surface-associated drug as part of the biphasic release profile. Complete release was observed in SIF, while only a 65 percent release was identified in SGF. (Fig-2).
Indirect method of radio-labeling using tri-carbonyl as conjugating agent

In the indirect method, at optimal reaction conductions, around 97% of radio-labeling yield was obtained (Fig.3A and 3B). The labeling was also validated with HPLC method, peak at 3.370 minutes is anticipated to be the labeled rifampicin peak (Fig.4).

Optimization of radiolabeling parameters of rifampicin with $^{99m}$Tc tricarbonyls

Maximum radio-labeling yield was achieved by increasing the amount of rifampicin to 500 µg, pH at 6.5-7, incubated at 80°C for 30 minutes in reaction 1 and 100°C for 40 minutes in reaction 2. When the preparation was stored at room temperature, there was no significant product degradation up to 12 h. Radionuclide ($^{99m}$Tc) used for labeling was passed through a low gamma energy filter made of lead, cadmium, and copper to reduce the radiation from $^{99m}$Tc and high energy photons from common impurities, such as Ru-103, I-131, and Mo-99. Single peak at 70th channel was found, which corresponds to 140 KeV energy of $^{99m}$Tc. Therefore, radionuclide ($^{99m}$Tc) used for labeling was found without any impurity.

Biodistribution of radio-labeled rifampicin in animals.

Throughout the duration of the trial, the rats were in good health. Animals “exhibited normal feed consumption” and was well-tolerated. There was no influence on body weight during the trial period, and the body weight was unrelated to dose amounts.

Figure 5 represents the distribution of labeled rifampicin in percentage administered dose per gram of organ (%AD/g). Approximately 2.7%, 1.24%, 0.58%, 0.5%, and 0.99% drug was found in lungs, liver, thyroid, kidneys, and stomach, respectively, at 30 minutes after oral administration of 4mg/kg rifampicin containing 100-200 µCi of activity. The highest percentage of radio-activity was found in the stomach because of the oral administration of labeled rifampicin, which acts as the source organ. At 4 and 8 h, the percentage of activity in the lungs starts to decrease. Subsequently, the radio-activity in the kidneys and liver starts to increase. The peak percentage of activity in the lungs was found at 30 min and decreased over 8 hours post oral administration of labeled rifampicin (Fig.5). Thus, attachment of $^{99m}$Tc to the rifampicin molecule does not affect the PK of rifampicin.

Animal pharmacokinetics and tissues distribution of PLGA nanoparticles loaded with rifampicin

In an animal study, a peak plasma concentration of 3µg/ml was found at 12 h and a trough concentration of 0.3µg/ml post-single-dose oral administration of rifampicin nanoparticles (Fig.6A). Tissue levels were obtained high in lymph nodes at 4.5µg/ml at 3rd hour and 12.6µg/ml at 6th-hour post single oral administration, spleen drug concentration was found 3.2µg/ml at 6th-hour post-dosing (Fig.6B).

Pharmacokinetics of rifampicin PLGA nanoparticles compared to conventional formulation in healthy human volunteers

The PLGA nanoformulation of rifampicin showed difference in release profile compared to convention formulation over 24 hours. The plasma level of rifampicin in nanoformulation was significantly higher over 5 to 15 hours, and drug levels in plasma were detectable over five days but were below the limit of quantification (BLQ) of 250 ng/ml (Fig.7).

Significant difference in some pharmacokinetic parameters and PK/PD indices were found between nanoformulation of rifampicin as compared to conventional rifampicin (Table. 5 and 6).

Biodistribution of PLGA loaded with radiolabeled rifampicin compared with conventional radiolabeled rifampicin

No focal uptake in any organ has been found in the conventional rifampicin arm, but blood pool activity was present at all scanning time points. Over the period, radioactivity in the body started to diminish and was completely dull 24 hours after administering the dose (Fig. 8A). Blood pool activity was not found in the nanoformulation group over 24 hours of post oral administration of single dose of 450mg rifampicin.
Nanoparticles were seen in different gut segments at different pre-defined time points and finally accumulated in the large intestine. (Fig. 8B). In the nanoformulation of the rifampicin arm, peri-splenic uptake was seen in all healthy volunteers (Fig. 8C).

Comparison of the temporal distribution of radio-pharmaceutical between conventional and nano-rifampicin formulations.

The number of counts in the nanoformulation group was found higher over 24 hours as compared to convention rifampicin group (Fig. 9).

Presence of percentage administrated activity in blood and urine in healthy human volunteers

Percentage activity of $^{99m}$Tc-rifampicin per gram of blood and urine in nanoformulation of rifampicin is significantly high in blood and less in urine as compared to conventional rifampicin (Fig. 10A and 10B)

Discussion

Patient noncompliance is a significant barrier to successful TB management. According to a recent meta-analysis, patients who missed 10% or more of their doses contributed to poor treatment outcomes (113). The risk of mortality, morbidity, and drug resistance increases dramatically in those who do not show adherence to the TB treatment (15). Noncompliance with TB treatment is multifaceted, but pill burden and dosing frequency play a significant role (114). As a result, there is an urgent need for new formulations and fixed dose combinations (FDC) that can provide all drugs in one pill with reduced dosing frequency.

