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Ebrahim, Salma

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FACULTY OF HEALTH SCIENCES

THE PREFORMULATION INVESTIGATION OF A COMBINATION ANTI-TUBERCULOSIS DOSAGE FORM.

Salma Ebrahim

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Pharmaceutical affairs)

Johannesburg, 1998

DECLARATION

I, Salma Ebrahim declare that this research report is my own work. It is being submitted for the degree of Master of Science in Medicine (Pharmaceutical affairs) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

S. EBRAHIM

.....4th.....day of.....June.....,1998.

Dedicated to my mum and dad, Ayesha and Abdool Hak Ebrahim, brother Nazeem Ebrahim and my husband Mohammed Ismail

ABSTRACT

Tuberculosis control in South Africa continues to be a major challenge with 90 000 new cases reported in 1995. Major contributory factors towards these epidemic proportions are patient non-compliance and the occurrence of drug resistant tuberculosis. Fixed drug combinations of anti-tuberculosis drugs have been reported to reduce the possibility of resistance arising to any one of the drugs in combination and to improve compliance. However, the combination anti-tuberculosis drugs available at present still suffer the disadvantage of patients having to take 6 tablets per day. Therefore, the purpose of this project was to investigate a formulation that would reduce this disadvantage.

The project consisted of two parts: First, to undertake a preformulation investigation and then, to formulate a powder combination of anti-tuberculosis drugs in the form of a sachet.

The purpose of this project was to undertake the preformulation investigation which involved establishing suitable assay systems and the study of the compatibility of rifampicin, isoniazid, pyrazinamide and ethambutol HCl when in a fixed combination formulation.

A high performance liquid chromatographic (HPLC) assay utilising ultraviolet detection was developed for the simultaneous determination of rifampicin, isoniazid and pyrazinamide. Since ethambutol HCl cannot be detected by UV absorption, an alternative HPLC assay method using electrochemical detection (ECD) was developed, which included the simultaneous determination of isoniazid, ethambutol HCl and rifampicin. The HPLC methods that have been developed are precise, accurate, rapid, and display good linearity and reproducibility.

Fourier Transform Infrared Spectroscopy was used to investigate the interactions between rifampicin, isoniazid, pyrazinamide and ethambutol HCl in a combination dosage form. The results suggest that no major interactions occurred between any of these drugs and that it was therefore feasible to formulate a combination sachet dosage form.

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1.0 INTRODUCTION

Tuberculosis accounts for more than 80% of all communicable diseases notified in South Africa and is regarded as one of the most serious health problems affecting the country. Tuberculosis affects all racial groups in South Africa, but the prevalence of the disease is by far greatest among the African Black and Coloured populations. The disease is especially of concern in the Western Cape, where reported incidence rates are up to three times higher than for other regions. The Coloured population in the Western Cape seems to be at high risk, with incidence rates in excess of 700 per 100 000. (Department of Health, 1992).The control of tuberculosis continues to be a major challenge in South Africa, since approximately 90 000 new cases were notified in 1995 and is exacerbated by HIV/AIDS in some areas (Department of Health, 1996).

Long term studies in many countries with short-course chemotherapy have been shown to be highly effective and the short-course therapy has become standard practice for pulmonary tuberculosis as well as the many forms of non-pulmonary disease (Collins, 1991). Inclusion of isoniazid, rifampicin and pyrazinamide is the key to a successful therapeutic outcome (Collins, 1991). Although chemotherapy is the single most effective tool in the management and control of tuberculosis world-wide, drug resistance remains a serious problem. An increase in the number of cases of drug resistant tuberculosis has been observed both locally and internationally, due mainly to inappropriate treatment and to the spread of HIV infection which may indirectly increase drug resistance since immuno-compromised patients are more susceptible to tuberculosis.

For these reasons, the caseloads for tuberculosis are increased beyond the capacity of existing health services and is resulting in lower cure rates and increased acquired drug resistance, (Kochi, 1993).

A further problem encountered with tuberculosis therapy is patient non-compliance. Therefore, supervision, or directly observed treatment (DOT), is essential to ensure compliance which requires that a responsible and well motivated person is present to ensure that the tablets are actually seen to be taken and swallowed. Fixed drug combinations makes supervision easier and ensures that patients cannot avoid taking all of the drugs prescribed. Directly observed treatment and combination anti-tuberculosis drugs have been shown to greatly reduce the possibility of drug resistance, (Collins, 1991).

The main reasons for the use of combinations of antibacterial agents therefore may be summarised as follows:

a) Combination anti-tuberculosis drug therapy achieves an additive or synergistic effect against a single organism. A resistant organism therefore may be successfully eradicated where a single drug is likely to be ineffective. Alternatively, smaller amounts of each agent may be used synergistically thereby reducing any dose-related toxic effects.

b) Combination anti-tuberculosis drugs prevents of the emergence of resistance.

c) Combination anti-tuberculosis drugs allow for the early treatment of a serious infection before a bacteriological diagnosis can be made or the causative organisms isolated and their antibiotic susceptibilities determined,(Gruneburg, 1980).

The Advisory Council for the Elimination of Tuberculosis (ACET) in the United States recommends that the initial treatment of tuberculosis should include all four drugs to help prevent the occurrence of more cases of drug-resistant tuberculosis so as to reduce treatment failure and the further transmission of tuberculosis (CDC, 1993). Similarly, Tahaoglu, (1994) also found that the high initial resistance in Turkey had a negative impact on the success rates of anti-tuberculosis treatment and it was therefore mandatory to begin anti-tuberculosis treatment in routine practice with at least four first line drugs, in which ethambutol is used instead of streptomycin because of its high resistance.

In South Africa, the current treatment regimens as recommended by the Department of Health requires first, an intensive phase of 2 months treatment in newly diagnosed adults and second, that the re-treatment adults should consist of rifampicin, isoniazid (INH), pyrazinamide and ethambutol HCl. Using the fixed drug combination of rifampicin, INH, and pyrazinamide, a patient under 50 kg will have a daily intake of 4 tablets and additionally two tablets of ethambutol which makes a total of at least 6 tablets per day, (Department of Health, 1996). This quantity may also be one of the major contributory factors toward non-compliance and highlights the need for a single combination dosage form of rifampicin, isoniazid, ethambutol HCl and pyrazinamide.

Poor patient compliance which is one of the biggest problems with any illness requiring long-term medication (Schlossberg, 1983), may be exacerbated in the case of tuberculosis because of the large number of tablets that need to be taken.
In view of the problems encountered with poor compliance and drug resistance, a study was undertaken to develop a fixed combination anti-tuberculosis dosage form.

Prior to the development of a dosage form, it is necessary to understand the physical and chemical properties of each of the drug molecules so as to identify approaches for the development of novel formulations (Wells, 1988).

The second step, is to develop a simple analytical method for the quantitative measurement of the different drug molecules (Wells, 1988). Several distinct problems occur in the analysis of drugs. These may entail interference from excipients during the assay and the sensitivity of the method to determine minute amounts of degradation products of the raw materials. Further problems that need to be resolved later, include the analysis of the drugs and their metabolites in body fluids (Pryde et al, 1979).

In this research report, a preformulation study of a fixed combination anti-tuberculosis dosage form consisting of rifampicin, isoniazid, pyrazinamide and ethambutol HCl is presented.

1.1 ASSAY DEVELOPMENT STRATEGY

To assist in the selection of suitable HPLC detection methods, a literature survey was carried out for methods that were previously developed and for the physico-chemical properties of the active drugs that will be used in the combination dosage form.

From the study of the literature a number of methods were identified for the assay of anti-tuberculosis drugs. Ajiboye et al. (1993), used a thermometric titration method. However, this method was not useful for assaying Rifater^{R} (a combination drug of rifampicin, isoniazid and pyrazinamide).

It was shown by Altomare et al. (1990), that isoniazid and pyrazinamide can be directly determined in human plasma by reversed phase HPLC. This method, however, does not determine the other co-administered anti-tuberculosis drugs such as rifampicin and ethambutol HCl. On the other hand Walubo et al. (1994) developed an assay that can simultaneously detect pyrazinamide, rifampicin and isoniazid with its hydrazine metabolites in human plasma by HPLC. However, this method is inconvenient since it uses two different mobile phase systems for the assay of rifampicin, isoniazid and pyrazinamide. Furthermore, ethambutol HCl could not be assayed since it has no significant ultraviolet (UV) absorption. Subsequently, an assay for determination of ethambutol HCl in human plasma and urine by HPLC with fluorescence detection was developed by Breda et al. (1996). However, this method is not suitable for the detection of any of the other anti-tuberculosis drugs.

It was therefore apparent that a single assay for the rifampicin, isoniazid, pyrazinamide, ethambutol combination anti-tuberculosis dosage form needed to be developed. However, since ethambutol HCl exhibits low UV absorption an alternative method of detection would have to be investigated. Jane et al. (1985) developed an HPLC method for basic drugs using non-aqueous ionic eluents using electrochemical detection, giving rise to the possibility that this method could be modified for assaying ethambutol HCl.

Therefore, for the first part of this study, the aim was to develop a single solvent system, HPLC assay method using UV detection for the rifampicin, isoniazid and pyrazinamide and then to develop an HPLC assay method using electrochemical detection for the determination of rifampicin, isoniazid and ethambutol HCl.

A further aim, was to study possible interactions of the anti-tuberculosis drugs when in combination. Drug interactions in combination could have deleterious effects on bioavailability from a powder dosage form as has been shown by Li Wan Po and Mroso (1984). Results from such a study are necessary for designing the combination dosage form.

Furthermore, the successful formulation of a stable and effective solid dosage form depends on the careful selection of the excipients used to facilitate administration, promote the consistent release for bioavailability of the drug, and to protect it from degradation.

Different types of thermal analysis can be used to investigate and predict any physicochemical interactions between different drugs in a formulation and also can be applied to the selection of suitable chemically compatible excipients (Wells, 1988). The method of Differential Scanning Calorimetry (DSC) commonly used, enables the study of possible incompatibilities in a relatively short time (Lund, 1994b). However, problems associated with the interpretation of DSC thermograms, does not make this a reliable technique, (Van Dooren, 1983).

The use of conventional HPLC or thin layer chromatography to monitor for drug or excipient incompatibilities would normally involve storage of the mixtures at elevated temperatures and humidity, and then assaying for drug content at different time intervals. This process is relatively long and is usually undertaken during stability studies at the time of registration of drugs for patient use.

However, Ker'c et al. (1992) demonstrated that Fourier transform infra-red (FT-IR) spectra could give qualitative and quantitative data about interactions of drugs. Also French and Morrison (1965), demonstrated that infrared spectroscopy can be utilised to identify complex formation of drugs in pharmaceutical dosage forms.

Hence, interactions of all the active ingredients were studied with the aid of Fourier transform infra-red spectroscopy.

1.2 PLACE IN THERAPY OF ANTI-TUBERCULOSIS DRUGS

1.2.1 RIFAMPICIN

Rifampicin is a bactericidal antibiotic with a wide spectrum of activity. However, resistance can develop rapidly and for this reason it is usually given in conjunction with other agents. Uses include the treatment of leprosy, tuberculosis, staphylococcal infections, brucellosis, and legionnaires' disease and for prophylaxis of haemophilus and meningococcal meningitis. (Reynolds, 1996a).

1.2.2 ISONIAZID

Isoniazid is a hydrazide derivative which is the mainstay of primary treatment of pulmonary and extrapulmonary tuberculosis. It is administered with other anti-tuberculosis agents such as rifampicin and pyrazinamide. Isoniazid is also used in high risk subjects for the prophylaxis of tuberculosis. (Reynolds, 1996b).

1.2.3 PYRAZINAMIDE

Pyrazinamide is used as a part of multi-drug regimens for the treatment of tuberculosis, primarily in the initial 8-week phase of short-course treatment (Reynolds, 1996c).

1.2.4 ETHAMBUTOL HYDROCHLORIDE

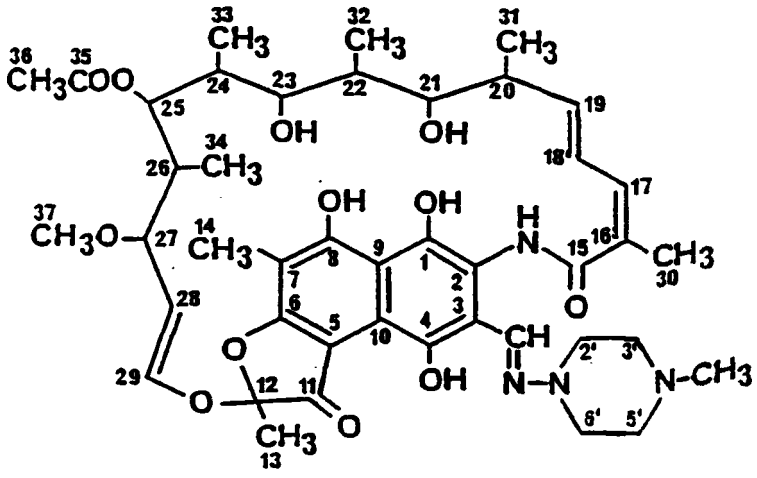
Ethambutol hydrochloride is used with other anti-tuberculosis agents in the primary treatment of pulmonary and extrapulmonary tuberculosis to suppress emergence of resistance to the other agents used in the regimens. It has also been used as a component of regimens for the treatment of opportunistic mycobacterial infections. (Reynolds, 1996d).

1.3 PHYSICOCHEMICAL PROPERTIES OF ANTI-TUBERCULOSIS DRUGS

1.3.1 RIFAMPICIN

Rifampicin is the USAN name for the compound, Rifampicin is the international nonpropriety name. Rifampicin is designated by IUPAC rules as 2,7-(Epoxypentadeca[1,11,13]trienimino)naphtho[2,1-b]furan-1,11(2H)-dione,5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[N-(4-methyl-1-piperazinyl)formimidoyl]-21-acetate.

Formula and molecular weight



$C_{43}H_{58}N_4O_{12}$ Mol.Wt.= 822.95

Figure 1.1 Structural formula of rifampicin

Spectra

The UV spectrum of rifampicin, recorded on a Perkin Elmer model 4000-A spectrophotometer in aqueous phosphate buffer pH 7,38 exhibits absorption maxima as shown in Table 1.1.

TABLE 1.1 Ultraviolet absorption of rifampicin

λmax nm	ϵ
237	33200
255	32100
334	27000
475	15400

The variation of the UV spectrum of rifampicin with pH (figure 1.2) indicates the presence of an ionizable function.

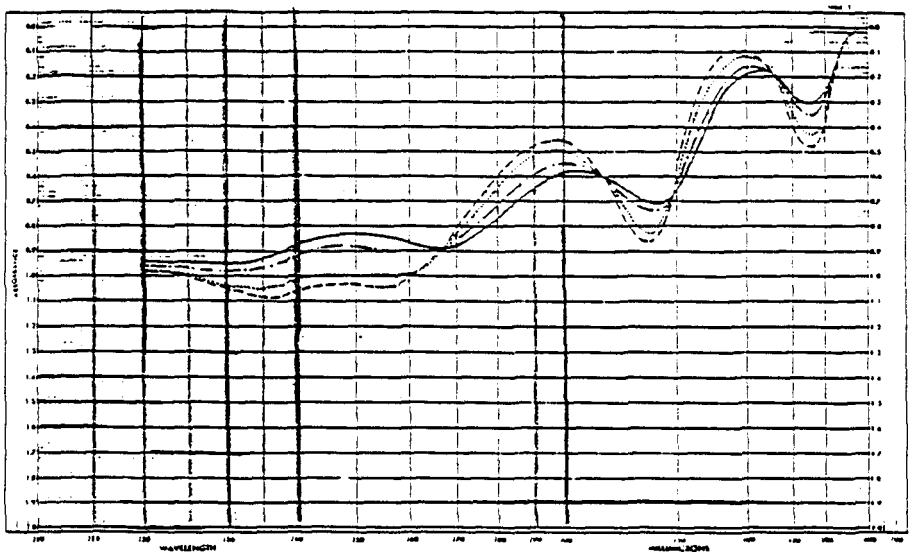


Figure 1.2 UV spectrum of rifampicin in methanol-water (2:3) solution at different pH's.

