Development and Validation of Analytical Methods for Newer Antitubercular Formulation along with Pharmacokinetic Study

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By

Mr. Devangkumar Bhagvandas Tandel

Enrollment No.: 169999901010

Under Supervision of

Dr. Kalpana Patel,

Ph. D. Supervisor, Professor & Vice-Principal

Pharmaceutical Quality Assurance Department

Anand Pharmacy College

Anand, Gujarat, India.

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1. Title of Thesis and Abstract

1.1. Title of Thesis

Development and Validation of Analytical Methods for Newer Antitubercular Formulation along with Pharmacokinetic Study

1.2. Abstract

Mycobacterium tuberculosis, which most frequently infects the lungs, is the cause of the life-threatening dangerous disease known as tuberculosis (TB). *Streptomyces mediterranei* is used to make the semi-synthetic antimicrobial rifampicin. It exhibits a broad spectrum of antibacterial activity, including activity against different Mycobacterium species. Some people who take antituberculosis medications have hepatotoxicity, which can end in rapid liver failure and death. Such occurrences restrict the therapeutic application of medications, which contributes to treatment failure and may result in antibiotic resistance. To overcome the above problems inclusion of herbal bioenhancer like Quercetin improves the bioavailability and reduces the adverse effect associated with the present tuberculosis treatment. In view of the aforementioned facts, the goal of the research study was to improve rifampicin's dissolution when combined with quercetin as a bioenhancer. Hence, two formulation development approaches for combination were liquisolid compacts delivered by oral route and targeted delivery to lungs via drug powder inhaler. Using Design Expert software, the crucial characteristics of liquisolid compact, solubility and percent cumulative drug release were further examined, and an optimum batch was then chosen based on the desirability function. The combination of Rifampicin and Quercetin being novel none of the analytical and bioanalytical methods were reported. Hence, analytical methods UV-spectrophotometric (Q-absorbance ratio and PLS chemometric), HPTLC were developed. Also, bioanalytical method was developed for estimation of drugs in rat plasma, lung's homogenates and BAL fluid using UPLC-MS/MS and HPLC technique. Dry powder inhalers (DPIs) are highly effective for delivery to lungs. Polymeric nanoparticles are advantages in for delivering drugs to the lungs due to their physicochemical stability, better drug loading efficiency, and, most significantly, their capacity to target drugs precisely to the site of action. Additionally, polymeric nanoparticles for respiratory administration, characteristics like size of particles, shape, and porosity might be easily modified. With the use of 3^2 full factorial designs, RIF and QUE nanocrystals formulated were then tested for in-vitro functionality and in-vivo respiratory pharmacokinetic. For pharmacokinetic study quick and accurate HPLC-PDA technique was

developed and validated according to USFDA guideline for quantification of quercetin and rifampicin loaded nanoparticle formulation in rat plasma, lungs homogenate and BAL fluid.

2. A brief description on the state of the art of the research

Dissolution of the drug, which is largely influenced by drug solubility, has an impact on bioavailability(1,2). The rate limiting step of the absorption process is dissolution since the majority of recently developed drugs are insoluble in water [BCS (class-II)](3). As a result, absorption is inadequate. For improving oral bioavailability, a very promising alternative for quick and thorough medication dissolution is Liquisolid technology(4). It is a straightforward, easily scaled-up, and inexpensive formulation technique for enhancing the rate of dissolution of poorly water-soluble drugs(5). The drug particles in the Liquisolid compact are dissolved in a non-volatile liquid vehicle before being combined with the right carrier and coating excipients to create a non-adherent, free-flowing, and compressible powder mixture by being caught within the excipients' intrinsic matrix. A coating of carrier particles begins to form once the liquid drug has completely saturated the interior matrix. The surplus liquid that was left on the carrier material's surface is further absorbed by the coating layer, leaving the entire system dry and free-flowing(6). The liquisolid approach has produced encouraging results for a variety of medications. Therefore, the Liquisolid compacts that contain insoluble drugs encourage dissolution by facilitating the wetting process by increasing the net effective surface area, which leads to an increase in the availability of the drug for dissolution media(7).