Our center has worked on PLGA nano-formulation of anti-tubercular drugs for the treatment of pulmonary tuberculosis (10). The nanoformulation has been evaluated for oral and inhalation administration in animals and a phase one ascending dose study is ongoing (14). A sustained release profile and better therapeutic effect were been seen (14). However, there is not a single human study of this or similar formulation for the evaluation of pharmacokinetics, pharmacodynamics and biodistribution profile. Before conducting Pharmacodynamics studies in humans, we require complete evaluation of safety, pharmacokinetics and biodistribution profile in humans (16). Therefore, we have elucidated the biodistribution pattern of PLGA loaded rifampicin nanoparticles in humans under gamma camera by using radiolabeled rifampicin. We exclusively tested rifampicin in this investigation because we wanted to get accurate results free from drug interference. Another reason for using only rifampicin was that it is used to treat *Meningococcal meningitis, Methicillin-Resistant Staphylococcus Aureus (MRSA), Neisseria gonorrhoeae, Haemophilus influenzae*, and *Legionella pneumonia* infections in addition to tuberculosis.

Radio-pharmaceuticals have an emerging role in diagnosis, therapeutic, or in both (theranostics). They have also been used in biodistribution and mass-balance studies to see the fate of a drug in a living system non-invasively. In this study, we labeled rifampicin by indirect method with $^{99m}$Tc to track the distribution of nanoparticles in healthy human volunteers. The various parameters have been standardized to obtain the highest radiochemical purity. When 500 $\mu$g of rifampicin, 15mCi activity, pH ranges between 6.5-7, was incubated at 80°C for 30 minutes in step one and at 100°C for 40 minutes in step two, more than 97 percent radio-labeling yield was obtained. Animals were used to evaluate the labeled rifampicin for polarity and distribution pattern changes. The labeled rifampicin was found in every organ, with the highest concentration in the stomach, followed by the lungs, liver, and intestines, which is consistent with previous findings (17).

In our study, we have evaluated rifampicin PLGA nanoparticles because we wanted to study how the drug will behave when given as a nano-formulation. This information would be applicable to other anti-tubercular drugs too. A significant difference in plasma level of rifampicin between the 5 to 15 hours of oral administration in nanoformulation of rifampicin group compared to conventional group (Fig. 7). In nanoformulation group, the plasma levels of rifampicin were detectable over five days but were below the limit of quantification (BLQ) of 250 ng/ml. We have done the confirmatory animal PK and biodistribution studies, in which, again, we got the plasma level of the drug in BLQ. The reason for limited sustained release could be dissipation of nanoparticles with feces after 24 hours which is average gut transit time (18). In addition to this, significant
drug concentration was observed in lymph nodes and spleen on the day sixth after administration. This presence of drug could be attributed to the smaller sized (100 nm or less) nanoparticles, absorbed through the para-cellular spaces from GI track\(^{1920}\). Further in lymph nodes the drug concentrations detected was also high i.e. up to 12 \(\mu\)g/ml on 6\(^{th}\)-day. The possible explanation could be the denominator effect as the lymph nodes harvested from each animal were 200-300 \(\mu\)gs of weight. A significant difference in the PK parameters in the two groups have been found, as elimination constant in nanoformation has been decreased, which could have increased the other parameters like \(t_{1/2}\), AUC, AUMC, and MRT\(^{21}\). The significant difference in AUC/MIC and percentage T>MIC could be because of sustained release behavior of nanoformulation.

The free movement of nanoparticles throughout the GIT indicates major proportion of nanoparticles remained un-absorbed. Nanoparticles were started to get accumulated in the distal parts of the GI tract from the 5\(^{th}\) hour of oral administration, which is in concordance with gastro-illic transit time. Lucia et al. 2019 have done an ex-vivo study of PLGA-PEG nanoparticles loaded with a fluorescent dye, rhodamine B, and the anti-cancerous drug paclitaxel. In that study, they saw the nanoparticles were in the gut lumen and started accumulating in the large intestines as time increased after oral administration.

They have also explored the absorption pattern of nanoparticles in the GI track, persistent luminal localization of nanoparticles has been seen at different time points\(^{22}\). Same pattern has been seen in our study, we found nanoparticles were get accumulated in large intestines gamma camera and showed the release of drugs from there. The higher counts in the intestines and lower counts in blood and urine over the 24 hours in the nano-formulation group as compared to the conventional group confirm the accumulation of nanoparticles in the large intestines.

In conclusion, our study has shown the bio-distribution and pharmacokinetic profile of PLGA nanoparticles in humans. Nanoparticles get accumulated into distal parts of the gut from where the encapsulated drug is released in a sustained release manner. Although the sustained release of seven days was not observed in healthy human volunteers, an increase in pharmacokinetic parameters in the nanoformulation group could be utilized to decrease the dose of ATT for the treatment of pulmonary tuberculosis.

Acknowledgment:

This work was funded by ICMR, New Delhi, India.

Conflict of interest

We don’t have any conflict of interest.

Data availability and statement

Research data are not shared


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Fig.docx available at https://authorea.com/users/580337/articles/621446-bio-evaluation-of-poly-lactate-co-glycolic-acid-plga-nanoparticles-loaded-with-radio-labeled-rifampicin

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