Infrared spectra

The infrared spectra for rifampicin is shown in Figure 3.15 (A).

Ionization constants

The pK_a values for rifampicin have been determined spectrophotometrically and potentiometrically in solution in water and in methylcellosolve-water (4:1) and are shown in Table 1.2. Rifampicin exists in aqueous solution as the zwitterion with an isoelectric point equal to 4,8. Rifampicin ionizes in non-aqueous solvents, i.e. in glacial acetic acid, the basic piperazine nitrogen can be titrated with perchloric acid. (Gallo et al, 1976).

TABLE 1.2 Ionization constants of rifampicin

	pKa	Pk MCS	Attribution
proton lost	1,7	3,6	hydroxyl at C-8
proton gained	7,9	6,7	piperazine N-4

Solubility

Slightly soluble in water, ethanol, and ether, freely soluble in chloroform, soluble in ethyl acetate and methanol.

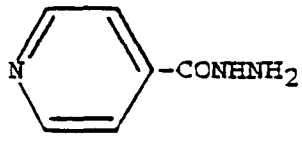
Melting point

185° with decomposition. (Moffat et al, 1986a).

1.3.2 ISONIAZID

4-Pyridinecarboxylic acid hydrazide.

Formula and molecular weight



$C_6H_7N_3O$ Mol.Wt. 137.14

Figure 1.3 Structural formula of isoniazid. (Brewer, 1977).

Ultraviolet spectra

Aqueous acid - 266nm (A^1_1 = 390 a)

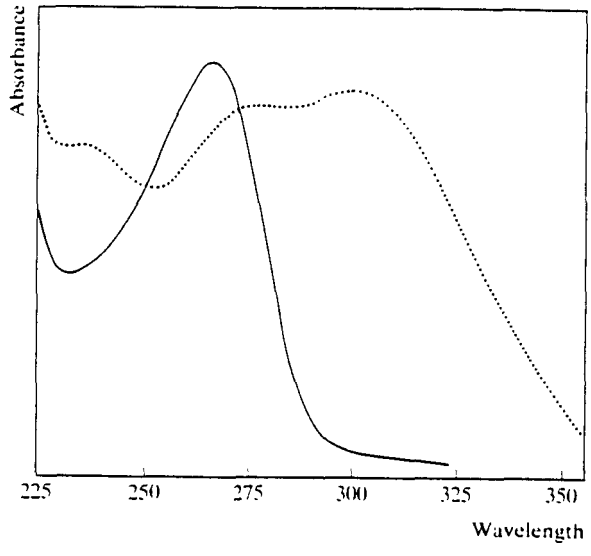


Figure 1.4 UV spectrum of isoniazid

Infrared spectra

The infrared spectra for isoniazid is shown in Figure 3.18 (A).

Dissociation constant

pK_a 1.8, 3.5, 10.8 (20°).

Solubility

Soluble in water and methanol.

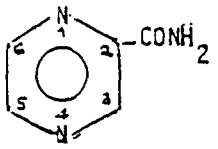
Melting point

140° to 142° . (Moffat et al, 1986b).

1.3.3 PYRAZINAMIDE

Pyrazinecarboxamide

Formula and molecular weight



$C_5H_5N_3O$ Mol. wt. = 123:11

Figure 1.5 Structural formula of pyrazinamide. (Felder, 1983).

Ultraviolet spectra

Aqueous acid- 269nm (A^1_1=659 a)

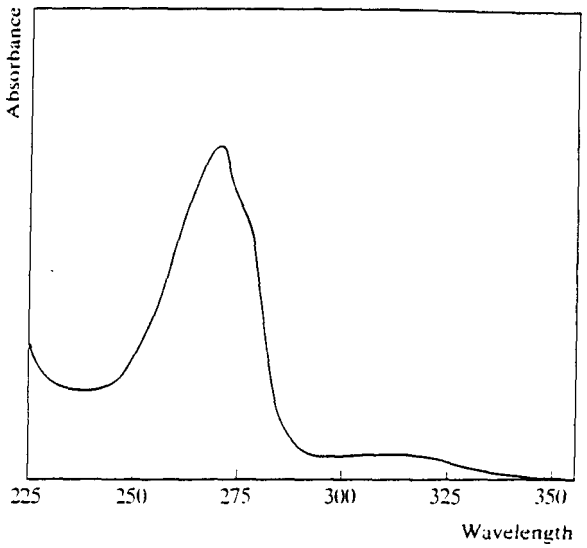


Figure 1.6 UV spectrum of pyrazinamide

Infrared spectra

The infrared spectra for rifampicin is shown in Figure 3.21 (A).

Dissociation constant

pK_a 0.5

Solubility

Soluble 1 in 60 of water and 1 in 110 of ethanol; soluble in chloroform and ether.

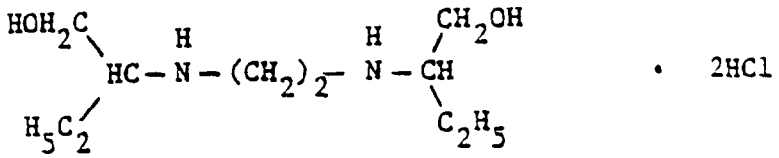
Melting point

188° to 191°. (Moffat et al, 1986c).

1.3.4 ETHAMBUTOL HYDROCHLORIDE

d-2,2'-(ethylenediimino)-di-1-butanol.

Formula and molecula weight



$C_{10}H_{24}O_2N_2(2HCl)$ M.W. = 277.5

Figure 1.7 Structural formula of ethambutol HCl. (Lee, 1978).

Ultraviolet spectra

No significant absorption, 230 to 360nm.

Infrared spectra

The infrared spectra for ethambutol HCl is shown in Figure 3.22 (A).

Dissociation constant

pK_a 6.3,9.5 (20°)

Solubility

Soluble 1 in 1 of water, 1 in 850 of chloroform, and 1 in 9 of methanol; very slightly soluble in ether.

Melting point

199° to 204°. (Moffat et al, 1986d).

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Isoniazid, was obtained from Marsing & Co.Ltd. while pyrazinamide, rifampicin and ethambutol HCl were obtained from Themis Chemicals Limited (Appendix C, D&E). HPLC grade water was obtained from a MilliQ system (Millipore, S.A.). Other reagents used were: acetonitrile and methanol (Hipersolv, BDH), ammonium perchlorate was obtained from BDH (South Africa) and all other chemicals were of analytical grade. The LC Lichrosphere reverse phase C8 column (5μm, 250 x 4 mm) was obtained from Merck (Pty) Ltd.

2.2 METHODS

2.2.1 THE DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYTIC METHOD UTILISING UV DETECTION

HPLC APPARATUS AND CHROMATOGRAPHIC CONDITIONS

HPLC assays were performed with a Beckman 126 pump, a Rheodyne injector and a diode array detector. Analyses were performed on a LC Lichrosphere reverse phase C8 column (5μm, 250 x 4 mm).The mobile phase consisted of acetonitrile (100%), in bottle A and 5mM phosphate buffer pH3.3, in bottle B. The flow rate was 1ml/min with a gradient run. The eluate was detected at 250nm. (Appendix A).

SAMPLE PREPARATION

STOCK SOLUTIONS:

A stock solution of the rifampicin, isoniazid, pyrazinamide and ethambutol HCl was prepared as follows: weighed out 60mg of rifampicin, 30mg of isoniazid, 150mg of pyrazinamide and 100mg of ethambutol HCl and dissolved in 100ml methanol to make a solution with the following concentrations: Rifampicin 600 μg/ml, INH 300 μg/ml, pyrazinamide 1500 μg/ml and ethambutol HCl 1000μg/ml.

STANDARD SOLUTIONS:

For the calibration curve, the following solutions were made:

Solution 1: rifampicin - 600μg/ml; isoniazid - 300μg/ml; pyrazinamide - 1500μg/ml; ethambutol HCl - 1000μg/ml, pipette out 10ml of the stock solution.

Solution 2: rifampicin - 300μg/ml; isoniazid - 150μg/ml; pyrazinamide - 750μg/ml; ethambutol HCl - 500μg/ml, pipette out 5ml of the stock solution and make up to volume with methanol in a 10ml volumetric flask.

Solution 3: rifampicin - 150μg/ml; isoniazid - 75μg/ml; pyrazinamide - 375μg/ml; ethambutol HCl - 250μg/ml, pipette out 2.5ml of the stock solution and make up to volume with methanol in a 10ml volumetric flask..

Solution 4: rifampicin - 90μg/ml; isoniazid - 45μg/ml; pyrazinamide - 225μg/ml; ethambutol HCl - 125μg/ml, pipette out 1.5ml of the stock solution and make up to volume with methanol in a 10ml volumetric flask.

Solution 5: rifampicin - 60μg/ml; isoniazid - 30μg/ml; pyrazinamide - 150μg/ml; ethambutol HCl - 62.5μg/ml, pipette out 1ml of the stock solution and make up to volume with methanol in a 10ml volumetric flask.

2.2.2 THE DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYTIC METHOD UTILISING ELECTROCHEMICAL DETECTION

HPLC APPARATUS AND CHROMATOGRAPHIC CONDITIONS

A Hewlett Packard 1050 HPLC system was used with a Rheodyne (20μl) loop. The LC Lichrosphere reversed phase C8 column (5μm, 250 x 4 mm) was used at ambient temperature. The mobile phase consisted of 200mM ammonium perchlorate in 75% methanol and 25% water pH 9.3. The analytes were measured using a Hewlett Packard 1049 A electrochemical detector with an applied potential of 1 Volt in the amperometric mode. (Appendix B). The samples were integrated by a HP5890 Chemstation from the external standard calibration table.

SAMPLE PREPARATION

STOCK SOLUTIONS:

A stock solution of the rifampicin, isoniazid, pyrazinamide and ethambutol HCl was prepared as follows: weighed out 60mg of rifampicin, 30mg of isoniazid, 150mg of pyrazinamide and 100mg of ethambutol HCl and dissolved in 100ml buffer solution to make a solution with the following concentrations: Rifampicin 600 μg/ml, INH 300 μg/ml, pyrazinamide 1500 μg/ml and ethambutol HCl 1000μg/ml.

STANDARD SOLUTIONS:

For the calibration curve, the following solutions were made:

Solution 1: rifampicin - 600μg/ml; isoniazid - 300μg/ml; pyrazinamide - 1500μg/ml; ethambutol HCl - 1000μg/ml, pipette out 25ml of the stock solution.

Solution 2: rifampicin - 450μg/ml; isoniazid - 225μg/ml; pyrazinamide - 1125μg/ml; ethambutol HCl - 750μg/ml, pipette out 18.75ml of the stock solution and make up to volume with buffer in a 25ml volumetric flask.

Solution 3: rifampicin - 300μg/ml; isoniazid - 150μg/ml; pyrazinamide - 750μg/ml; ethambutol HCl - 500μg/ml, pipette out 12.5ml of the stock solution and make up to volume with buffer in a 25ml volumetric flask..

Solution 4: rifampicin - 150μg/ml; isoniazid - 75μg/ml; pyrazinamide - 375μg/ml; ethambutol HCl - 250μg/ml, pipette out 6.25ml of the stock solution and make up to volume with buffer in a 25ml volumetric flask.

2.2.3 COMPATIBILITY STUDY BETWEEN ACTIVE INGREDIENTS USING FOURIER TRANSFORM INFRARED SPECTROSCOPY

APPARATUS AND SAMPLE PREPARATION

Samples were prepared in KBr tablets and FT-IR spectra of each of the drugs individually and in combination were carried out in an Impact 4000 FT-IR Spectrometer. Differential spectra of the combinations were determined and interactions were evaluated by comparing the differential spectra to that of the individual compounds.

KBr tablets were prepared by intimately grinding 2g of KBr and 0.02g of active ingredient with a mortar and pestle. For combination studies, 0.02g of each active ingredient was utilized.

3.0 RESULTS

3.1 ASSAY DEVELOPMENT

3.1.1 HPLC ANALYTICAL METHOD UTILISING UV DETECTION

3.1.1.1 *PERFORMANCE OF HPLC SYSTEM*

Figure 3.1 shows a chromatogram for the separation of rifampicin, isoniazid, and pyrazinamide. The run time for this method of analysis is 15 minutes with retention times as follows: isoniazid, 4.5 minutes; pyrazinamide, 5.4 minutes and rifampicin, 9.1 minutes.

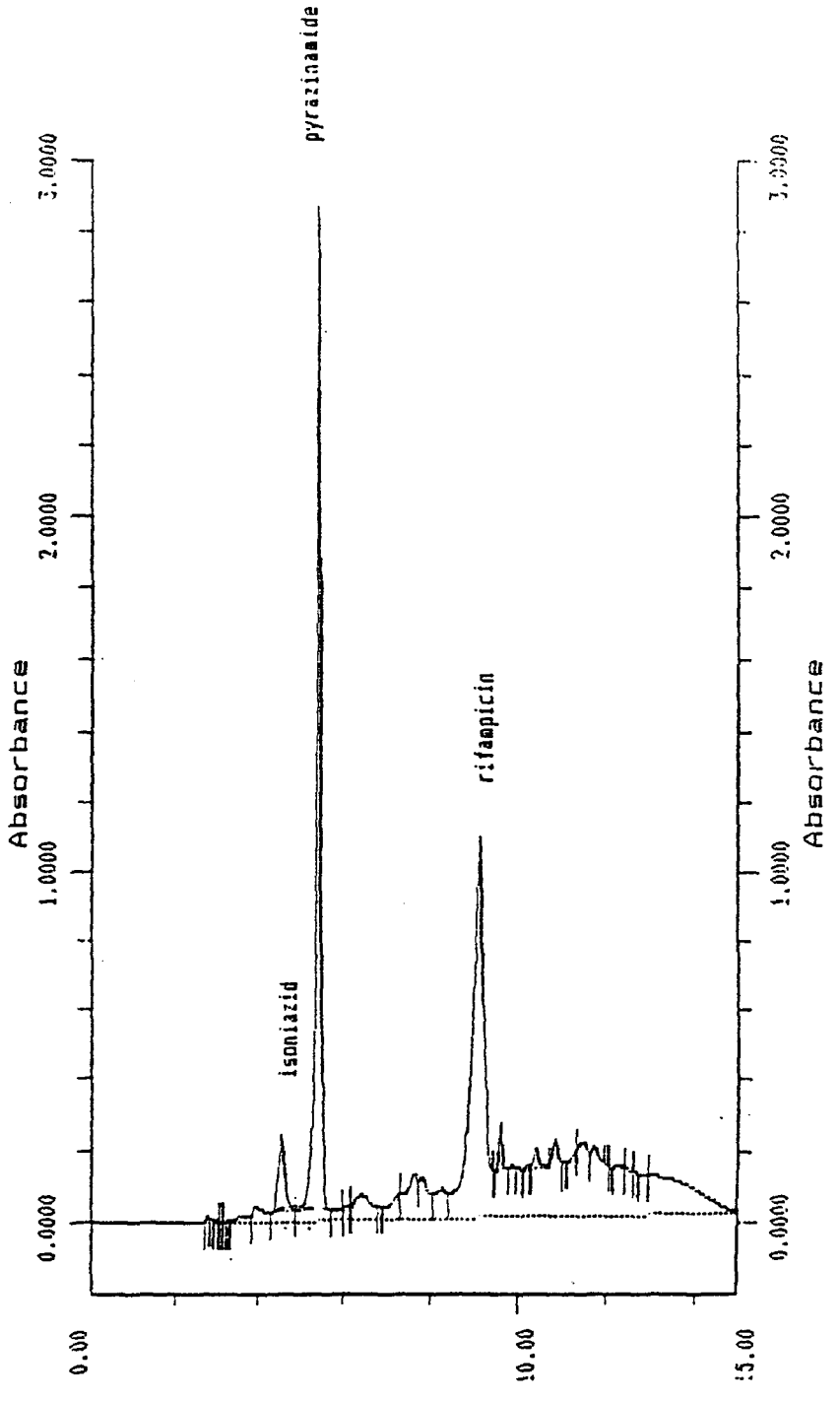


Figure 3.1 UV chromatogram of rifampicin, isoniazid and pyrazinamide

3.1.1.2 *SPECIFICITY*

The specificity of the method was examined under conditions listed in Appendix A. The individual elution profiles are shown in Figures 3.2, 3.3 and 3.4 for rifampicin, isoniazid and pyrazinamide respectively. In comparison with the chromatogram in Figure 3.1 it can be seen that rifampicin, isoniazid and pyrazinamide can be detected as separate distinct peaks with similar retention times and the presence of shoulder peaks in Figures 3.2, 3.3 and 3.4 are the result of a gradient run.

