ISBN: 978-81-909047-9-7, p-ISSN:2249-2569, e-ISSN:2320-2955

"THE GNOSIS"
DIGITAL EDITION

INTERNATIONAL RESEARCH JOURNAL OF HUMANITIES, ENGINEERING & PHARMACEUTICAL SCIENCES

(An International Registered Research Journal)

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IJHEPS/JANUARY 2016

TABLE OF CONTENT

ARTICLE	TITLE/AUTHOR
1	CONDITION OF TEACHING ENGLISH IN RURAL & BACKWARD AREAS OF CHANDIGARH, INDIA & IT'S MALADIES
2	HPLC-ASSAY OF RIFAMPICIN IN HUMAN PLASMA: APPLICATION FOR PROTEIN BINDING STUDY

ARTICLE

1

CONDITION OF TEACHING ENGLISH IN RURAL & BACKWARD AREAS OF CHANDIGARH, INDIA & IT'S MALADIES

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Abstract:
With the globalization and the present day demand of the job market, it has become not only important but mandatory to be equipped with the language called English. It would not be wrong to say that the use of this language is going to make the things different for the youth and the people who seek better earning and living opportunities anywhere in the globe. It has become the prerequisite for the success. However, it is observed that the students living in the rural and the other backward areas of the society are not fully equipped with the use of this language.
Teachers and teaching facilities in such areas are very less or in other words they are not up to the level that is required. Moreover, the teachers are also not able to inculcate the environment that will help in the teaching learning of the language. Teaching this language should be targeted in such a way that it should facilitate the student and make him independent enough to use it efficiently and effectively to find himself settled in the situation.
Lack of infrastructure and the modern teaching aids are one of the major flaws of the system. On the other hand lack of innovation and efficiency on the part of the teacher make this problem worse. This study aims at finding the prevailing conditions of the schools in rural areas and neglected colonies of Chandigarh and the effective tools that can be used to make the condition of rural youth good in this arena. The need of the hour is to take onus of the responsibility to teach English to the youth and devise such techniques to yield the better results. The responsibility of the teacher has increased from the old pedagogy of lecture method to use new techniques to make the students confident enough to use it for their survival in the present day job culture.

Introduction

Education is the major activity that enables the individual to live his life in a balanced way to achieve the goals of life. Today with the change in the objectives of the education the main focus of education, today lies in the development of the learner to make him capable enough to earn good and lead a healthy life thereafter. Education enables the man to get a good job, in other words the main objective what a lay man thinks is to get a job after finishing the studies. Education is considered as the most important invention of mankind (Sekhon, singh, sukhwinder & kaur, Amardeep, Philosophical and sociological bases of education, Kalyani Publishers, Jallandhar,2010,pp-1.1)

In today's environment where people struggle for the job, it is the primary responsibility of the education, in fact school to make the student capable of getting job according to his caliber. It has been observed that in today's society when competition is increasing daily, English has become the most important language to grab a good job. It has also become a tool to succeed in the professional life today. The people having good knowledge of this language in addition to the subject matter of their respective field take them to the next level very soon. It opens several opportunities for them.

On the contrary the people particularly from the rural and backward areas are having insufficient knowledge of English language are still struggling for the better opportunities. Despite of their good hold on their work area, they are not able to get good success in the professional life. The earning opportunities in their life are restricted to the local level with less money and facilities at all. English is considered as the most important tool to tackle the job market. It is therefore of utmost importance to examine the system in which people live for several years hoping for the best future. Yes, it is the education system that shoulders the responsibility to create the competent workforce. Education can create the prudent and skilled youth, so that one can lead a healthy life in the years to come.

It has been made mandatory for the state to provide free and compulsory education to all under Right to Education. (http://mhrd.gov.in/rte) It is of course a big step toward realizing the dream of educated India. But if we see the real picture of the market, it compels us to think on the issues that why most of the people are not able to secure a good job? Why most of the employees are not able to sustain and grow in the jobs they have? With the globalization and the present day demand of the job market, it has become not only important but mandatory to be equipped with the language called English. It would not be wrong to say that the use of this language is going to make the things different for the youth and the people who seek earning and living opportunities anywhere in the globe. It has become the prerequisite for the success.

This analysis has been done keeping in mind that despite of good teaching learning facilities under state and private owned schools, still the youth is not properly equipped with the use of this language. The students passing out from the schools in rural and backward areas lack in the skills to present themselves in English, in other words they are less proficient in the use of English. May it be writing, speaking, reading or listening, in this language the students hesitate to express anything confidently.

Methods:
In order to analyze the condition of teaching English in rural and backward areas in the union territory of Chandigarh, two schools one in village Raipur Khurd and other in sector 43, Chandigarh , that caters to the need of the nearby slum colony and the people of humble background of the area were chosen. Students at Government senior secondary school Raipur Khurd and Sarv hitkari Vidya Mandir were questioned on the pre designed questionnaire, and some students were interviewed also, to access the condition regarding the teaching learning process in the school and other correlated socio economic aspects that can influence this process directly or indirectly. More than 120 students were included in this survey that reflects the root causes of the problem under consideration.

The Outcomes:
After recording the answers of the students from the questionnaire following outcomes can be postulated. The observations are explained categorically in the following headings.