Recent research has shown that pulmonary drug delivery is a non-invasive and alluring method for treating a variety of illnesses, particularly those that damage the lungs. Pulmonary drug administration may be more advantageous than parenteral or oral approaches(8). A quick start to the drug's effect is ensured by the enormous lung tissue contact area and its plentiful bloodstream, which create the optimal condition for faster drug penetration into the circulatory system(9). Additionally, a customised delivery system to the pulmonary significantly raises the therapeutic ratio by lowering potentially serious detrimental effects and increasing effectiveness of therapy(10). Additionally, the lungs exhibit no or little first pass metabolism due to their very modest local metabolic activity. Another target for respiratory tract infections therapy is alveolar macrophage. Potential particle-based pulmonary systems to increase bioavailability of drugs and treatment effectiveness for the deep lung is significant(11).

3. Problem Definition

In 2021, an unexpected 10.6 million new instances of tuberculosis were reported. Geographically speaking, Africa and Asia bear the brunt of tuberculosis(12). Nearly 40% of

tuberculosis cases worldwide are reported in China and India. From the literature review on the subject related to Tuberculosis and their available therapies, the challenges associated with the current therapies were identified, and from the review emerged the research problem to be addressed(12).

Rifampicin, a broad-spectrum antibiotic, belongs to the first line of treatment. It acts by binding to the β-subunit of RNA polymerase, the enzyme responsible for transcription and expression of mycobacterial genes, resulting in inhibition of the bacterial transcription activity and thereby killing the organism(13). An important characteristic of rifampicin is that it is active against actively growing and slowly metabolizing (non-growing) bacilli. Rifampicin is a BCS Class II drug according to Biopharmaceutical Classification System (BCS), with low water solubility and high permeability, showing variable bioavailability due to its in vivo dissolution and is subject to food effects(14).

Current therapies for Tuberculosis cause hepatotoxicity in certain people prompting intense liver disappointment, which brings about death. Such instances limit the clinical utilization of medications, adding to treatment disappointment that conceivably causes drug opposition(15). Additionally, these drugs might cause adverse effects such as neurotoxicity, ototoxicity, tetchiness, nephrotoxicity, GI poisoning and CNS damage. RIF's bioavailability is also only about 50–60%, making formulation strategy for boosting solubility and subsequently bioavailability a necessity(15). In order to combat the aforementioned problems, using a herbal bioenhancer like quercetin will increase bioavailability and lessen the negative effects of the existing medical therapy, and Quercetin has additionally anti-inflammatory and anti-oxidant effect, reported as per literature(16,17).

Hence, the aim of the present investigation was to design, fabricate and evaluate of Liquisolid and Dry powder inhaler formulation of Rifampicin and Quercetin to treat systemic and pulmonary tuberculosis.

4. Objective and Scope of work

In line of the above notion, the overall objectives of the research study are summarized as:

1. Development and Validation of HPTLC method for simultaneous estimation of Rifampicin and Quercetin using DoE in robustness study.
2. Development and Validation of Chemometric-assisted (PLS) UV-spectrophotometric method and Q-absorbance ratio method for simultaneous estimation of Rifampicin and Quercetin in developed formulation.

3. Formulation and Optimization of Rifampicin and Quercetin Loaded Liquisolid Compact: In-Vitro and In-Vivo Study using DoE approach.
4. Rifampicin and Quercetin Dry powder inhaler formulation for Pulmonary Tuberculosis: Optimization, In-vitro and In-vivo characterization using DoE approach.
5. Bioanalytical method Development and Validation for Rifampicin and Quercetin in rat plasma using UPLC-MS/MS.
6. Bioanalytical method Development and Validation for Rifampicin and Quercetin in rat's lung homogenate, BAL fluid and blood plasma using RP-HPLC.

Scope of the research work:

With reference to the above-mentioned objectives the research study focuses on development of two newer anti-tubercular formulations, analytical and bioanalytical methods. By developing the liquisolid formulation to improve the dissolution profile of both drugs ultimately to improve and enhance the absorption and bioavailability at the site of action. By preparing Dry powder inhaler formulation we can effectively deliver the drug to the alveoli portion of lung to improve the targeted therapy, reduce dose and its frequency, reduce side effects, heal the damaged lung tissue, effective management and eradication of organism from the lungs. After clinical trials and fulfilment of other regulatory requirements, the developed formulation may prove to be a boon to the society at large for the complete treatment of the most contagious disease Tuberculosis.