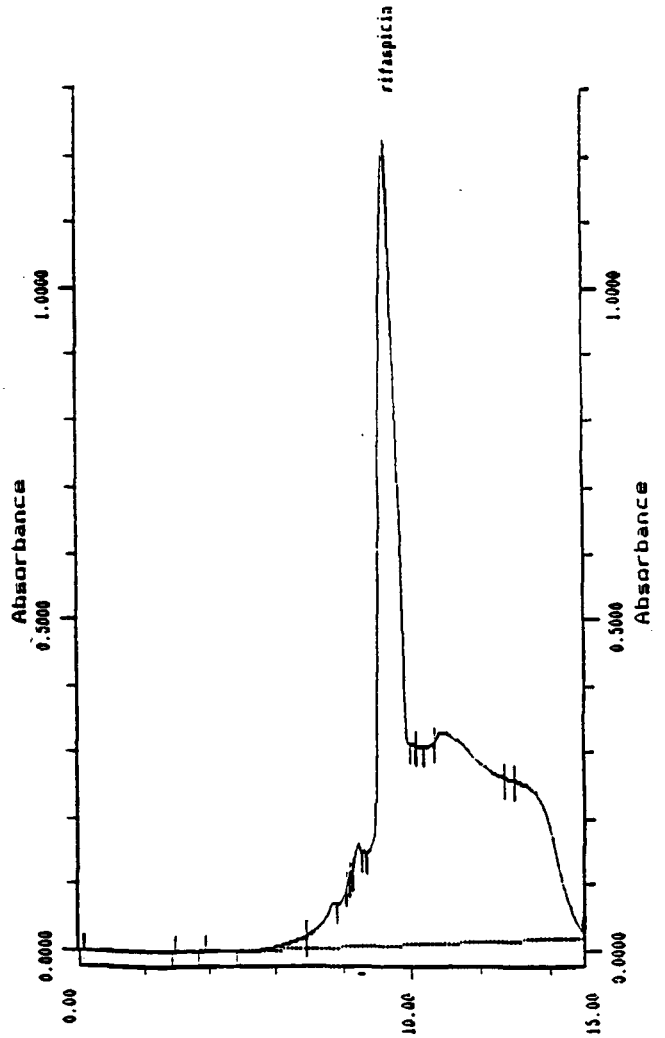


Figure 3.2 UV chromatogram of rifampicin

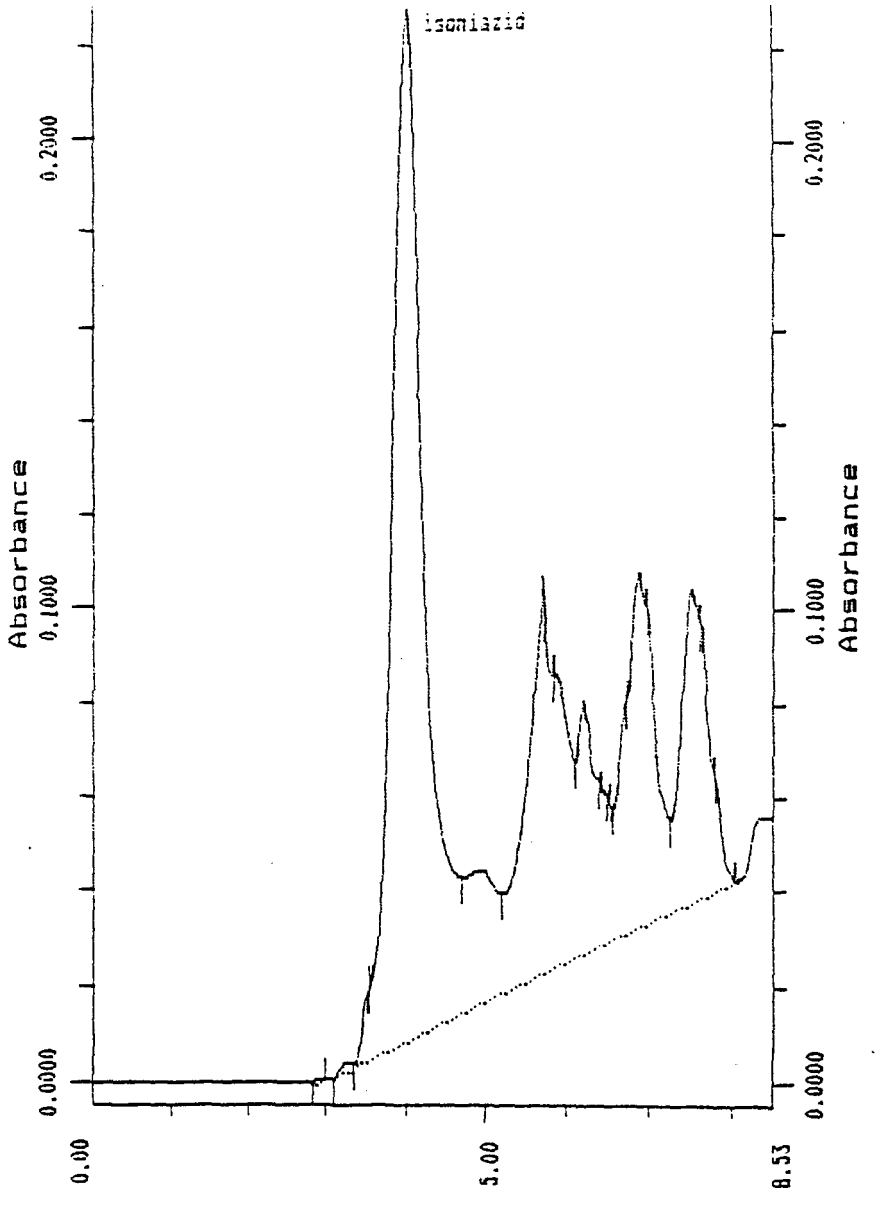


Figure 3.3 *UV chromatogram of isoniazid*

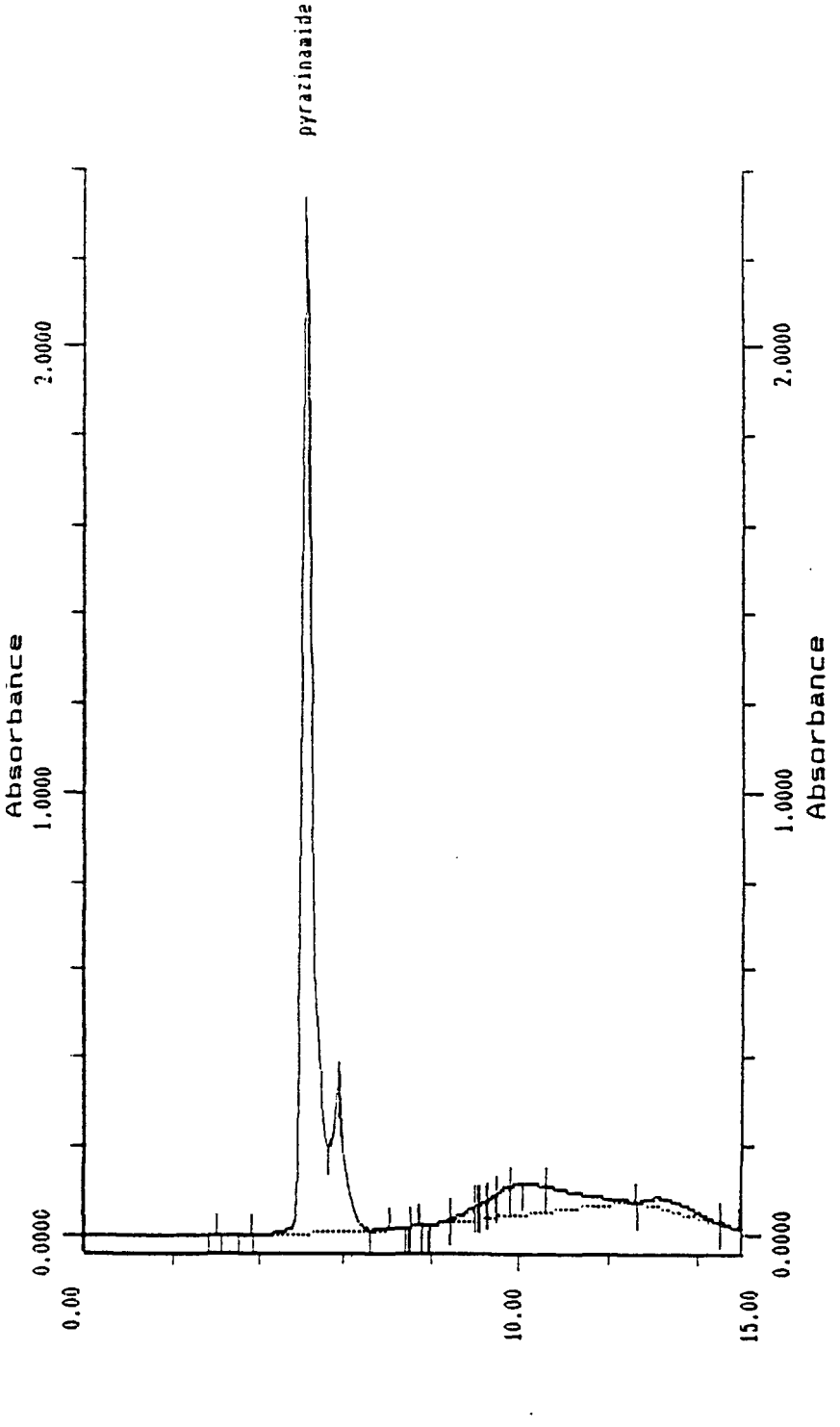


Figure 3.4 UV chromatogram of pyrazinamide

3.1.1.3 *LINEARITY*

Rifampicin, isoniazid, and pyrazinamide were assayed over the concentration ranges as mentioned in section 2.2.1. The calibration curves are plotted in figures 3.5, 3.6 and 3.7 and show a good line-fit correlation for rifampicin, isoniazid, and pyrazinamide respectively. The correlation coefficients for rifampicin, isoniazid and pyrazinamide are 0.99571; 0.99635; and 0.98885 respectively.

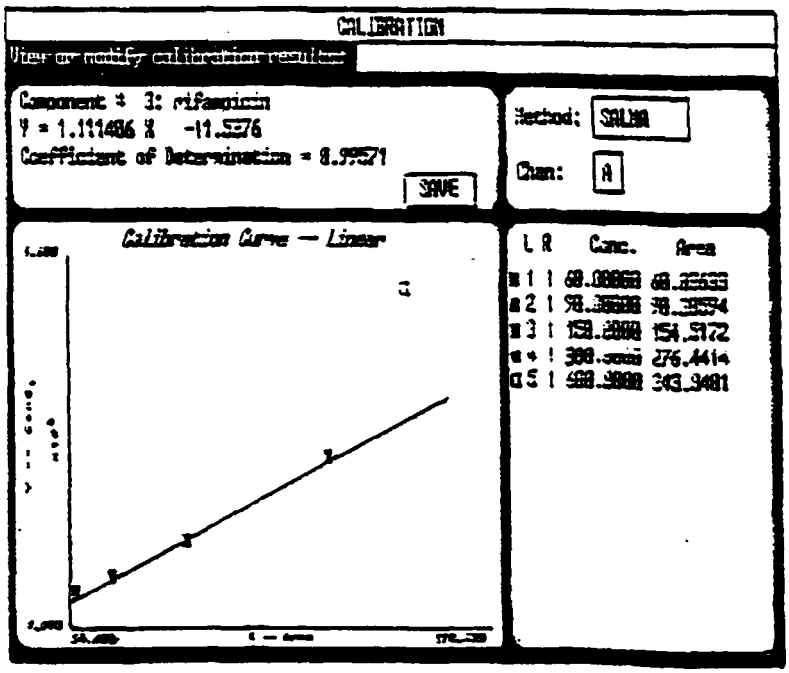


Figure 3.5 Calibration curve of rifampicin (UV detection)

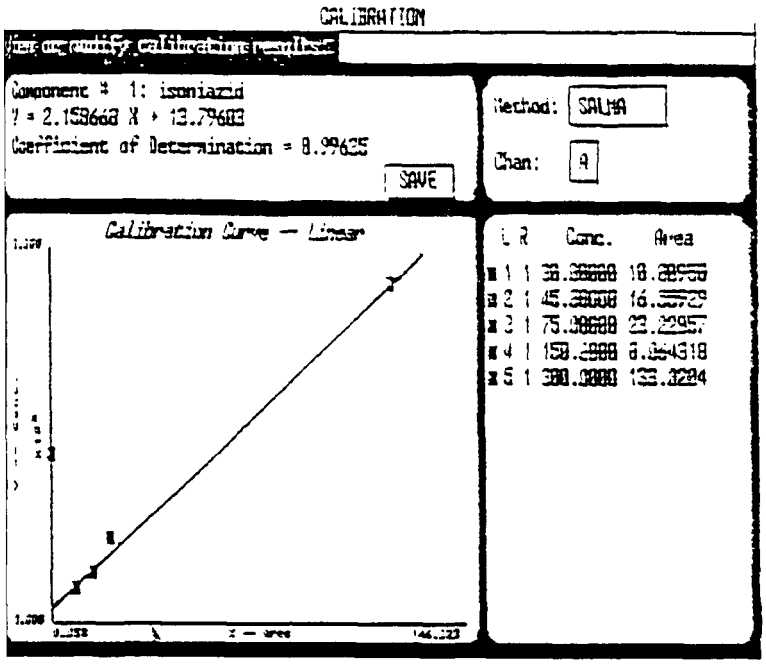


Figure 3.6 Calibration curve of isoniazid (UV detection)

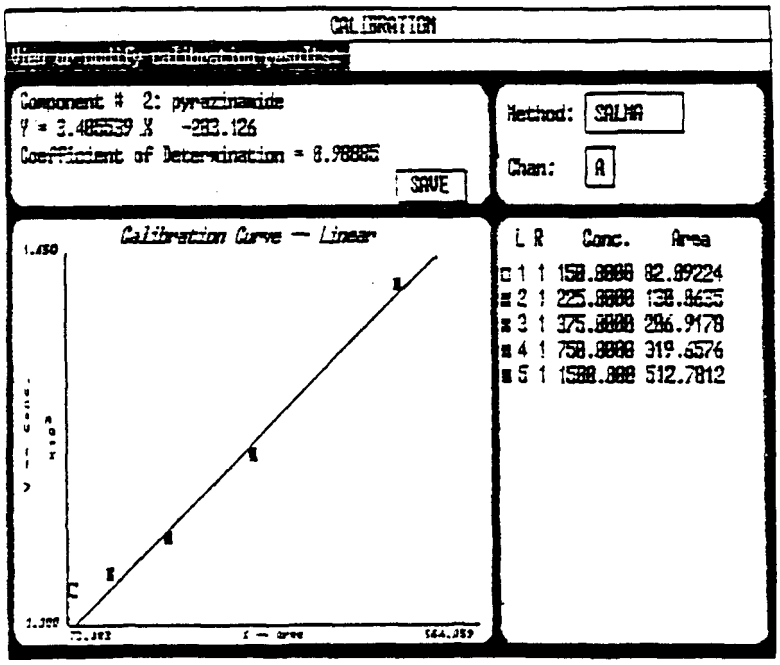


Figure 3.7 Calibration curve of pyrazinamide (UV detection)

3.1.1.4 *PRECISION*

INTRA-ASSAY VARIABILITY

The method was tested for intra-assay variability by replicate analysis of samples with known concentrations of rifampicin, isoniazid, and pyrazinamide. Assays were performed using a standard solution containing the following concentrations: Rifampicin 300μg/ml, isoniazid 150μg/ml, and pyrazinamide 750μg/ml. From the peak areas obtained, the concentrations were determined from the calibration curves in figures 3.5, 3.6 and 3.7, and the results for the intra-assay variations are shown in table 3.1.