Infrastructure:
Infrastructure is one of the basic requirements of any educational institution. It is the basic indicator of the outcomes of the system. If the infrastructure of the institution is not so good it cannot yield good results. It cannot produce best students. It has been recommended by Right to Education (R.T.E) and Sarv Siksha Abhiyaan to provide better infrastructure to the students so that they can study easily and comfortably.(http://ssa.nic.in) The government, according to this act has the responsibility to provide good infrastructural facilities so that a proper and congenial environment can be created to give right education. The schools in questions are good from the basic infrastructure aspect, which means they have proper and sufficient room arrangements; there is a connection of electricity provided by the electricity department. There are proper washrooms. (http://ssapunjab.org) But if we talk about the teaching learning process of language, and that of a foreign language proper language lab is required. Language lab is the important setup where students practice and learn the basics of any language. It is although an important set up in the school. A language lab with computer systems and proper software is of great use for the students.

Policies:
1) Policies, rules and regulations framed by the government help to execute the programs for the benefit of the people. Right to Education under Sarv Siksha Abhiyaan proposes for free books to all the students studying in the government schools, aided schools and local body schools of the areas. There is a provision of providing free text books to the children till they complete their elementary level by the government. No charge will be taken from the students or their parents to ensure the essence of the law i.e. “free education for all.” This is of course a good initiative by the government to shoulder the financial burden of the poor. (http://ssa.nic.in) However, in the survey it is found that the students of government school are getting free books from the school. The books distributed are in Hindi medium it means that the books given to the students for their study are in Hindi language except the books given for the subject of English. The students read all other subjects in Hindi, may it be social science, general science, mathematics? At elementary level each student studies seven subjects, out of which only one book is in English. All other books are in Hindi language, this promotes the Hindi language and on the other hand unconsciously this policy takes English language to the back seat. Only 14.28% of the material is given in English language that itself is a big blow to the study of English. Out of 125 students who were asked questions 9 students were not able to write their names in English language properly. It comes out to be 7.2% of the sample. If the present condition is evaluated, it comes up with the result that English is not given its due place in the curriculum.
2) There is another policy under R.T.E that child cannot be failed till he finishes elementary class. It says that the child should not be failed or should be promoted to the next class till he reaches the ninth standard.(http://mhrd.gov.in) This rule makes the situation worse for this subject called English This rule was actually made to combat the negative and suicidal tendency of the students in the elementary level education. Secondly it also makes the statistics of the department nice to see and evaluate. When all the students will pass the examination till eighth standard, it will yield hundred percent of the result and a good boast for the department to say that all the enrolled students get passed. It is another easy way to ensure total education. Most of the students are aware of this rule that they will be promoted continuously without putting many efforts. This condition gets worse when the students take undue advantage of the said rule. They sit as mute spectators in the class without any participation. This has brought down the motivation level of the students who work hard and also promotes the students who are doing no efforts at all. They are not so active to work on their own to equip themselves with the study and particularly the knowledge of English.

Environment
Environment is such a thing that helps to make the things happen. It is aptly said that it is very important and difficult also to make the environment. Once the environment is created it helps the people to create and develop in it. An environment that fosters student’s growth and quality learning is dependent on high quality teaching and effective classroom management (Reddy, sambasiva.K, &Naidu, Jagmohan, Education Planning and classroom planning, Arpan Publisher, Delhi, 2014,pp-91.)

Environment in the school: it is found that the environment in these schools tend towards Hindi. Hindi is treated as the first language and as discussed earlier also everything is taught in this language that keeps the proficiency of the students studying here in English language less as compared to the Hindi language. As Chandigarh is Punjabi (one of the languages of India) speaking area, most of the students as well as teachers are from Punjab and speak Punjabi well. If we observe the total environment we can find out easily that the teachers and students interact in Hindi and Punjabi languages. Most of the subjects like mathematics, science, social studies etc are taught in Hindi medium. These observations bring about the fact that Hindi is the forerunner in these schools as compared to the language called English. In the government school only English language is taught in English. Sarvhitkari Vidya Mandir a school run by trust is teaching mathematics, science, social studies in English language, but the interaction between teacher students is in Hindi. It defeats the purpose of an English medium school. Here in these school's teachers explains each and every thing in Hindi language and sometimes it goes to the extent by explaining the concepts in Punjabi language. These attitudes of the teachers at schools make the students reluctant to grasp the things in English language. Mostly teachers do not come regularly in the school.

Attendance:
The attendance of teachers is mostly less in the cases where the village or the school area is far away from their residence. They prefer to take leave or in other words spend less time in the school premises. Some time it is observed that the teacher takes very casually to conduct the class and make the things happen.

On the other hand, students from the humble background often miss the school for the one reason or the other. It is observed that the students coming from the daily wagers family stay home for the petty jobs to earn and support their families.

Environment at Home:
Home is one such place where a student stays maximum time. It is therefore mandatory for this study to find out the environment at their respective homes. It is found that most of the students coming to these two schools are from the families where parents are not highly educated. They are also dependent on the tutors and other well educated neighbors or relatives to take care of the studies and related tasks of the children. Having a graduation degree (in some cases) but not attachments with the studies make the condition of the parents pathetic to take care of their children. Most of the parents are illiterate or have gone up to the school level long ago. They are not able to generate the environment for the child to study even. It would be wrong to expect the environment from such parents where the child can practice in English. It is here that a lay man fails, he fails to generate environment and in turn the very base of the child that should be strong enough under the strong supervision of the parent remains weak and the blame game of parents and teacher starts to escape their responsibilities. It becomes difficult for the child to sustain a good study and particularly in English language as expected and demanded by the job market. Parents prefer to work rather than caring their children. Sometimes they take their children to work for the petty jobs to earn better for the day. Parents should be educated about the today's need and should be made aware so that they can at least help the children by giving and facilitating the environment that can help the learning of English.