5. Original contribution by the thesis

The entire work in this synopsis , as well as thesis is original. Extensive literature review was done to identify the challenges associated with the existing treatment for tuberculosis and approaches which can resolve them. The combination of Rifampicin and Quercetin is new and no one has performed any research work on this combination. None of the analytical and bioanalytical methods were reported so, the research study was to develop and validate Q-absorbance UV-spectrophotometry, PLS-chemometric and HPTLC analytical methods with validation as per ICH guideline. Additional bioanalytical method was developed and validated using USFDA guidance by RP-HPLC and UPLC-MS/MS method for estimation of developed formulation. Design, development and evaluation of Liquisolid compact and Dry powder inhaler formulation for Rifampicin and Quercetin in combination to treat, cure and effective management of tuberculosis was probably yet not investigated by any other researcher.

6. Methodology of research, results / comparisons

6.1. Design of Experiment

Literature survey was done to identify and determine the quality target product profile (QTPP), critical quality attributes (CQA), manufacturing procedures for Liquisolid and Dry powder formulation, various process and formulation attributes having effect on product CQA. For Liquisolid formulation investigation were done by preliminary screening for various non-volatile solvents, carrier and coating material. Different preliminary experiments were performed for selection of non-volatile solvents which may directly influence the dissolution profile of drug and by applying D-optimal mixture design the composition of non-volatile solvents were optimized. Different ratio of carrier and coating material were investigated to adsorb the liquid on powder based on liquid load factor and its capacity. For Dry powder formulation, optimization was done by preliminary screening for various polymer and surfactant. Different preliminary experiments were performed for selection of polymer and surfactant. To optimize the concentration of polymer and surfactant including process parameters like homogenization speed 3^2 full factorial design was applied. The compatibility of the drug-excipients was checked before the optimization of various process and formulation variables for both formulations. Finally, the developed formulations were investigated by developed analytical methods in pharmaceutical formulation and in various biological fluids.

6.2. Analytical Methods

Three analytical methods Q-absorbance ratio UV-spectrometric, PLS-chemometric and HPTLC methods were developed for estimation of Rifampicin and Quercetin in developed formulations.

6.2.1. HPTLC method

The present research study focuses on the development of the high-performance thin-layer chromatographic method using the design of experiment approach in robustness study for the simultaneous estimation of Rifampicin and Quercetin. Chromatographic separation was performed on Aluminium plates precoated with silica gel 60 F_{254} using chloroform : methanol : formic acid : ethyl acetate : benzene (4:1.8:0.3:1.7:1, v/v/v/v/v) as optimized mobile phase. A fractional factorial design was applied for the robustness study and the independent variables selected were mobile phase composition (A), solvent front (B), chamber saturation time (C), and wavelength (D). It was statistically revealed that the volume of ethyl acetate affected the R_f of both drugs resulting in stricter control of ethyl acetate volume compared to the other three variables. R_f for Rifampicin and Quercetin was 0.35 and 0.71 at 347 nm. The linear

concentration range was 100–600 ng/band for both drugs. The % recovery ranged between 95.25% and 96.71% for Rifampicin and 95.33%–96.86% for Quercetin. The method was validated by determination of linearity, precision, accuracy, and specificity according to Q2(R1) guidelines, and the % relative standard deviation values were less than 2% for both drugs. A simple, accurate, and reproducible high-performance thin-layer chromatographic method was developed, for routine quality control testing of pharmaceutical formulation and acid degradation study of Rifampicin(18,19).

6.2.2. Chemometric-assisted (PLS) spectrophotometric method

To simultaneously determine quercetin and rifampicin in raw material and liquid-solid formulation, a numerical technique built on spectroscopic data associated with partial least squares (PLS) multi - variate measurement is suggested in this research work. Rifampicin and quercetin spectra were captured at concentrations that fell within the 2–10 µg/ml linear range for each drug. They were used to compute 25 simulated mixtures containing 16 calibration and 9 validation sets, with wavelength spacing of λ of 15 nm in hydrochloric acid (0.1 M) and phosphate buffer pH-6.8. The wavelength ranges were 200 to 630 nm. Based on the validation and calibration data's (RMSE) root mean square errors, the models' appropriateness was determined. Recovery studies (%) and relative prediction errors were used to compare and contrast the analytical capabilities of various chemometric techniques. The recovery study's findings showed that this technique was successfully used to formulate pharmaceuticals without excipient interference. The suggested technique is quick, easy to use, and can be utilised as a replacement for traditional analytical tools in the formulation and quality control of pharmaceuticals.