TABLE 3.1 Precision Studies: Intra-Assay Variability for rifampicin, isoniazid and pyrazinamide

Sample No.	Assay of Rifampicin μg/ml	Assay of Isoniazid μg/ml	Assay of Pyrazinamide μg/ml
1	296.17	135.96	750.48
2	307.03	138.43	741.42
3	342.13	138.03	768.88
4	325.76	137.01	766.07
5	323.5	138.17	750.73
Mean	318.92	137.52	755.52
Standard deviation	15.91	0.92	10.36
%Relative standard deviation	4.99	0.67	0.014
Range	296.17-342.13	135.96-138.43	741.42-768.88

3.1.1.5 *SYSTEM SUITABILITY TEST*

System suitability parameters give an indication of column performance and also shows that the method employed is suitable for the analysis being carried out. These parameters were calculated from the chromatogram in figure 3.1.

3.1.1.5.1 Resolution

$(R)_S = 2\ Z/\ (W_A + W_B)$

where: $(R)_S$ is resolution

Z is the retention time of species B - the retention time of species A

W_A & W_B are the peak widths of species A & B

Table 3.2 Peak resolution for isoniazid, rifampicin, and pyrazinamide (UV detected)

Peak name	Resolution R_s
Isoniazid	0.790
Rifampicin	1.156
Pyrazinamide	0.646

From the chromatogram in figure 3.1 it is evident that the peaks are well resolved. A value of > 1 indicates good resolution. While, the results in table 3.2 indicate that the resolution for isoniazid and pyrazinamide are not optimal they are sufficient for the purposes of this assay. Further improvements can be obtained for example by increasing the column length.

3.1.1.5.2 Selectivity and Capacity factor

Selectivity factor

$$\alpha = \{(t_r)_B - t_m\}/\{(t_r)_A - t_m\}$$

where: α is selectivity factor

$(t_r)_B$ & $(t_r)_A$ are the retention times of species B and A

t_m is the retention time of the non retained species

Capacity factor

$$k' = \{t_r - t_m\}/t_m$$

where: k' is the capacity factor

t_r is the retention time of species

t_m is the retention time of the non retained species

Table 3.3 Column selectivity and capacity factor for isoniazid, rifampicin, and pyrazinamide (UV detected)

Peak name	Capacity factor k'	Column Selectivity α
Isoniazid	1.077	1.55
Rifampicin	3.154	0.3958
Pyrazinamide	1.469	2.53

A value of >1 for both parameters indicates that the peaks do exist discretely. However, rifampicin has a column selectivity factor of 0.3958 which may be improved by varying the type of column or solvent system used in this assay technique.

3.1.1.5.3 Efficiency

$N=16(t_r/w_b)^2$ at base line

$N=5.545(t_r/w_{b1/2})^2$ at half peak height

where: N is efficiency

t_r is the retention time of species

w_b is the peak width at its base

$w_{b1/2}$ is the peak width at half of peak height

Table 3.4 Efficiency for isoniazid, rifampicin, and pyrazinamide (UV detected)

Peak name	Efficiency N
Isoniazid	115.81
Rifampicin	602463
Pyrazinamide	163.55

This parameter gives an indication of column performance and its value may range from tens to thousands of units and it is specific for a particular drug.

3.1.1.5.4 Asymmetry

Asymmetry = b/a. Asymmetry is a measure of peak symmetry and indicates whether the peaks are symmetrical, fronting or tailing. The results below, indicate that perfectly symmetrical peaks were obtained.

Table 3.5 Peak asymmetry for isoniazid, rifampicin, and pyrazinamide (UV detected)

Peak name	Asymmetry
Isoniazid	1
Rifampicin	1
Pyrazinamide	1

3.1.1.6 *APPLICABILITY*

In view of the performance of the HPLC system, specificity, linearity, and precision, no problems are envisaged in the applicability of the method.

3.1.1.7 *OTHER EXPERIMENTAL FINDINGS*

Phenelzine sulphate, anhydrous caffeine, and quinine were investigated to find a suitable internal standard. None of the compounds produced a sufficiently quantifiable or stable peak that could be used as an internal standard.

3.1.2 HPLC METHOD UTILISING ELECTROCHEMICAL DETECTION

3.1.2.1 *PERFORMANCE OF HPLC SYSTEM*

Figure 3.8 shows a chromatogram for the separation of rifampicin, isoniazid, and ethambutol HCl. The run time for this method of analysis is 10 minutes with retention times as follows: isoniazid, 2.515 minutes; ethambutol HCl, 2.815 minutes and rifampicin, 6.490 minutes.

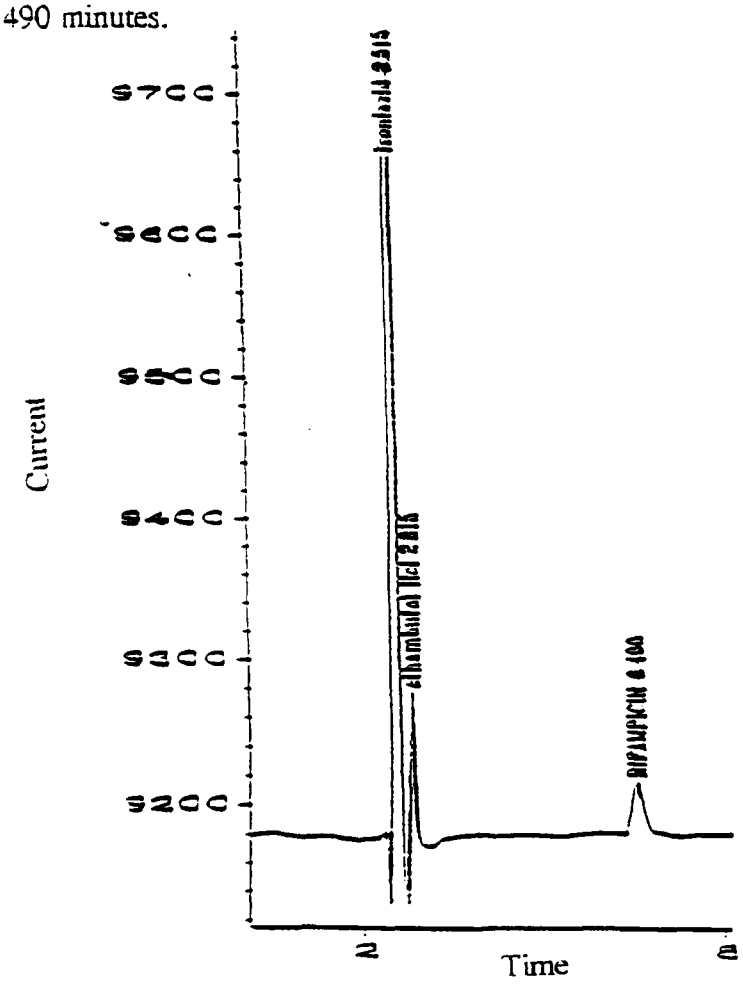


Figure 3.8 Electrochemically detected chromatogram of the solution of isoniazid, ethambutol HCl and rifampicin.

3.1.2.2 *SPECIFICITY*

The specificity of the method was examined under conditions listed in Appendix B. The individual elution profiles are shown in Figures 3.9, 3.10 and 3.11 for rifampicin, isoniazid and ethambutol HCl respectively. In comparison with the chromatogram in Figure 3.8 it can be seen that rifampicin, isoniazid and pyrazinamide can be detected as separate distinct peaks with similar retention times.