Teaching Methodology:
Teaching methodology helps a lot to make the process of teaching learning interesting and easyfor the students. It has been
observed in the study that the teachers in the schools are following the age old teaching methodologies that are not sufficient
and effective in today's time. It has been proved by the psychologists that there are three types of learners. There are three
main types of learning styles: auditory, visual, and kinaesthetic. Most people learn best through a combination of the three
types of learning styles, but everybody is different. Proper mixture of right type of teaching is required to make the concept
clearer. (http://www.learningrx.com/types-of-learning-styles-faq.htm#sthash.naolfNBN.dpuf)

Auditory Learners: Learning by Hearing:
Auditory learners listen to the things explained to them, then read about them. Reciting information out loud and having music in the background may be a common study method. Other noises may become a distraction. They need a relatively quiet place. (http://www.learningrx.com/types-of-learning-styles-faq.htm#sthash.naolfNBN.dpuf)

Visual Learners: Learning by Seeing:
Visual learners learn best by looking at graphics, watching a demonstration, video, movie or reading. For them, they look at charts and graphs, understand the things, but they may have difficulty focusing while listening to an explanation. (http://www.learningrx.com/types-of-learning-styles-faq.htm#sthash.naolfNBN.dpuf)

Kinaesthetic Learners: Learning by touching or feeling:
kinaesthetic learner process information best through a "hands-on" experience. Doing an activity, can be the easiest way for such learners to learn. Sitting still while studying may be difficult, but writing things down makes it easier to understand. Practicing the things or working on the miniature model can facilitate learning for them. (http://www.learningrx.com/types-of-learning-styles-faq.htm#sthash.naolfNBN.dpuf)
It can be assumed from the above discussion that different set of teaching methodologies are required in a class. No one methodology can easily help the students understand the things properly. It is therefore necessary for the teacher to use wisely the combination of predefined set of teaching methodology while dealing with any particular topic. There are several teaching methodologies available or discovered to help the children understand the topics. While interviewing the students it has been observed they are taken very less to the smart class. Teachers use mostly lecture method or chalk and talk method to teach their subjects. Teachers are either reluctant or unaware of the different teaching methodologies. Good teaching methodologies help to participate and proper involvement of the students in the class that in turn facilitates the proper learning. Learning by playing, learning by doing, buddy system, quiz, demonstrations, field tours, movies are some of the important teaching methodologies that make the teaching learning process interesting. Generally some people assume that some subjects are tough.

English language taught as subject:
It is found in this study during survey that learning English is tough, as it is tough subject. Moreover, English is taught as a subject in the schools. Teachers fail to teach it as a language. Although, English has been given as the status of second language in the syllabus, but still students are not able to do well in this language. Students cram to pass the exam. Teachers tell the students from the perspective of exam only. The main concentration is on exam rather than knowledge. At last the result is students pass in the exams but fail to use it in the rest of life.

Poor curriculum:
Analysis and change in age old curriculum is necessary to bring novelty to it. Children love to see new things, so instead of changing the whole subject matter something new can be added to it.(Dash, B.N, The Learner Nature and Development, Dominant Publishers, Delhi, 2005, pp-194) The very basic of the language is missing in the curriculum. Students are not taught about the phonic sounds of each alphabet and they are not aware of the sounds made by the combinations of the different consonants, vowels etc. Phonetics is the branch of linguistic science or language which deals with the study of speech sounds. It is infact the most important contribution made by the science of linguistic. (Sachdeva, M.S,Modern teaching of English, Bharat book centre,tendon publication, Ludhiana,pp-87) Stress is on the major concepts only and not on the basic language. Grammar is taught as chapters and enough practice is not demanded from the students. It has been observed that the exercises at the back of the lessons are given and the students keep on writing these questions from the keys or guides. Less emphasis is laid on the rules of grammar and vocabulary. Explanation is not ample so that the students can use the concepts easily thereafter in their life. The major emphasis is made on the quantity of education, in the sense that maximum people can be covered and maximum lessons can be downloaded to them.

Teaching Aids or Devices:
Teaching aids as the name indicates are the helping material that helps to facilitate the teaching learning process. It can help the teacher to explain the concept at one side and also make the understanding prompt and easy. Teaching aids are also called as teaching devices. Teaching devices are those devices which a teacher uses while teaching a lesson in order to make teaching understandable to learners. With the help of these devices or aids new knowledge is made stable in the minds of students permanently. For this purpose teacher performs some activities that are additional in the class. (Pearson) It is found that the teachers teach English with limited teaching aids. Although it is clear that the teachers in these schools are qualified and capable enough to handle the assigned students. There is only one smart class in these schools that hampers their regular turn to come to the class and have the proper studies. Sometimes students do not get time to sit and do practice for month even. Moreover teachers do not teach the students with the latest available technology and teaching aids. Modals, movies, internet, educational tours, real life situations can be used as teaching aids. Mostly chards with pictures and all other descriptions like sound of the word, etc is missing in the classes. These help the students to understand the things in better way. Teachers in rural schools use less teaching aids. Modern teaching aids are still a dream for the rural students. Teachers in the city schools are using more and modern teaching aids as compare to the teachers in the rural schools. It takes longer times to understand the concept.