6.2.3. Q-absorbance ratio spectrophotometric method

The method for determining rifampicin and quercetin simultaneously in combination in two dissolution media pH-6.8 phosphate buffer and 0.1 M Hydrochloric acid is described in current study as being straightforward, sensitive, quick, precise, accurate and affordable. Utilizing the proportion of absorbances at two chosen wavelengths one being λ_{max} of one of the two components and the other being isoabsorptive point is the Q-absorbance ratio method. At 420 and 411 nm, respectively, in pH-6.8 phosphate buffer and 0.1 M Hydrochloric acid, rifampicin and quercetin exhibit an isoabsorptive point. The second wavelength, which corresponds to quercetin's λ_{max} in pH-6.8 phosphate buffer and 0.1 M Hydrochloric acid, is 368 and 367 nm. According to the ICH guideline (Q_2R_2), the methods were validated for accuracy, linearity, precision, detection limits and quantification limits. Both Rifampicin and Quercetin showed linearity between 2–12 µg/ml. Utilizing the absorbances proportion at the Quercetin's λ_{max} and

isoabsorptive point, the drug concentrations were calculated. The recovery trials using the conventional addition methodology further confirmed the accuracy and validity of the suggested method. Because the liquisolid excipients did not interfere with the measurement and, it was effectively used and applied to pharmaceutical dosage forms. Statistics and recovery studies have been used to validate the research findings(20).

6.3. Liquisolid compact formulation of Rifampicin and Quercetin

The main goal was to develop liquisolid compacts containing rifampicin and quercetin for enhanced gastrointestinal absorption and dissolution behaviour. Propylene glycol, PEG 200, and Tween 20 were chosen as ideal non-volatile liquid carriers to formulate the required formulations because of their increased drug solubility. In order to create a free-flowing, compressible powder, the liquisolid formulations were then combined with a carrier and coating material. In order to create liquisolid powders with good flow ability, Avicel pH-102, Aeroperl 200, and Aerosil 200 demonstrated good liquid retention potential values, demonstrating their efficiency as solid carrier and coating materials. The medication and carrier had no discernible interaction, according to FT-IR spectra. The DSC and PXRD experiments proved that the crystalline form of the drugs was absent from the liquisolid powders. Furthermore, the improved drug dissolving performance in liquisolid systems revealed that the drug had changed into a molecular or amorphous state. The capacity of non-volatile liquid vehicles of liquisolid systems to increase the gastro intestinal absorption and dissolution rate of Rifampicin and Quercetin was disclosed by in-vivo rat pharmacokinetic experiments, which also showed an improvement in drug absorption from formulation(21).

6.4. Dry Powder Inhaler formulation of Rifampicin and Quercetin

The current study mainly focuses on localized lung delivery of an antitubercular Rifampicin and Quercetin employing dry powder respirable nanoparticles as a non-invasive respiratory approach to target the alveoli macrophage. By using the rotary evaporation process, poly (lactic acid-co glycolic acid) nanoparticles loaded with quercetin and rifampicin were formulated and to optimize, a (3^2) full-factorial approach was used. Morphology, entrapment efficiency, particle size, and investigation of drug-excipient incompatibility of the optimized nanoparticles were all performed. An Andersen cascade impactor was also used to check the optimized formula's aerosolization performance. The optimized formulation was also tested for in-vivo pulmonary study. The entrapment efficiency was found to be >75% for both medications, and the optimized nanoparticles of rifampicin and quercetin had irregular shapes with particle sizes of 281 and 278 nm, respectively. The 70% favourable fine particle fraction in the optimized formula ensures that powders could be inhaled. The $AUC_{0\text{-}24h}$ area under the curve for

customized nanoparticles was 1.5 times greater than the one for pure medicine, demonstrating that lung tissue is where they prefer to concentrate in vivo. Additionally, compared to pure drug, the formulation showed improved prolonged drug residency in the lung for up to 24 hours after inhalation. Inhalable nanoparticles of rifampicin and quercetin may be a potential therapeutic strategy for enhancing patient adherence to therapy while also helping to effectively eradicate tuberculosis(22).