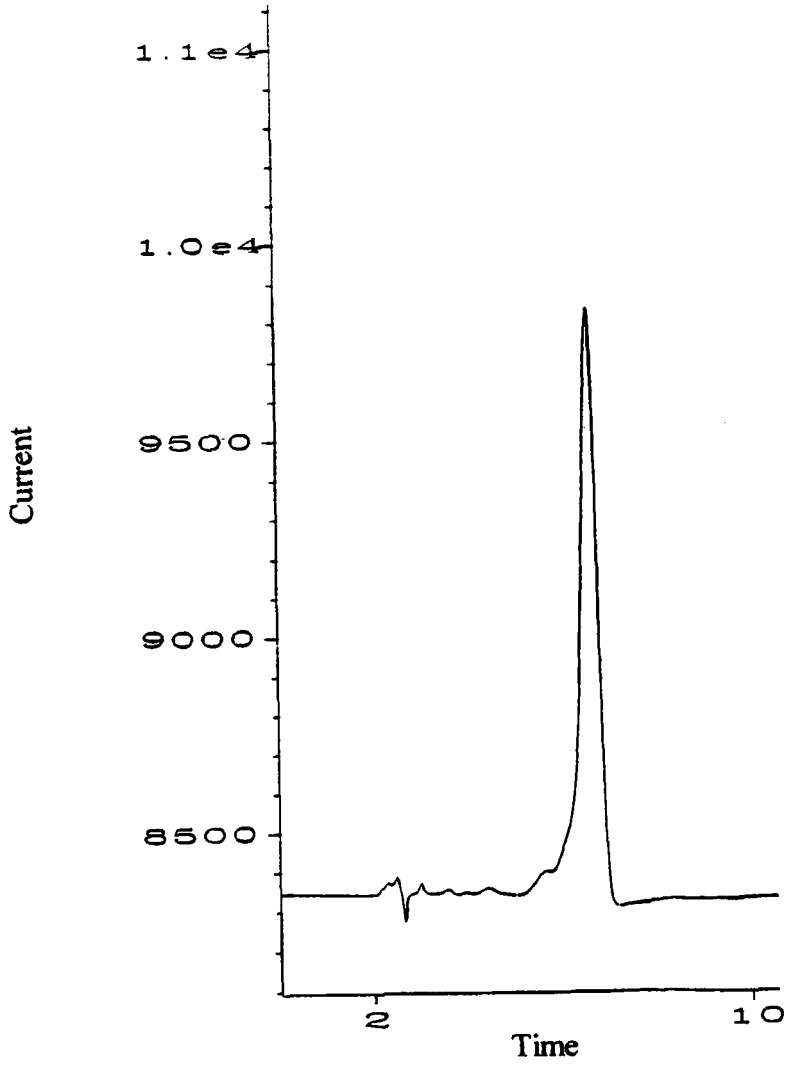


Figure 3.9 ECD chromatogram of rifampicin

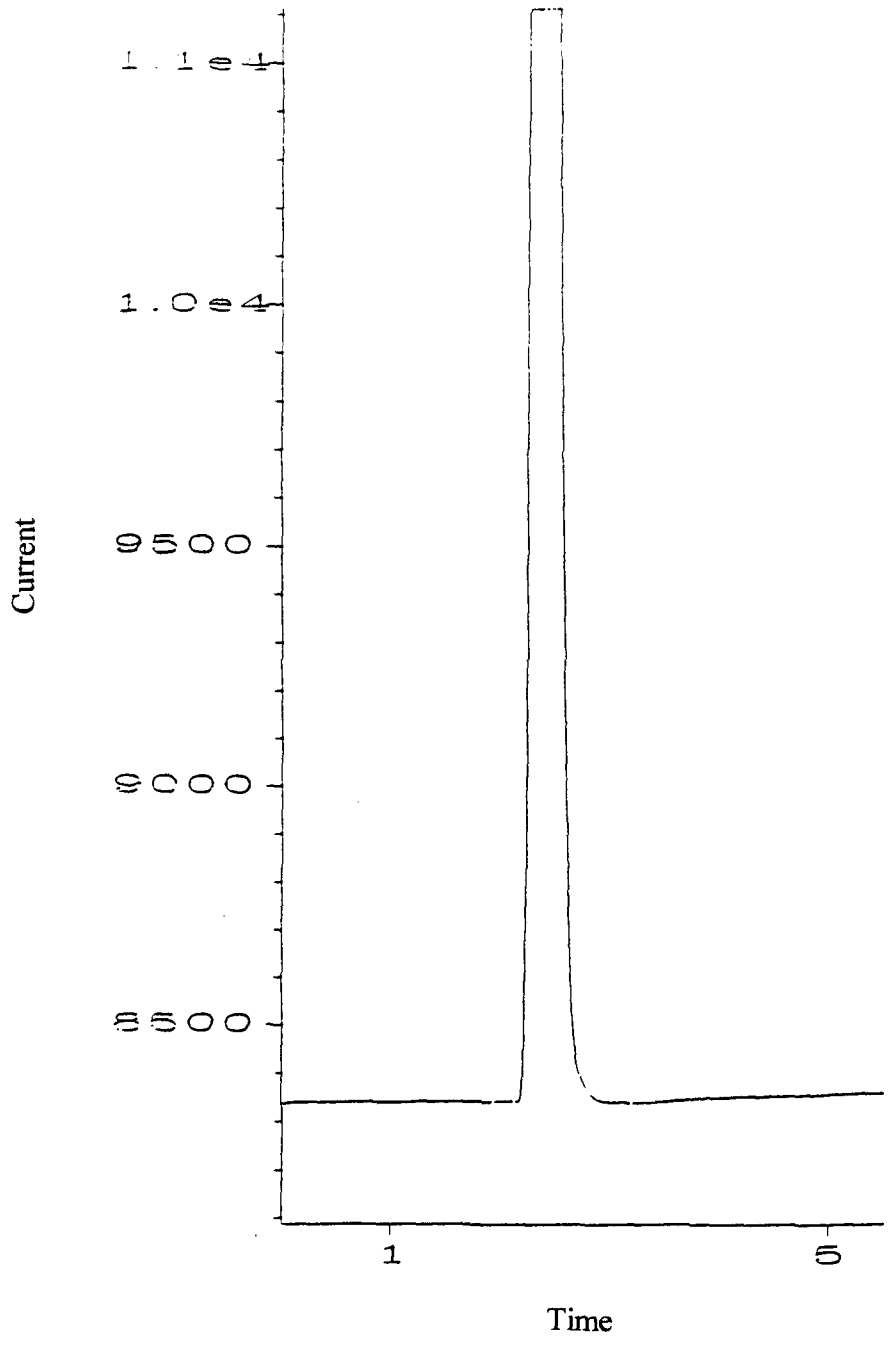


Figure 3.10 ECD chromatogram of isoniazid

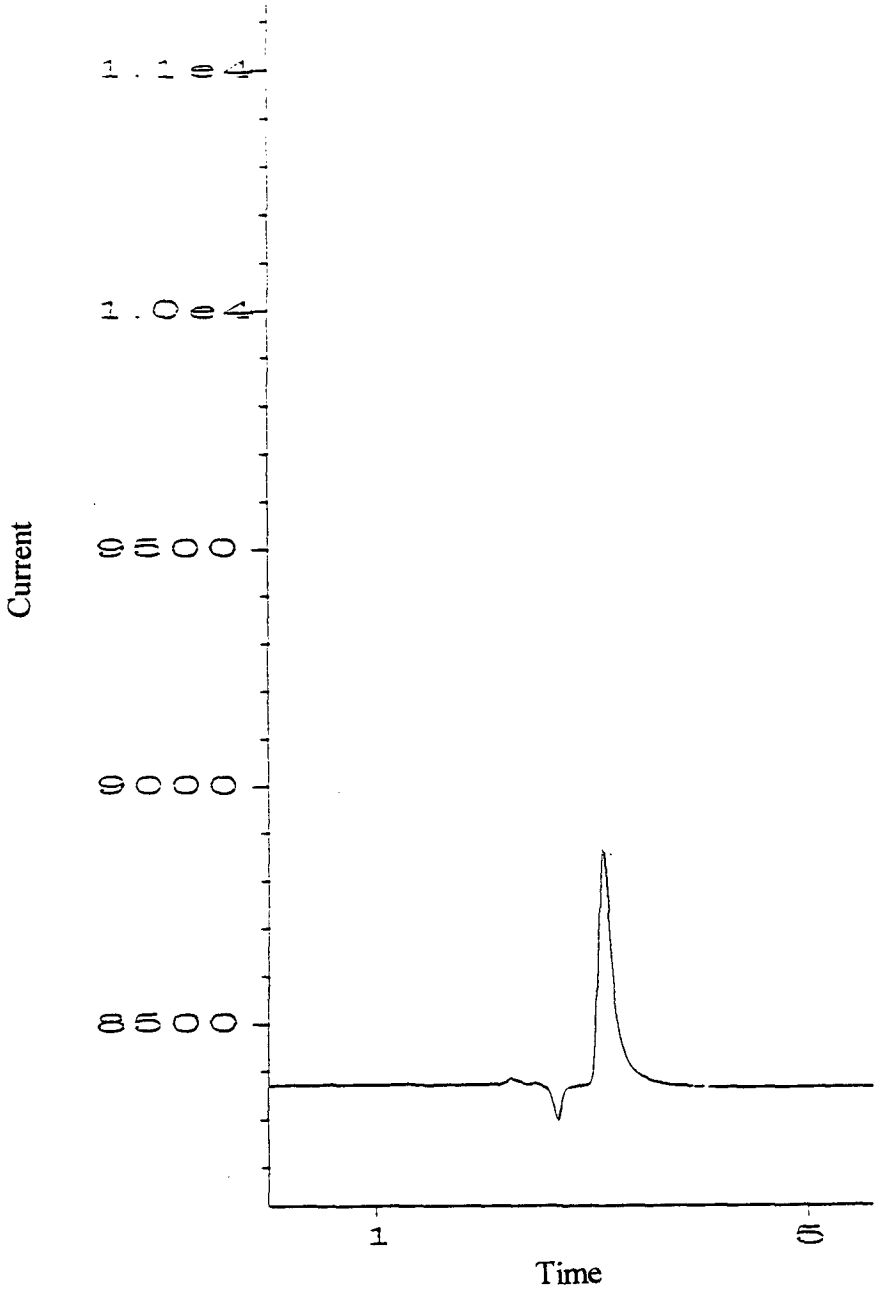


Figure 3.11 ECD chromatogram of ethambutol HCl

3.1.2.3 *LINEARITY*

The calibration curves are plotted in figures 3.12, 3.13, and 3.14 and show a good line-fit correlation for isoniazid, ethambutol HCl and rifampicin over the concentration ranges as indicated in section 2.2.2. The correlation coefficients for isoniazid, ethambutol HCl and rifampicin, are 0.990; 0.982; and 0.985 respectively.

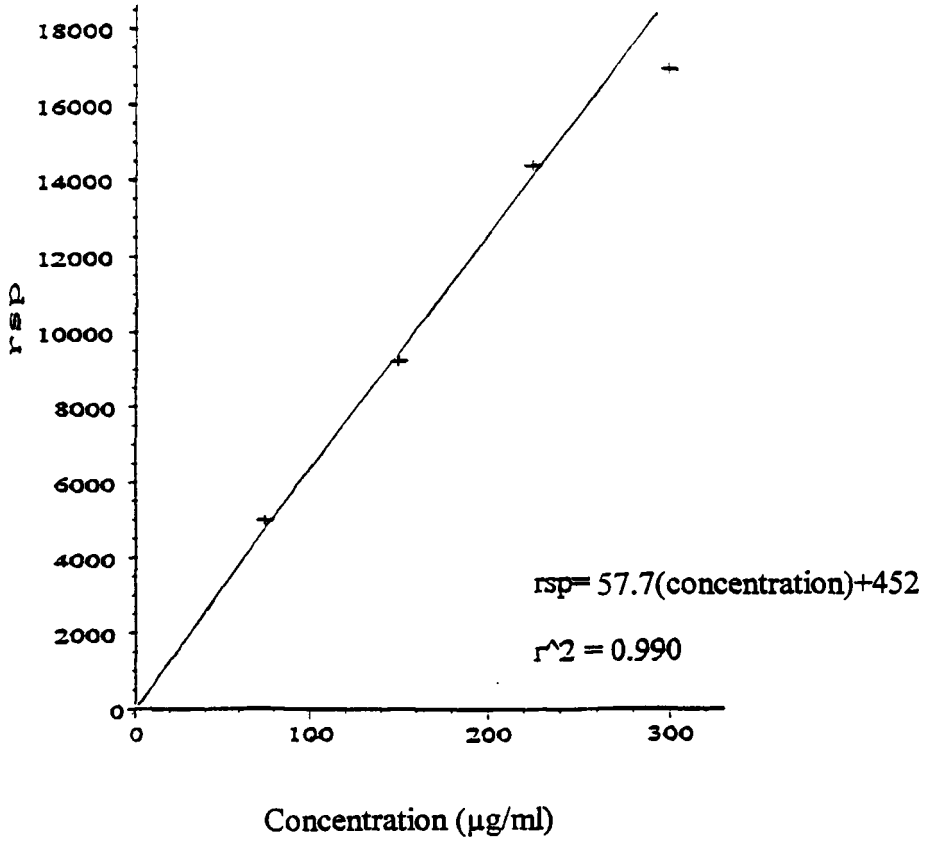


Figure 3.12 Calibration curve of isoniazid (electrochemical detection)

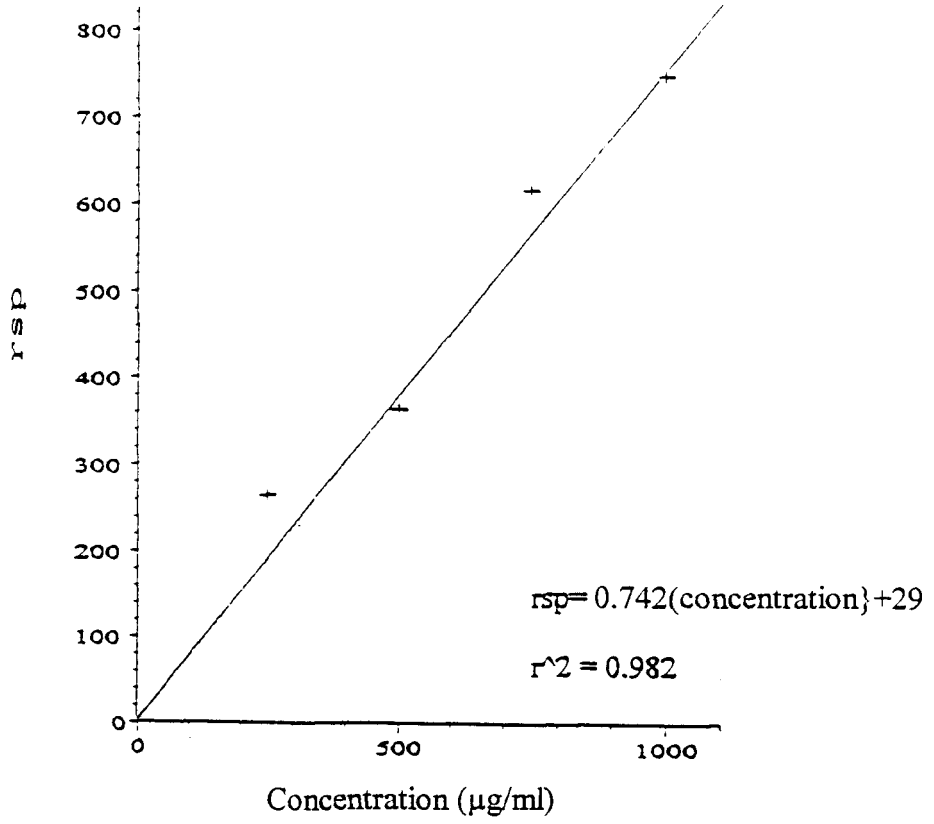


Figure 3.13 Calibration curve of ethambutol HCl (electrochemical detection)

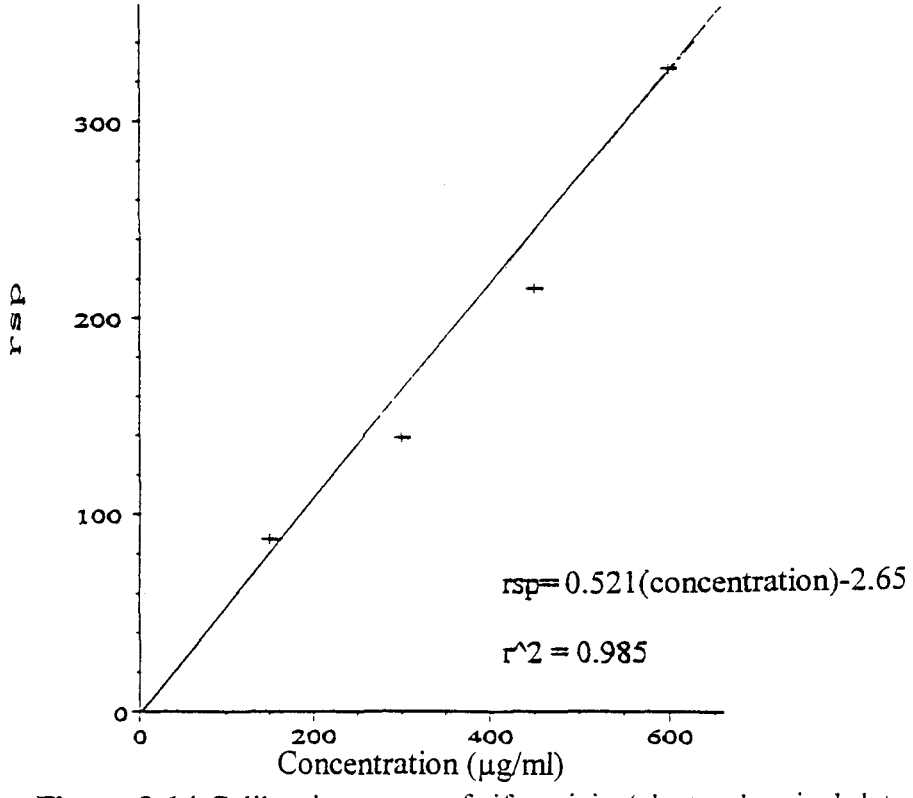


Figure 3.14 Calibration curve of rifampicin (electrochemical detection)

3.1.2.4 *PRECISION*

INTRA-ASSAY VARIABILITY

The method was tested for intra-assay variability by replicate analysis of samples with known concentrations of rifampicin, isoniazid, and ethambutol HCl. Assays were performed using a standard solution containing the following concentrations: Rifampicin 600μg/ml, isoniazid 300μg/ml, and ethambutol HCl 1500μg/ml. From the peak areas obtained, the concentrations were determined from the calibration curves in figures 3.12, 3.13 and 3.14, and the results for the intra-assay variations are shown in table 3.6.

TABLE 3.6 Precision Studies: Intra-Assay Variability for rifampicin, isoniazid and ethambutol HCl (ECD)

Sample No.	Assay of Rifampicin μg/ml	Assay of Isoniazid μg/ml	Assay of Ethambutol HCl μg/ml
1	592.34	291.92	988.50
2	610.26	296.86	1112.01
3	614.26	286.34	1006.22
4	596.52	294.02	987.36
5	608.47	288.06	987.92
Mean	604.37	291.44	1016.40
Standard deviation	8.43	3.84	48.33
%Relative standard deviation	1.40	1.32	4.75
Range	592.34-614.26	288.06-296.86	987.36-1112.01

3.1.2.5 *SYSTEM SUITABILITY TEST*

System suitability parameters give an indication of column performance and also shows that the method employed is suitable for the analysis being carried out. These parameters were calculated from the chromatogram in figure 3.8. Calculations shown below, were carried out as shown in section 3.1.1.5.

3.1.2.5.1 Resolution

Table 3.7 Peak resolution for isoniazid, rifampicin, and ethambutol HCl (ECD)

Peak name	**Resolution R_s**
Isoniazid	0.158
Rifampicin	2.145
Ethambutol HCl	0.308

From the chromatogram in figure 3.8 it is evident that the peaks are well resolved. A value of >1 indicates good resolution. While, the results in table 3.7 indicate that the resolution for isoniazid and ethambutol HCl are not optimal, they are sufficient for the purposes of this assay. Further improvements can be obtained for example by increasing the column length .

3.1.2.5.2 Selectivity and Capacity factor

Table 3.8 Column selectivity and capacity factor for isoniazid, rifampicin, and ethambutol HCl (ECD)

Peak name	Capacity factor k'	Column Selectivity α
Isoniazid	0.143	1.955
Rifampicin	1.95	0.143
Ethambutol HCl	0.28	6.976

Capacity factor and selectivity are a measure of the separation capabilities of the column used. A value greater than 1 for both parameters indicates that peaks exist discretely. However, not all the values calculated above are optimal yet, from the chromatogram in figure 3.8 it is evident that the peaks do exist discretely. Further improvements can be obtained for example by increasing the column length or adjusting the solvent system.

3.1.2.5.3 Efficiency

Table 3.9 Efficiency for isoniazid, rifampicin, and ethambutol HCl (ECD)

Peak name	Efficiency N
Isoniazid	25.3009
Rifampicin	26.9569
Ethambutol HCl	31.00

This parameter gives an indication of column performance and its value may range from tens, to thousands of units and it is specific for a particular drug.

3.1.2.5.4 Asymmetry

Table 3.10 Peak asymmetry for isoniazid, rifampicin, and ethambutol HCl (ECD)

Peak name	Asymmetry
Isoniazid	1
Rifampicin	1
Pyrazinamide	1

The above results indicate that perfectly symmetrical peaks were obtained.

3.1.2.6 *APPLICAPABILITY*

In view of the performance of the HPLC system, specificity, linearity, and precision, no problems are envisaged in the applicability of the method.

3.1.2.7 *OTHER EXPERIMENTAL FINDINGS*

Different concentrations of sodium perchlorate in 25% water and 75% methanol were investigated for suitability as solvent systems for the separation of rifampicin, isoniazid, and ethambutol HCl. The concentrations were 100mM, 250mM, and 500mM sodium perchlorate solution. However, the peaks were poorly resolved. Further investigations showed that better separation was achieved with the ammonium perchlorate as a solvent system.

Phenelzine sulphate, anhydrous caffeine, codeine phosphate, atropine and quinine were investigated to find a suitable internal standard. None of the compounds produced a sufficiently quantifiable or stable peak that could be used as an internal standard.

3.2 COMPATIBILITY STUDY BETWEEN ACTIVE INGREDIENTS UTILISING FOURIER TRANSFORM INFRARED SPECTROSCOPY

Table 3.11 shows a number of different combinations of rifampicin, isoniazid, pyrazinamide and ethambutol HCl. The results from the FT-IR interaction studies are shown in figures 3.15-3.26.

TABLE 3.11 Different combinations of drugs used in FT-IR interaction studies

rifampicin +isoniazid	rifampicin +pyrazinamide	rifampicin +ethambutol HCl
isoniazid +pyrazinamide	isoniazid +ethambutol HCl	isoniazid +rifampicin
pyrazinamide +isoniazid	pyrazinamide +ethambutol HCl	pyrazinamide +rifampicin
ethambutol HCl +isoniazid	ethambutol HCl +pyrazinamide	ethambutol HCl +rifampicin

No significant differences between the differential FT-IR spectrum of any of the drug combinations in comparison to the individual drugs were found. The spectra for the different combinations in comparison with the individual drugs are shown in Figures 3.15-3.26. These results show that it is unlikely for physical interactions between any of the drugs in combination to be occurring, since the major bands (peak maxima and minima) in the infrared spectra have been retained for each drug even when in combination.