Maladies:
It is clear from the above discussion that the condition of teaching learning process and particularly English in the rural areas is really a big problem. It can be improved in the rural & backward areas by adopting some simple but stringent measures.

1. There should be proper infrastructure, like proper rooms, teaching aids in the schools that can be used to help the teacher for smooth teaching learning process.

2. To improve the proficiency in English it is necessary to generate environment where the interaction can be done in English, this helps to improve the language and build confidence. The
3. Teacher should always motivate the students so that they can understand and interact in this language effectively.
4. The books other than languages should be given in English language so that it helps the student to study more and more English and interact positively.
5. There should be soft skills classes in the schools to initiate the subject and in this way students can understand English language effectively.
6. Drills should be conducted in the classes in the presence of teacher so that he can correct the students if they go wrong
7. It would be better if the staff of the institution interact in English.
8. English period should be conducted in English language only. Teacher should encourage the students in English and also interact in English.
9. Videos and cartoons in English language should be shown in the classes and children should be motivated to watch the cartoons and children movies in English language.
10. Practice exercises and workbooks should be increased along with the doubt removal sessions.
11. There must be English lab in each school and students should be given free access to the lab so that they can practice the language software and develop the said skill in the stipulated times.
12. Quiz, debates and declamation contests, writing contests should be conducted in the school and the participation of the students should be encouraged. These competitions should be made of national importance
13. English club should be made and dramatics poetics should be made compulsory for the students.
14. Fear of foreign language should be remove by teaching systematically i.e. by starting with sounds (Phonics), words, vocabulary, sentences should be made compulsory and in specific order only.
15. Special incentives or awards should be kept to improve this skill.
16. Teaching aids and methodologies should be used so that the student gets involved in the class activities and become proficient in the language.
17. Government should concentrate both on quality and quantity. It must be taken care that the quality should always be the priority than the quantity. If the quality is compromised there will be no encouraging results. The pass outs will not be able to get good opportunities.

Conclusion

It is evident from the above discussion that is based on the questions asked from the students from the questionnaire attached below that the condition of teaching English is not good in the rural areas. All the observations have been made from the primary data which has been asked to the students through the pre designed questionnaire and the interviews conducted. With the problems in the facilities like English language laboratory, lack of environment at school and home, less practice on the language, it is really very difficult to improve the condition of teaching English in a way that it helps to generate the best output that can be measured and that shows positive results.

It is observed that the government is focused on the quantity of education, which means the state is focusing more on the quantity i.e. the number of students enrolled in the schools rather than quality where the state is...?

It has been found that the entire process needs to be checked and verified again. Teachers should be set responsible for the duties and should conduct the classes in such a way that it helps the students to generate interest and make them confident enough to use English in their daily routine. Teacher's job should be made performance linked. Head of the department or school should supervise the institution in a very positive way so that a good environment can be generated. The importance of English should be downloaded to the students effectively, so that the students can understand the importance of English and start using it wisely.

It is quite possible to make the things positive and effective for the students. It is very important for the care takers, policy makers and the people implementing these policies to do justice and faithfully implement the things in such away so that it can help the masses and produce the desired results.

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Student's Questionnaires':

1.	I can understand what is being taught in the class.	Y/N
2.	I enjoy English lessons in my class.	Y/N
3.	I like to communicate in English in all the classes.	Y/N
4.	I like to communicate in English in English class.	Y/N
5.	I think pronunciation is important in this language.	Y/N
6.	I can read easily all the text books in English Language.	Y/N
7.	I spend more time to do English home work than other subject.	Y/N
8.	I wish to be taught in English all the subjects.	Y/N
9.	I think learning English is important for my future	Y/N
10.	I am satisfied with the style of teaching English in my class	Y/N
11.	I would like to know each and every thing in English.	Y/N
12.	I wish to learn English even after I start working in society.	Y/N
13.	My parents help me in doing my English work	Y/N
14.	My teacher spares no efforts to teach me English	Y/N
15.	I go to smart class thrice in a week	Y/N

THE END

ARTICLE

2

HPLC-ASSAY OF RIFAMPICIN IN HUMAN PLASMA: APPLICATION FOR PROTEIN BINDING STUDY

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Abstract:

A rapid and sensitive HPLC-PDA method was developed for the quantification of rifempicin in human plasma using chlorzoxazone as internal standard. The analyte and internal standard were extracted from the plasma sample using a liquid-liquid extraction method. Chromatographic separation was achieved on a Thermo (250 × 4.6 mm, 5 µm) column with a mobile phase consisting 2mM sodium acetate buffer and acetonitrile (50:50, v/v with); at a flow rate of 1.0 ml /min. The assay was validated with a linear range of 2.5 – 40 µg /ml for rifampicin using 200 µL of plasma sample. The intra- and inter-day assay precision ranged from 1.20% to 3.90% and 0.30to 4.2%, respectively, and intra- and inter- day assay accuracy was between from 0.2 to 4.0% and -0.4% to 2.4%, respectively. The method was successfully applied to the protein binding studies of rifampicin in human plasma.