6.5. Bioanalytical UPLC-MS/MS method

UHPLC-MS/MS was utilised to quantitatively analyse Rifampicin and Quercetin loaded Liquisolid compact in rat plasma. The UPLC Acquity C_{18} (1.7 µm, 2.1 x 15 mm) column and 0.5 ml/min were used to separate the analyte. In a low-pressure gradient mode, A: 0.1 % formic acid in water and B: 0.1 % formic acid in acetonitrile as the mobile phase. Sample pre-treatment was performed by protein precipitation technique with methanol and acetonitrile (1:1) from rat plasma. As an internal standard (IS), the analyte was found by tracking precursor-to-product ion transformations of 823 → 791.3 m/z for rifampicin, 303 → 257 m/z for quercetin, and 138→121 m/z for isoniazid in MRM mode. For precisions, accuracy, linearity, specificity and analyte recovery, the proposed technique was validated and were determined to be within the acceptable range. After oral administration of Liquisolid compact and pure drug solution, a almost equivalent extent of absorption was found between liquisolid and marketed formulation, the method's suitability for determining the pharmacokinetic profile of each drug was tested(23,24).

6.6. Bioanalytical RP-HPLC method

A rapid and sensitive reversed-phase high-performance liquid chromatography (HPLC) method using protein precipitation extraction technique has been developed for the quantitative determination of Rifampicin and Quercetin in rat plasma, BAL fluid and lung homogenate. Ofloxacin was used as Internal standard. The method was validated according to US FDA guidelines. Separation was achieved using a Phenomenex Luna-C_{18} (150 × 4.60 mm, 5 µm) column and mobile phase composed of disodium hydrogen phosphate buffer 20 mM, adjusted to pH 4.5 with orthophosphoric acid : methanol in a ratio of 35:65 (v/v) showing retention time 3.54, 5.23 and 8.18 min for Ofloxacin, Quercetin and Rifampicin, respectively. The combination of 500 µL of acetonitrile and 500 µL of methanol showed extraction recovery, >85 % for all the drugs from three different biological fluid. This extraction procedure afforded clear samples resulting in convenient and cost-saving procedure and showed good linear relationship ($r > 0.9958$) between peak area ratio and concentration. The results of pharmacokinetic study showed that significant amount of Rifampicin and Quercetin from

prepared Dry Powder Inhaler formulation was reached in to the lungs compared to the blood circulation. In conclusion, the developed RP-HPLC method with simple ultraviolet detection offered a number of advantages including good quantitative ability, wide linear range, high recovery, short analysis time as well as low cost(25,26).

7. Achievements with respect to objectives

The oral route is the most preferred means of the drug administration due to the higher patience compliance and low cost of production. The drug must be in solution form for absorption through gastrointestinal tract when given orally. In case of poorly soluble drugs like Rifampicin and Quercetin, dissolution is the rate-limiting step in absorption process. The concept of liquisolid compact was developed initially from powdered solution technology that can be used to formulate liquid medication. So, different non-volatile liquids, carrier and coating material etc. were screened and statistically analyzed for proper selection. A dry, non-adherent, free-flowing and compressible powder mixture was developed from liquid medication. And it was successfully evaluated for In-vitro and In-vivo study. The developed formulation shows more absorption compare to pure API and marketed formulation.

The dry powder inhalers (DPIs) are potential drug delivery systems that could locally introduce the anti-tuberculosis agent to the lung. Compared to the metered-dose inhalers (MDIs), DPIs are more environmentally friendly due to freedom from chlorofluorocarbon (CFC) propellants. Sustaining drug release by employing biodegradable polymers in the fabrication of particles could improve the therapeutic outcomes and reduce adverse effects. In the present study, various polymers like poly lactic co-glycolic acid (PLGA, 75:25), chitosan, Polyvinyl pyrrolidone were tried to entrap the drug and modulate the release profile of drug from formulation. Various surfactant like Tween 20, Poloxamer 407 were tried to suspend the nanoparticles. Nanoprecipitation and Rotary evaporation technique were applied to prepare nanoparticles. Finally, rotary evaporation technique with PLGA and Poloxamer 407 nanoparticles was prepared and the particle size found was desirable with good aerodynamic properties, with sustained release of drug up to 24 hr. Further the nanoparticles were successfully evaluated for In-vitro and In-vivo study. The developed dry powder formulation showed that of higher amount of drug reaches to alveoli with greater retention time, sustained release of drug from formulation. Other advantages includes reduction of dose, dose frequency and side effects associated with the drug.