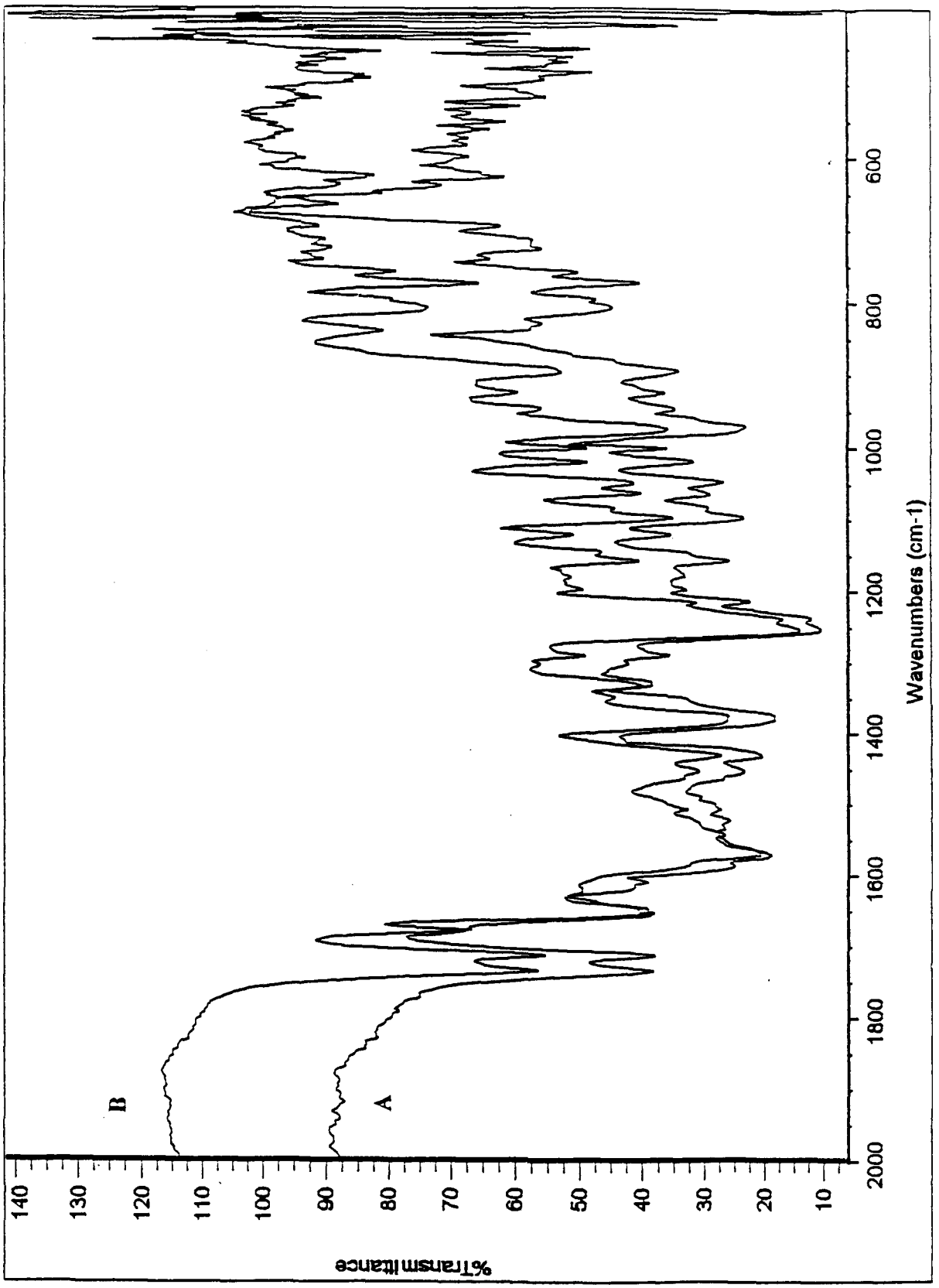


Figure 3.15 FT-IR spectrum of rifampicin (A) and differential spectrum of a physical mixture of rifampicin/isoniazid (B)

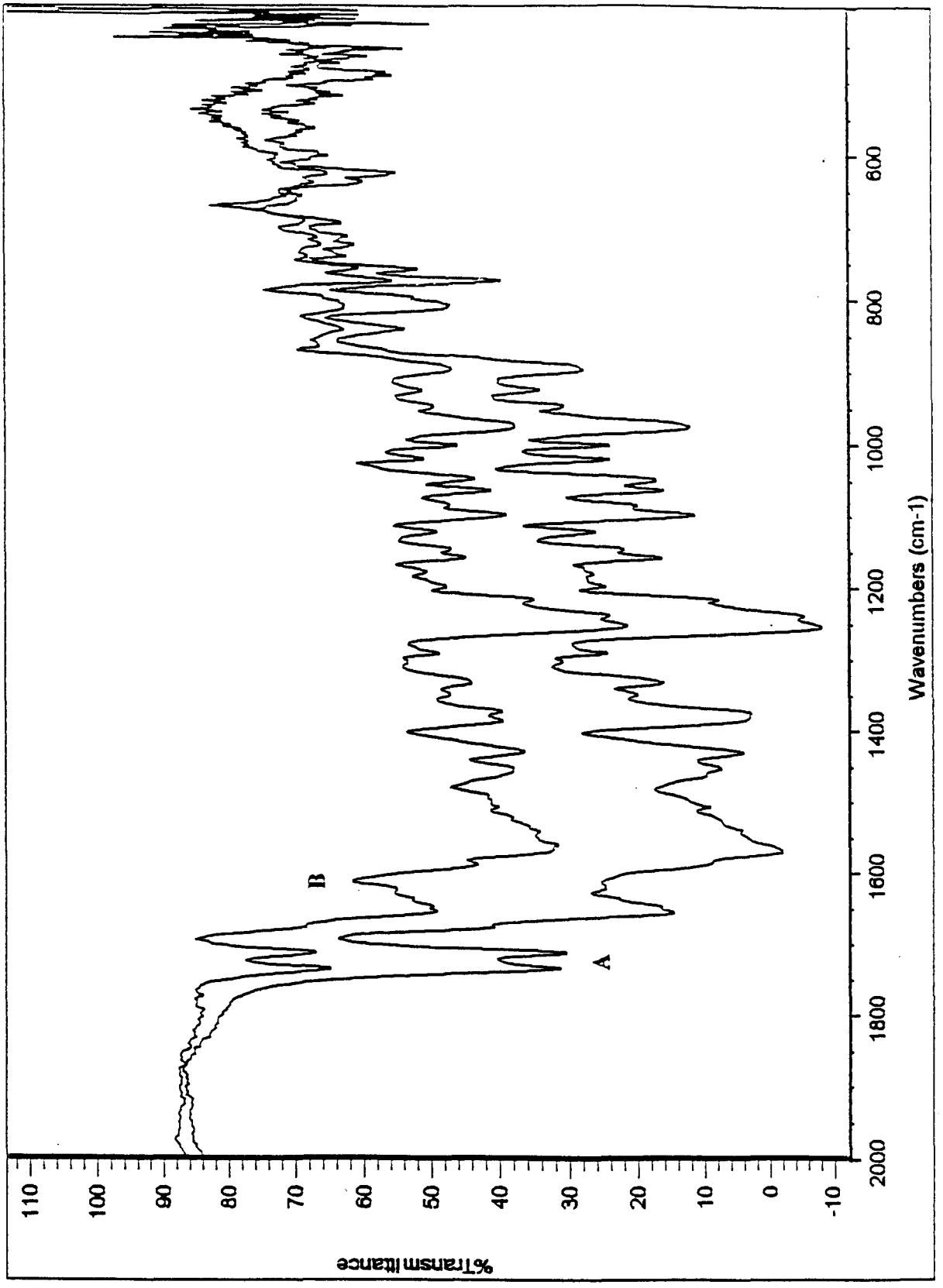


Figure 3.16 FT-IR spectrum of rifampicin (A) and differential spectrum of a physical mixture of rifampicin/pyrazinamide (B)

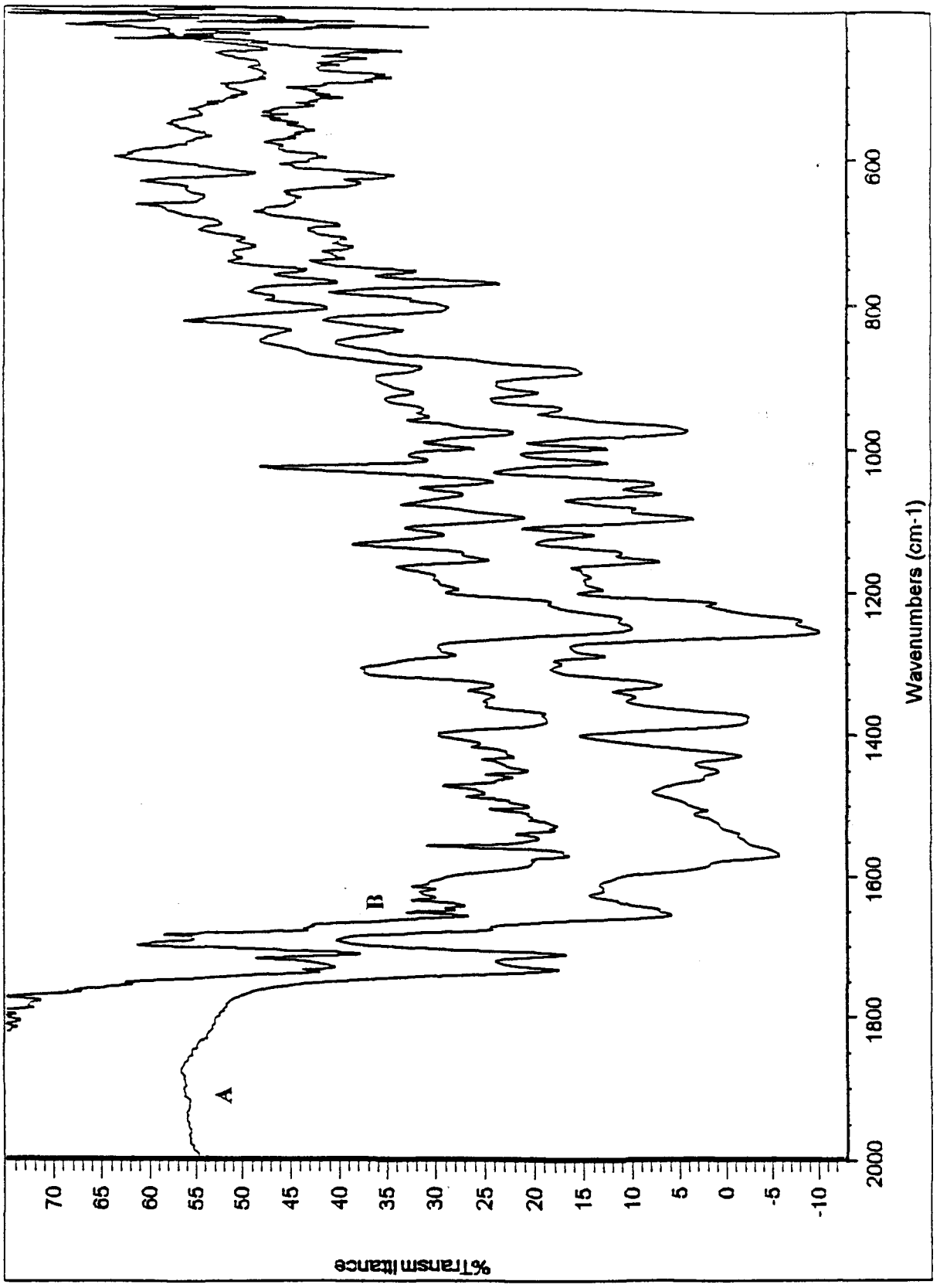


Figure 3.17 FT-IR spectrum of rifampicin (A) and differential spectrum of a physical mixture of rifampicin/ethambutol HCl (B)

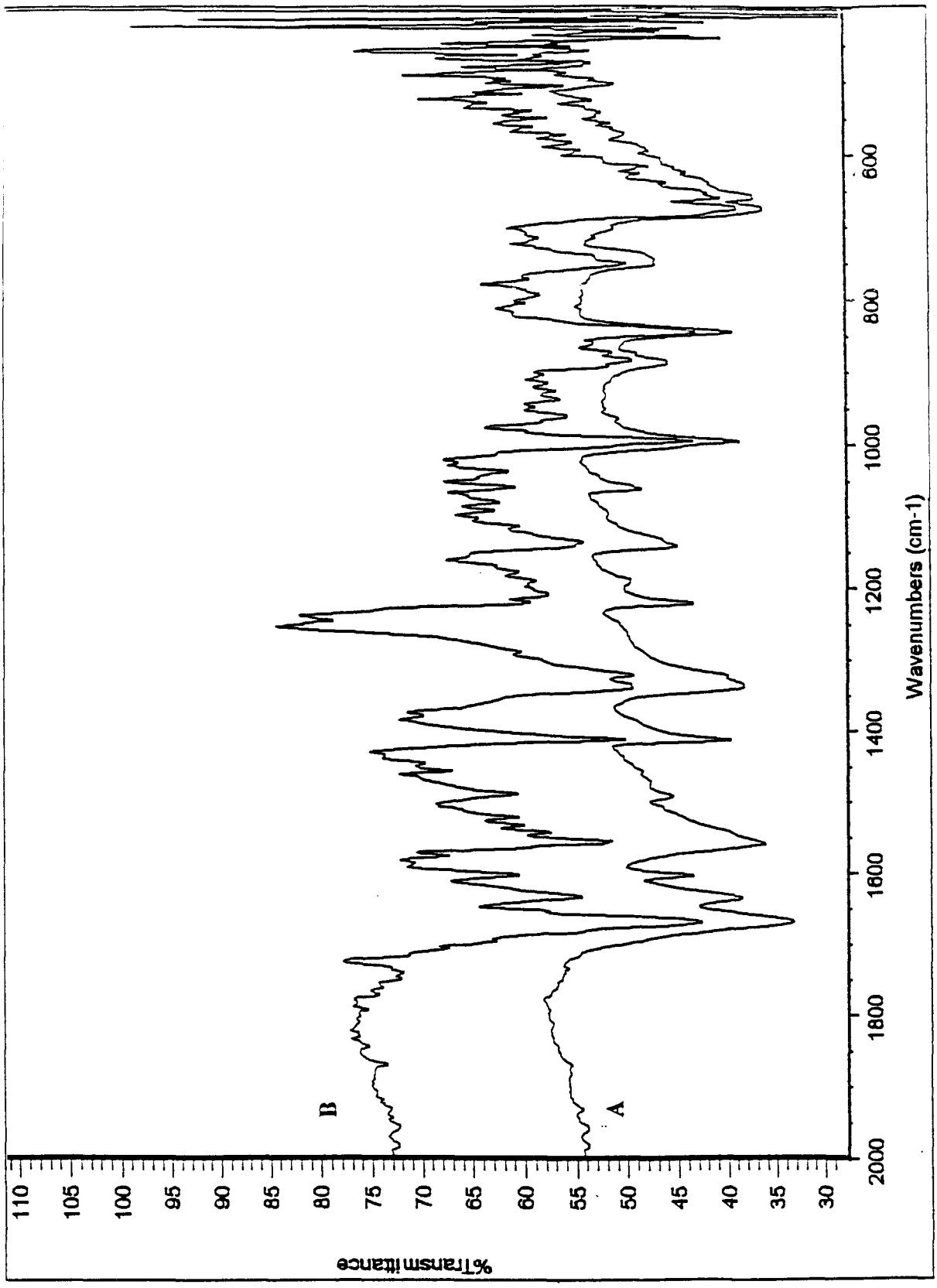


Figure 3.18 FT-IR spectrum of rifampicin (A) and differential spectrum of a physical mixture of isoniazid/rifampicin (B)