Introduction:

Tuberculosis (TB) is one of the most infecting human bacterial diseases caused by mycobacterium tuberculosis (Mtb) commonly affecting the lungs [1] Tuberculosis has recently emerged as a major health concern. Each year, approximately 2 million persons worldwide die of tuberculosis [2] [3]. TB epidemics ravaged much of Europe and North America, resulting in 800 to 1000 deaths per 100 000 per year. Today, approximately 9 million new cases of TB are identified per year. TB is caused by Mtb the single greatest cause of mortality due to a bacterial pathogen[4]. TB is an airborne disease. Mtb is spread by small airborne droplets, called droplet nuclei, generated by the coughing, sneezing, talking, or singing of a person with pulmonary or laryngeal tuberculosis. These minuscule droplets can remain air borne for minutes to hours after expectoration [5, 6].

Rifampicin (Fig. 1) is a group of complex, macrocyclic antibiotics produced by *Amycolatopsis mediterranei*. It inhibits DNA-dependent RNA polymerase in bacterial cells. It causes greater and earlier reduction in the number of Mtb. It is a main drugs to combat tuberculosis and is central to TB regimens in worldwide [7]. It is one of the most important first line anti-TB drugs [8]. Rifamipisin has been used for the treatment of TB, leprosy [9] [10] some types of osteomyelitis and endocarditis[11]. The drug is most regularly deployed in the cocktail of drugs used as first-line treatment for TB.

Several analytical and bioanalytical methods are already available for the determination of rifampicin in biological fluids and pharmaceutical dosage forms, including methods based on HPLC [12], [13], [14], [15], HPLC following solid phase extraction [16], UPLC [17], LC–MS/MS [18], [19] and MALDI–TOF [20].
There are, however, a few simple precise and rapid methods for determining the plasma concentration of RFP in patients, they are much less those using high performance liquid chromatography (HPLC). Most existing methods require tedious procedures or require special equipment. That is why the aim of this study was to standardize simple, precise selective, highly robust RP- HPLC method a method for determining the concentration of rifampicin in human plasma. This method was applicable for protein binding study in human plasma.

Experimental

Chemicals and Materials

Rifampicin and Chlorzoxazone (Internal standard, IS) were were obtained from Sigma Aldrich (St.Louis, MO). HPLC grade acetonitrile and methanol were from Sigma Aldrich Chemicals Pvt Ltd (Mumbai, India). Sodium acetate AR, glacial acetic acid AR, and ammonia solution (25%) were purchased from E Merck Pvt. Ltd (Mumbai, India). Dichloromethane (DCM) was obtained from Molychem. N-Pentane was obtained from Spectrochem pvt. Ltd. (Mumbai). Ethylenediaminetetraacetic acid disodium dehydrate (EDTA.Na_2.$2H_2O$) was purchased from USB Corporation, Cleveland. Potassium chloride was purchased from SRL pvt. Ltd Mumbai Ultrapure water was obtained from a Milli-Q PLUS PF water purification system. Heparin sodium injection I.P. (1000 IU/mL) was purchased from Biologicals E. Limited (Hyderabad, India). Human plasma was collected as per the guidelines of the institutional ethics committee.

Instrumentation and chromatographic conditions

The method was developed using a Waters HPLC system (Milford USA) consisted of a binary pump (model 515), auto sampler (model 717) connected to waters 2996 Photo diode Array detector. The separation was achieved on Thermo (250 × 4.6 mm, 5 µm) column with a mobile phase consisting 2 mM sodium acetate buffer and acetonitrile (50:50, v/v with pH-4); at a flow rate of 1 mL/ min. Detection range of PDA detector was 200-500nm. Detection was carried out with PDA at 334 nm wave lenght. Total run time was 14 min and

volume of injection was 50 µL, prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. Analysis was performed at ambient temperature. The data collection and analysis were performed using breeze - version 3.1 software.

Preparation of Stock and standard Solution

Rifampicin and IS stock solutions for CS samples were prepared in acetonitril (normal concentrations 1.0 mg/ml). CS samples were prepared by spiking respective stock solutions in blank humanplasma at concentrations of 2.5, 5, 10, 20 and 40 µg/ml. CS samples were prepared from a blank plasma pool. Rifampicin stock solution for QC was prepared separately. QC samples at three different concentrations (2.5 10 and 40 µg/ml as LLOQ, medium and high, respectively) were prepared separately in five replicates, independent of the calibration standards. Calibration curves were plotted as concentration of drugs versus peak area response. QC samples were prepared from different matrix pools on each day of analysis. All prepared plasma samples were stored at -15 ◦ C or below and all prepared stock solutions were stored at a 4 ◦C.

Sample Preparation

Calibration standard, QC and plasma-protein binding samples were extracted using liquid-liquid extraction method. An aliquot of sample mixture (200 µL) of blank human plasma mixed with 10 µl of internal standard (5µg/ mL). In 180 µL of plasma add 10 µL of rifampicin (stock cc) and 10 µL of IS. Vortex it for 2 min. Add 2 mL of extracting solvent [DCM: n-Pentane(50:50)].Again vortex it for 3 min. Centrifuge it for 5 min at 5000rpm.Snap freeze the plasma with the help of Liquid nitrogen. Take out the supernatant. Again after thawing the plasma add 2 mL of extracting solvent [DCM: n-Pentane (50:50)].Again vortex it for 3 min. Centrifuge it for 5 min at 5000 rpm. Snap freeze the plasma with the help of Liquid nitrogen. Take out the supernatant and mix both the supernatant. Dry it in the TurboVap LV. Reconstitute the residue with 200 µL of ACN. The eluate was collected and evaporated to dryness under vacuum in a speedvac concentrator (Savant Instrument, Farmingdale, USA). The residue was reconstituted in 200 µL of mobile phase and 50 µL was injected into the HPLC.