For quantification of Rifampicin and Quercetin various Analytical methods like Q-absorbance ratio and Chemometric assisted PLS UV-spectrophotometric and HPTLC method

were developed and validated according to ICH (Q_2R_2) guideline. Two Bioanalytical method, UPLC-MS/MS and RP-HPLC method were developed and validated according to USFDA guideline. The above methods were successfully applied for simultaneous estimation of both drug in formulation as well as biological fluid.

8. Conclusion

With the present investigations, it may be concluded that the first approach of formulation, liquisolid technique can be a tempting method for enhancing the dissolution profile of medications with high dose requirements and low water solubility. The drug release was enhanced by the addition of a non-volatile solvent and surfactant in a liquisolid compact. The use of the fractional factorial design approach has shown that different combinations of PEG 200, Propylene glycol, and Tween 20 have a substantial impact on drug release from the formulation. The better dissolution profile of the liquisolid compact was presumably caused by the medication being in an amorphous state or being molecularly disseminated in the liquisolid compact, according to the results of DSC and XRD investigations. The second approach for formulation development was DPI and rotary evaporation was used to successfully create PLGA nanoparticles that were rifampicin and quercetin loaded. When used as a DPI for lung administration, the formulated nanoparticles exhibited the desirable features. Additionally, in vitro, aerosol performance tests on the improved formulation indicated that the drug was deposited deep in the lungs. Additionally, in vivo tests demonstrated the extended lung residency time and prolonged release of the drug from the manufactured PLGA-nanoparticles at the infection site (lung), increasing the likelihood of effectively treating tuberculosis while also lowering dose frequency. Rapid, sensitive and selective various analytical and bioanalytical methods were developed and successfully employed for quantification of Rifampicin and Quercetin in pharmaceutical formulation and biological fluids.

9. Copies of papers published and a list of all publications

9.1. Papers Published

1. Tandel D, Patel K, Thakkar V, Gandhi T. Formulation and Optimization of Rifampicin and Quercetin Laden Liquisolid Compact: In-Vitro and In-Vivo Study. Int J Pharm Res Allied Sci. 2022;11(4):75-86. https://doi.org/10.51847/wibIR5NRzg
2. Tandel D, Patel K, Thakkar V, Gandhi T. Chemometric-Assisted UV-Spectrophotometric Method for Quantification of Novel Anti-Tubercular Liquisolid Formulation. Int J Pharm Res Allied Sci. 2023;12(1):17-25. https://doi.org/10.51847/W70eZEZtYs

3. Tandel D, Patel K, Thakkar V. Validated high-performance thin layer chromatographic method for simultaneous quantification of rifampicin and quercetin in liquisolid formulation using fractional factorial design in robustness study. Sep Sci plus. 2023;6: 2200087. https://doi.org/10.1002/sscp.202200087
4. Devang B. Tandel, Kalpana G. Patel, Vaishali T. Thakkar, Amar A. Sakure & Tejal R. Gandhi (2023): Bioanalytical Method Development and Validation for Determination of Rifampicin and Quercetin in Rat Plasma by UHPLC-MS/MS: Applications to Pharmacokinetic Study, Analytical Chemistry Letters, 13. https://doi.org/10.1080/22297928.2022.2162830

9.2. Patent Filed

Title: Liquisolid formulation of Rifampicin and Quercetin

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The Patent Office Journal No. 48/2022

9.3. Oral/Poster Presentation

- Devang Tandel (Oral Presentation) et al., 2020. "Development and Validation of UHPLC-MS/MS for Quantification of Novel Combination of Rifampicin and Quercetin in Rat Plasma", presented in Two-day International Conference on "Molecular structure and Instrumental Approaches" on Virtual platform, sponsored by Gujarat Council on Science and Technology, Govt. of Gujarat, on 26th – 27th November, 2020 organized by Department of Chemistry, School of Science, RK University, Rajkot.
- Devang Tandel (Poster Presentation) et al., 2022. "Inhaled Rifampicin And Quercetin Dry Powder Inhaler Formulation For Pulmonary Tuberculosis: Optimization, In-Vitro And In-Vivo Characterization", presented in AICTE sponsored International Conference on "Potential of Engineered Technology and Novel drug delivery system in health care" by Faculty of Pharmacy, Parul University on 9th & 10th December, 2022, Vadodara.

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