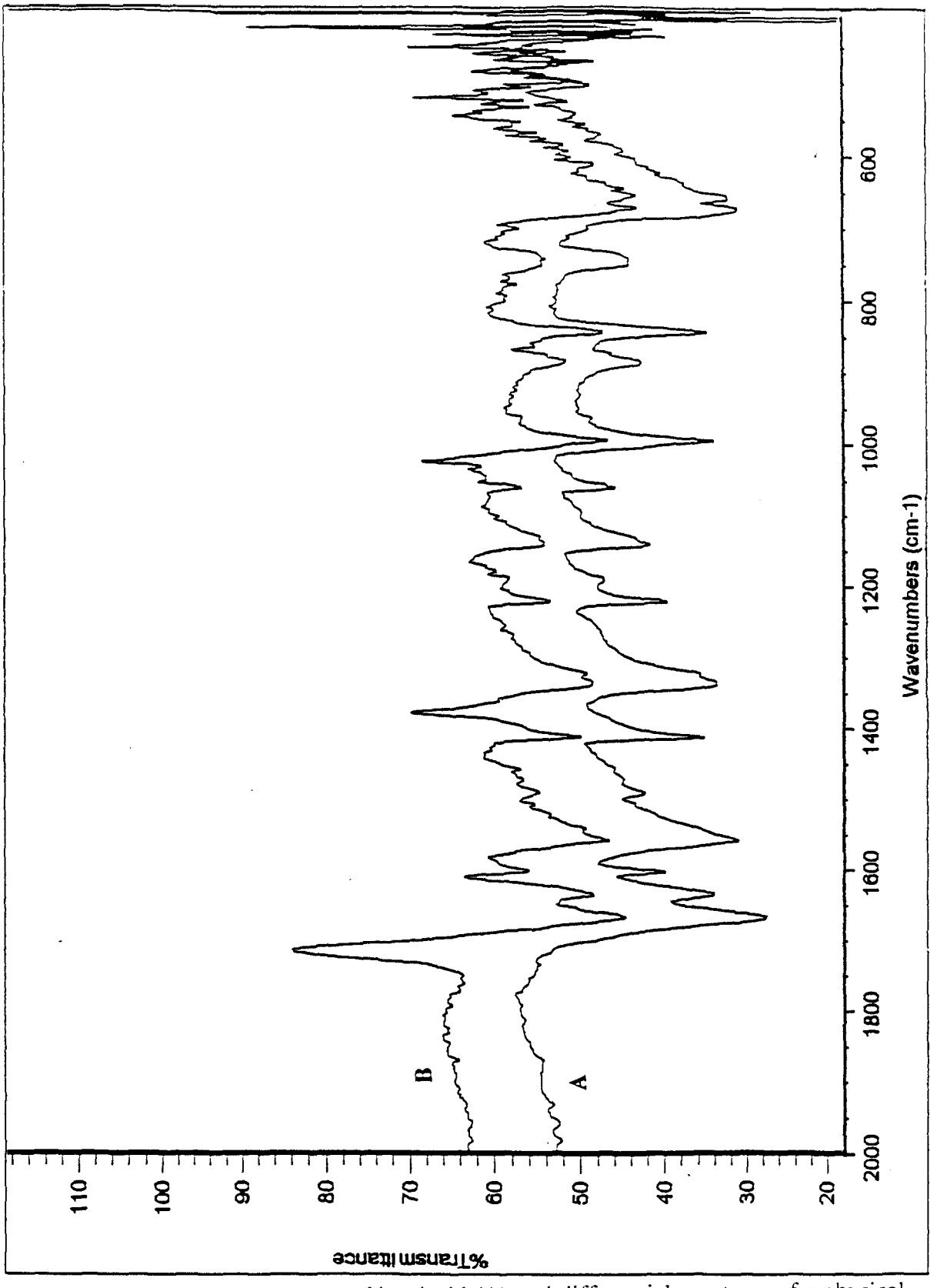


Figure 3.19 FT-IR spectrum of isoniazid (A) and differential spectrum of a physical mixture of isoniazid/pyrazinamide (B)

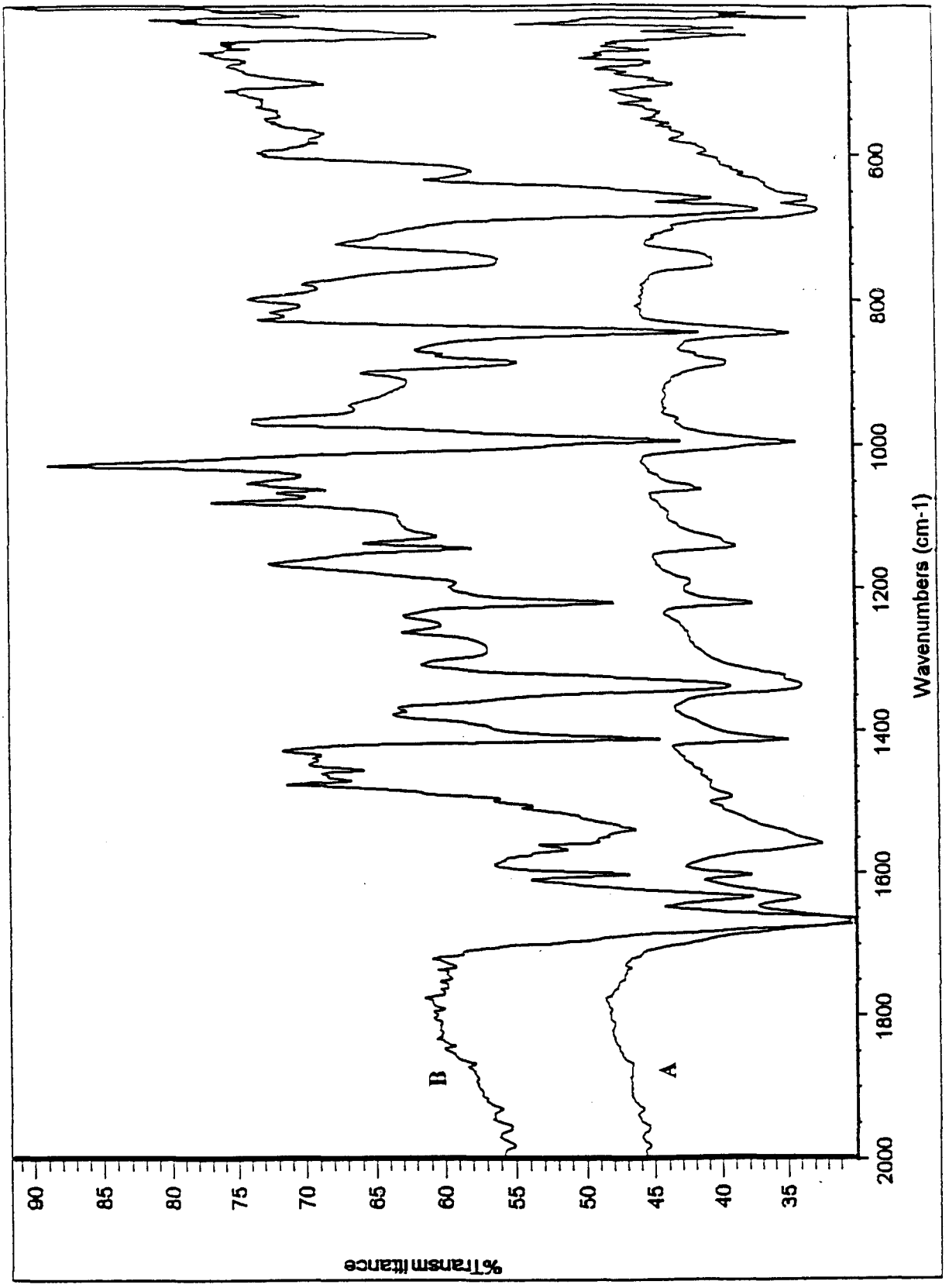


Figure 3.20 FT-IR spectrum of isoniazid (A) and differential spectrum of a physical mixture of isoniazid/ethambutol HCl (B)

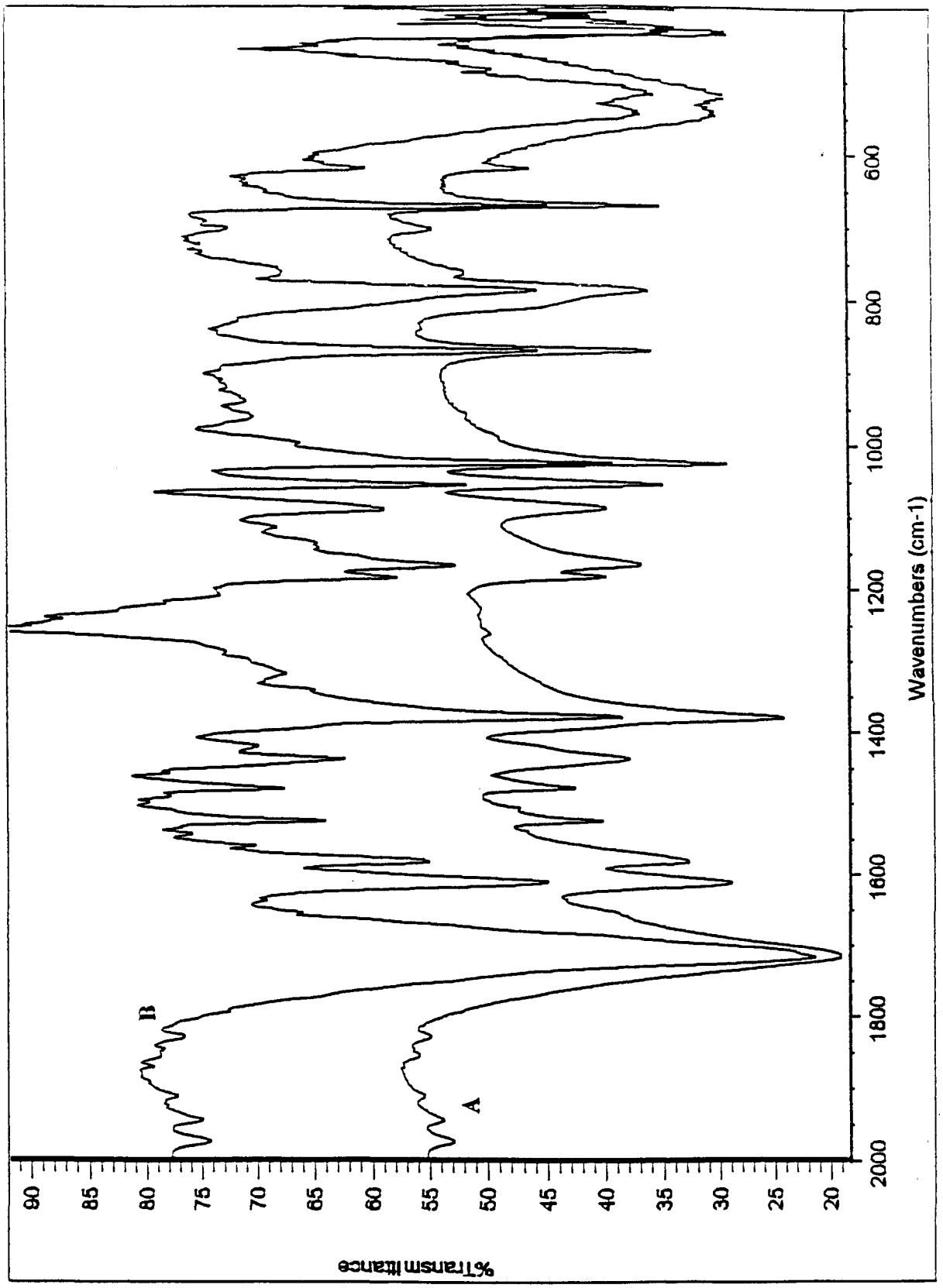


Figure 3.21 FT-IR spectrum of pyrazinamide (A) and differential spectrum of a physical mixture of pyrazinamide/rifampicin (B)

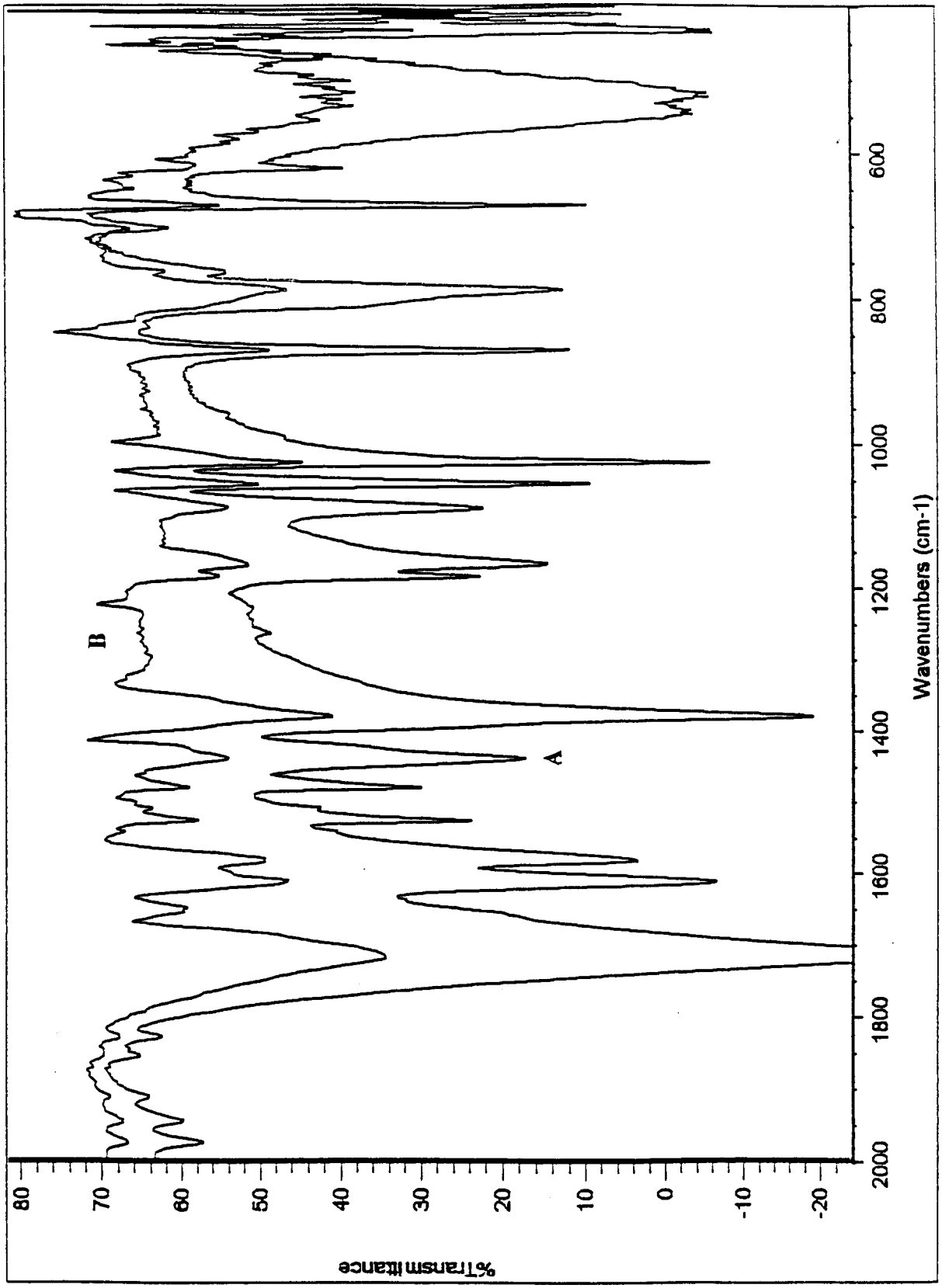


Figure 3.22 FT-IR spectrum of pyrazinamide (A) and differential spectrum of a physical mixture of pyrazinamide/isoniazid (B)