Recovery

The recovery of rifampicin and IS, was determined by comparing the responses of the analytes extracted from replicate QC samples (n = 5) with the response of analytes from post-extracted plasma samples at equivalent concentration. Recoveries of rifampicin were determined at QC low, QC medium and QC high concentrations, viz., 2.5, 10 and 40 µg/ml, the recovery of the IS was determined at a single concentration of 5 µg/ml.

Validation procedure

The method was validated to demonstrate the specificity, linearity, matrix effect, accuracy, precision and stability (Guideline For Industry, FDA) [21].

The specificity of the method was evaluated by analyzing blank plasma samples collected from six different rats to investigate the potential interferences in the liquid chromatographic peak region for the analyte and IS using the proposed extraction procedure and chromatographic conditions.

Linearity was tested at six concentration levels covering a range of 2.5 – 40 µg / ml. The calibration curve was obtained by plotting the peak area ratio (peak area analyte/peak area IS) versus concentration. The results were fitted to a linear regression analysis $y = mx + c$, a using weighing factor ($1/x^2$). The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at the LOQ, which was set at ± 20% . The lower limit of quantitation (LOQ) of the validation was assessed as the lowest concentration on the calibration curve that could be quantitatively determined with acceptable precision and accuracy within ± 20%. The LOQ was established based on six replicates on five consecutive days.

The matrix effect was evaluated in humanplasma by comparing the corresponding peak areas of the post extraction spiked samples to those of the standard solutions evaporated directly and reconstituted in mobile phase. Experiments were performed at the three QC levels. If the ratio was less than 85% or more than 115%, the matrix effect was regarded as being of significance.

Batches consisting of five calibration standards at each concentration and QC samples were analyzed on five days to complete the method validation. In each batch, QC samples at 2.5, 10 and 40 µg/ml were assayed in sets of five replicates to evaluate the intra- and inter- day precision and accuracy. The criteria for acceptability of the data included accuracy (% bias) and precision which must be within ± 15% of the nominal value and ± 20% at the LOQ. The accuracy was expressed as % bias:

$$\%\text{Bias} = (\text{observed conc.} - \text{nominal conc.}) \times 100 / \text{nominal conc.}$$

All stability studies were carried out at 2.5 and 40 µg/ml in five replicates. The freeze-thaw stability was determined after three freeze-thaw cycles (room temperature to -70 ± 10 °C). The post extracted auto-sampler

stability of rifampicin was examined at 4 °C for 72 h. The bench-top stability of rifampicin in biomatrix was evaluated at ambient temperature (25 ± 5 ºC) for 24 h. The long-term freezer stability was determined at -80 ± 10 °C over 30 days.

The method was validated in compliance with FDA guidelines, in terms of specificity, Selectivity, linearity, precision, accuracy, limit of quantification, limit of detection, robustness and other aspects of analytical validation.

Application of the method

Refampisin plasma protein binding in human plasma was performed at three different concentrations (2.5 and 40 ng/mL). The bound and unbound fractions of refampisin were separated from human plasma samples by ultra-filtration through the centrifuge micro partition system (Amicon, Centrifree device Inc., MA, USA) [22] [23]. Samples in duplicates (0.5 mL) were placed in Centrifree devices and centrifuged at 1500g for 10 min to collect approximate 10% (100 mL) of the original volume of plasma as ultrafiltrate. Non-specific binding of rifampisin was determined by spiking test concentration into 0.01 M phosphate buffer (pH 7.4) and applied same procedure as plasma. The in-vitro samples and their respective ultra-filtrates were analyzed by HPLC. The non-specific binding and plasma protein binding were determined using the following equations:

Non-specific binding (%)
= 100-[(conc. in buffer ultrafiltrate)×100/conc. in buffer]
Plasma protein binding (%)
=100-[(conc. in plasma ultrafiltrate) × 100/conc. in plasma].

Result and discussion

HPLC method development

Analysis of rifampicin is a challenge owing to its high polarity and small molecular size, which lead to poor retention on reversed-phase liquid chromatographic columns. The chromatographic conditions were optimized with respect to specificity, resolution, and time of analysis. Hence we started the development activity with C_{18} stationary phase of various manufacturers such as Zorbax, ODS (250 × 4.6 mm, 5 µm), Spherisorb ODS (250 × 4.6 mm, 5 µm) Symmetry shield C_{18} (250 mm × 4.6 mm, 5.0 µ), Phenomenex Luna C_{18} (250 ×4.6 mm, 5 µm), Spheri-5, CYANO column (30 × 4.6 mm, 5 µm) and Thermo (250 × 4.6 mm, 5 µm) column . The last two columns were found to be suitable. However, Thermo (250 × 4.6 mm, 5 µm) column was used as a good resolution and minimum elution time were obtained. The stationary phase was not only the parameter which could give better resolution. Mobile phase, pH and organic modifies also played very important role which leads the best separation.

Different mobile phases containing acetonitrile, methanol, water and buffer were examined. Initially the methanol was used as an organic modifier which gives the poor baseline with baseline drift. Hence the response for the rifampicin was reduced. To improve the resolution and response, acetonitrile was tried as an organic modifier. The base line was found good and response rifampicin was improved. Effects of pH (3–7) and ionic strength (5–50 mM) were investigated using phosphate and acetate buffer. It was found that at higher and lower pH the tailing of the rifampicin peak was more and also resolution was poor of the analyte. The effect of buffer concentration on the retention of rifampicin was also studied. The mobile phase containing acetonitrile: sodium acetate buffer 2 mM (50: 50 v/v, pH 7), was selected as optimal for obtaining well-resolved peaks with acceptable system suitability parameters. Flow rates from 0.5 to 1.5 mL min^{-1} were tested. Flow rates less than 0.5 ml/min led to an increase in retention times peak broadening and the time of analysis. High flow rates led to a remarkable increase in column pressure and decrease in resolution. It was found that 1 mL min^{-1} was optimal as it compromised between resolution and run time.

Effect of the wavelength on the response factor was observed over the wavelength range 200–500. The detection wavelength, 334 nm was found optimal due to the high absorbtivity at this wavelength for rifampicin (fig-2). Complete separation was achieved in < 14 min at ambient temperature (Fig. 3). The average retention times ± RSD % for rifampicin and IS were found to be 10 ± 0.10 (n = 10) and 12±0.10 respectively.

Sample extraction and recovery

The blank plasma was used as a surrogate matrix organic solvent precipitation using acidified acetonitrile or methanol and DMSO in methanol resulted in non-reproducible recoveries and interferences from the sample matrix with the chromatography of the analytes (data not shown). Protein precipitation was not a feasible option due to extraction of endogenous product in human plasma, they produced significant matrix effect. Solid phase extraction (SPE) was investigated using Oasis HLB, CN and C18 cartridges for optimizing the extraction procedure. But SPE method was more expensive and not reproducible. Subsequently, liquid- liquid extraction was investigated using [DCM: n-Pentane(50:50)] gave consistent results in terms of recovery of rifampicin and IS and also gave cleaner plasma sample extract. The use of 50 % DCM in n-entan during extraction provided the extraction strength of both analyte and IS in human plasma. This resulted in

considerable improvement in recovery of rifampicin. The absolute mean recovery of rifampicin and IS were 90.09% and 70.35% respectively. The %CV values are within the acceptable limits (<10%) table (3).

Validation procedures

Selectivity and specificity

Six lots of blank humanplasma were analyzed for the evaluation of selectivity and specificity. These samples did not show any significant interfering peaks at the retention times of either rifampicin the IS (Fig. 3).

Matrix effect

The adverse consequences of matrix effects on the results of quantitative HPLC analyses have been fully recognized and the assessment of matrix effects is becoming an integral part of method development and validation. The matrix effect for rifampicin at 2.5 and 40 µg/ ml concentration levels in human plasma was < ± 2%. Thus no significant matrix effect was observed.

Calibration curves

The peak area ratio of analyte to IS was linear over a concentration range of 02.5 -40 µg/ml for rifampicin. The calibration curve had a reliable reproducibility over the concentration range 2.5 -40 µg/ml. The average regression (n=3) was found to be 0.998 ± 0.0018. The lowest concentration with RSD < ± 20% was taken as the LOQ and this was found to be 2.5 µg/ml.

Accuracy and precision

Intra- and inter-assay precision was determined from the relative standard deviations (%RSD) of the quality control samples (LQC, MQC, and HQC). The intra-day assay precision and intra-assay accuracy were within the limits and ranges from 1.20% to 3.90% and 0.30 to 4.2%, respectively respectively (Table 1). The inter-day assay precision and accuracy ranged from 0.2 to 4.0% and -0.4% to 2.4%, respectively. Both intra and inter-day precision and accuracy were found to be within the accepted variable limits.

Stability

The predicted concentrations for rifampicin at 2.5 and 40 µg /ml samples deviated within the nominal concentrations in a battery of stability tests, viz., auto sampler stability (24 h), bench-top stability (6 h), repeated three freeze-thaw cycles and at -70 ± 10 ºC for 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

Application of the method

In-vitro plasma protein binding studies

The present in-vitro study was carried out to determine the extent of plasma protein binding of refampisin. Bound and unbound drug from spiked/treated plasma was separated by the ultra-filtration technique. Plasma protein binding of rifamicin was found 82.32%, 78.40% and 76.32% at 2.5, 10 and 40 µg/mL respectively. Concentration dependent human plasma protein binding was not observed within the selected concentration range (2.5–40 µg / mL). Non-specific binding of refampisin was found <5%.

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Conclusion

Bio analytical method was developed and validated in human plasma for the model drugs chosen. Effect of multiple freeze-thaw cycles on the extent of plasma protein binding of drugs determined after every freeze-thaw cycles. No significant difference was observed in the percent bound determined in fresh human plasma and plasma after multiple freeze-thaw cycles In summary, a sensitive, rapid and specific HPLC assay of rifampicin using the liquid-liquid extraction was developed and validated. A good linearity was obtained over the concentration range of 2.5 - 40 µg/ ml. Moreover, the assay demonstrate a high sensitivity with LOQ 2.5 µg/ ml using 200 µL volume of plasma sample and simple liquid-liquid extraction procedure. The method is accurate, precise, reproducible and was applied successfully protein binding study .

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Acknowledgements

The authors are thankful to Vice chancellor, King George's Medical University, Uttar Pradesh Lucknow, India, for his constant encouragement and support. We also acknowledge Indian Council of Medical Research (ICMR) for providing research fellowships to one of the authors.

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Table 1 Intra-day and inter-day assay precision and accuracy for refampicin

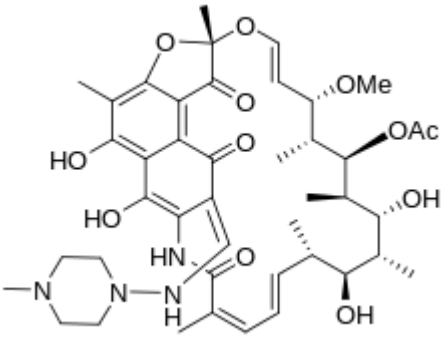
Concentration (µg/ml)	Accuracy (%Bias)		Precision (%RSD)	
	Intra-day	Inter-day	Intra-day	Inter-day
2.5	2.4	4.0	4.2	3.9
10	-0.4	2.0	2.4	2.6
40	1.4	0.2	0.3	1.2

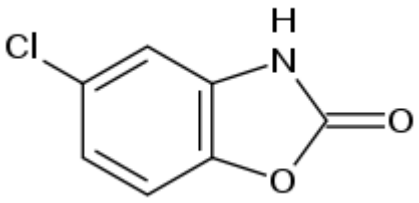
Table 2 Stability of refampicin in human plasma.

Nominal Conc.	Stability	Mean	S.D.	Precision (%RSD)	Accuracy (%bias)
2.5 µg/ml	Auto sampler stability	2.56	0.04	0.44	0.92
	Bench-top stability	2.59	0.12	1.73	-6.32
	Freeze-Thaw stability	2.48	.08	1.34	-0.56
	Long-term stability	2.53	0.14	1.09	4.9
40 µg/ml	Auto sampler stability	40.31	1.05	2.12	-0.68
	Bench-top stability	40.59	1.08	2.23	-5.34
	Freeze-Thaw stability	40.98	1.13	3.09	-0.6
	Long-term stability	39.56	2.11	1.98	3.49

Table 3 Recovery of rifampicin in human plasma

Sample	Concentration (µg/ml)	%Recovery	%CV
Human Plasma	2.5	92	4.0
	10	96	3.3
	40	94	3.2





Rifempicin (Analyte) Chlorzoxazone (IS)

Fig.1 Structure of Rifampisin (analyte) and Chlorzoxazone (IS)

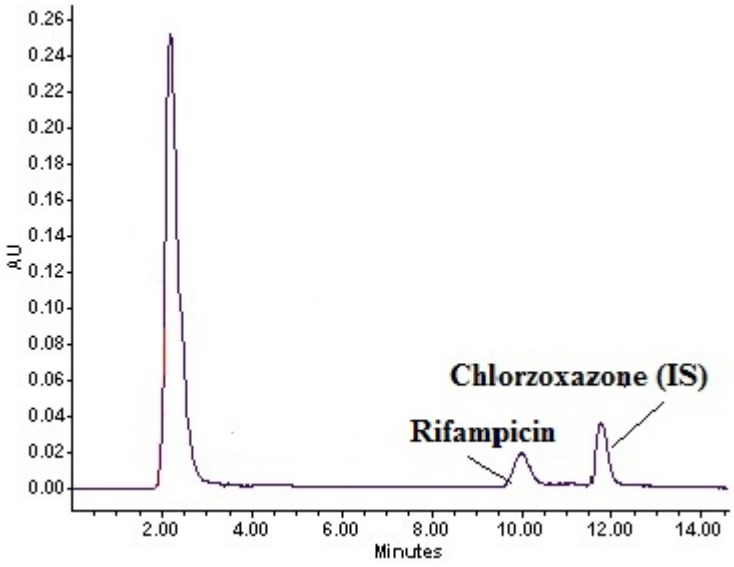


Fig.2 Representative chromatogram of analyte and IS after extraction of CS in human plasma

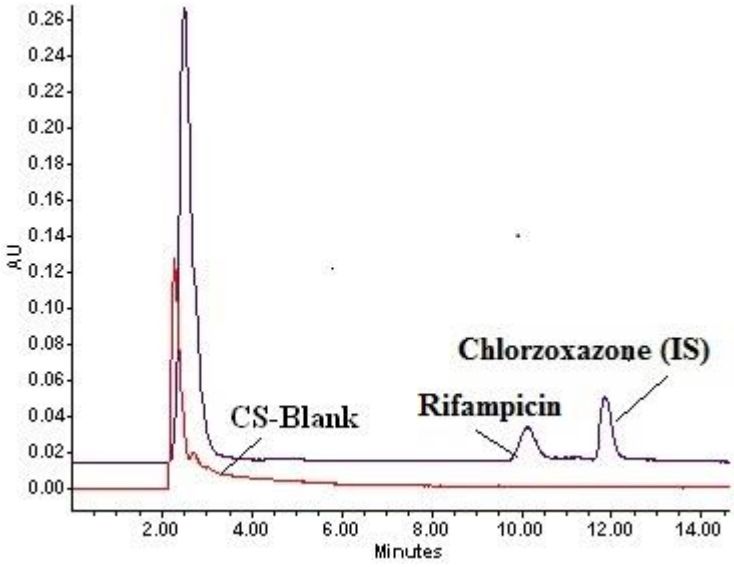


Fig.3 Overlay chromatogram of analyte and IS with CS blank

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