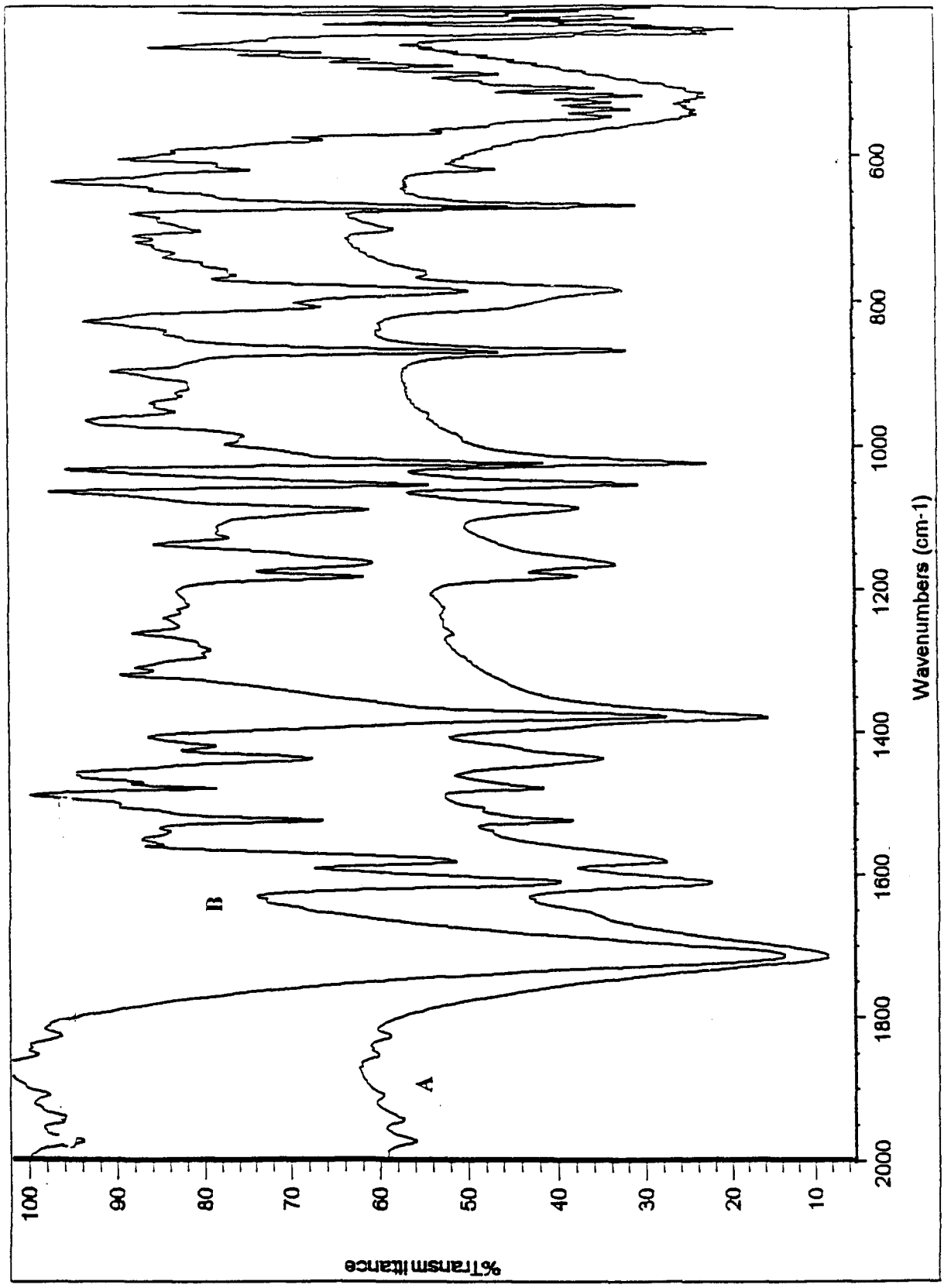


Figure 3.23 FT-IR spectrum of pyrazinamide (A) and differential spectrum of a physical mixture of pyrazinamide/ethambutol HCl (B)

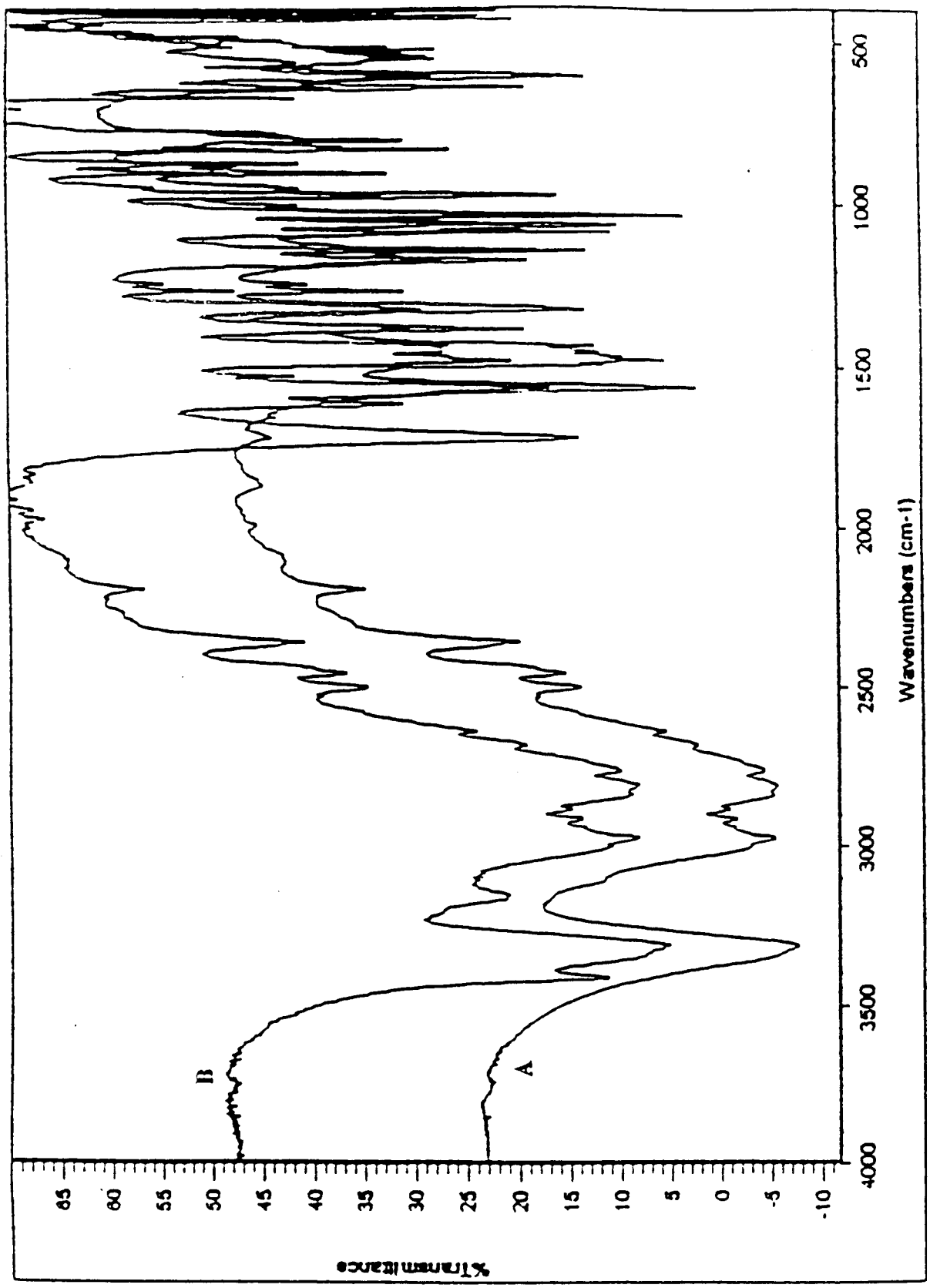


Figure 3.24 FT-IR spectrum of ethambutol HCl (A) and differential spectrum of a physical mixture of ethambutol HCl/pyrazinamide (B)

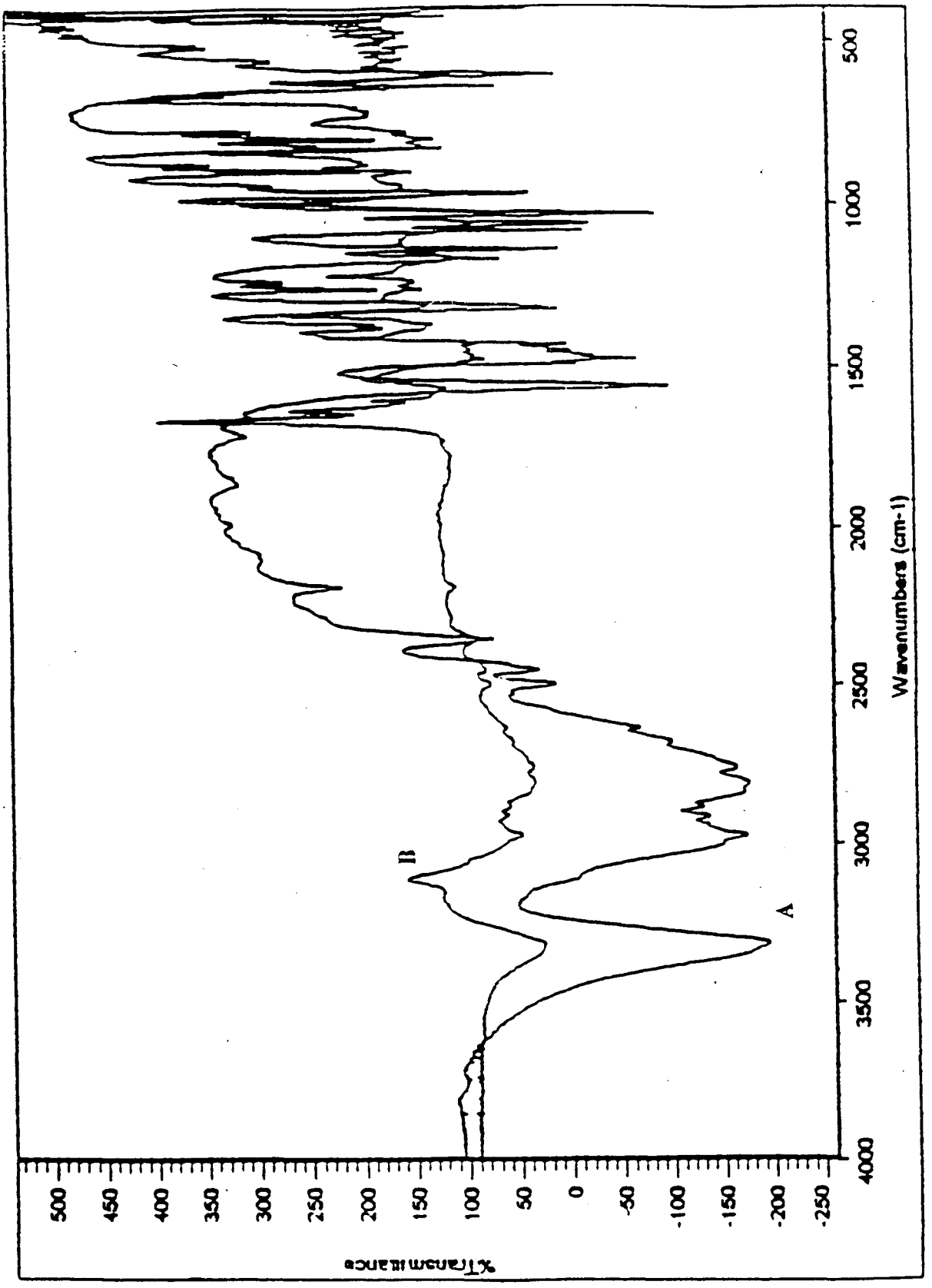
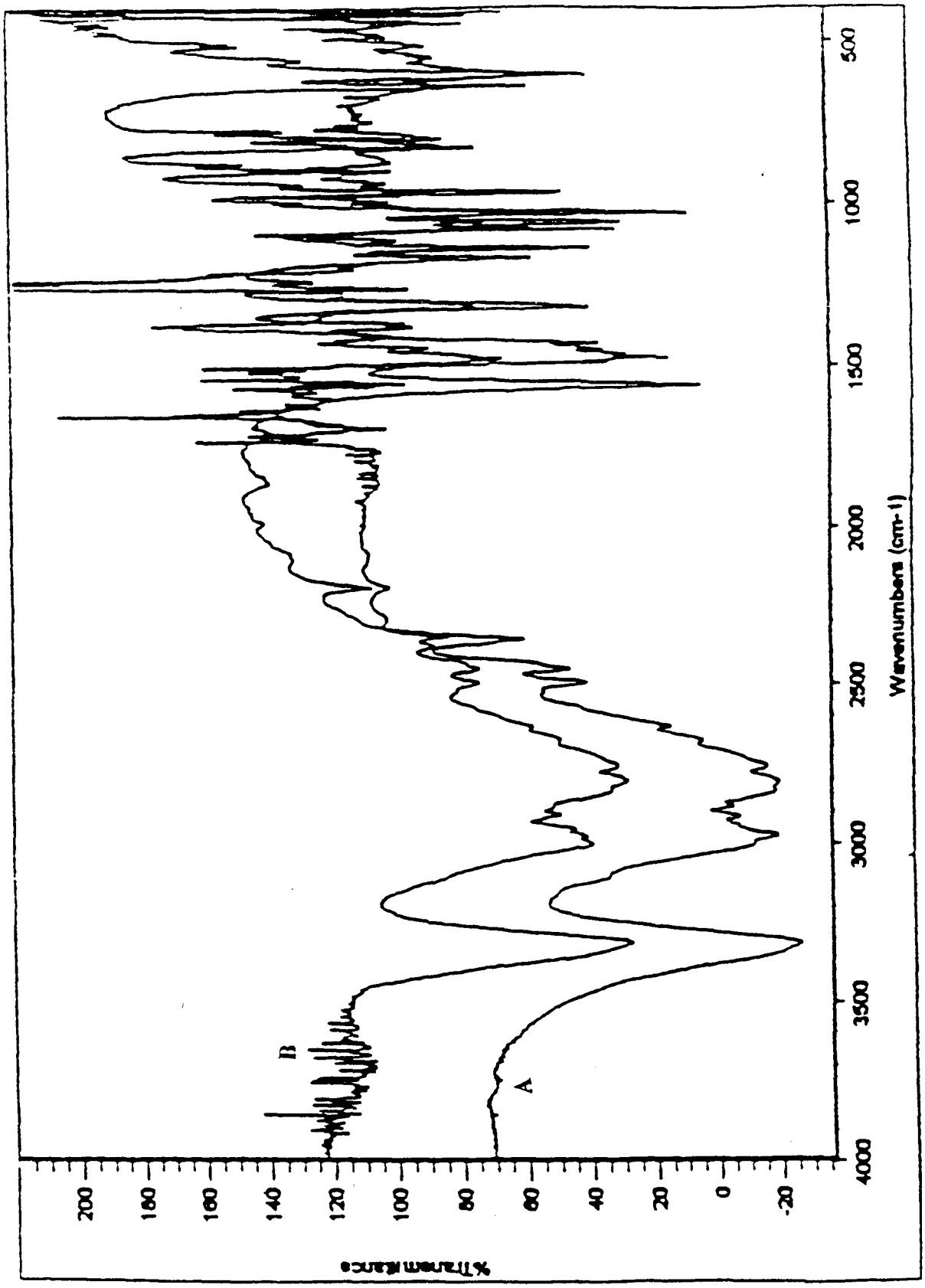


Figure 3.25 FT-IR spectrum of ethambutol HCl (A) and differential spectrum of a physical mixture of ethambutol HCl/isoniazid (B)



igure 3.26 FT-IR spectrum of ethambutol HCl (A) and differential

spectrum of a physical mixture of ethambutol HCl/rifampicin (B)

4.0 DISCUSSION

The aim of this study was to develop an HPLC assay method for the detection of rifampicin, isoniazid, pyrazinamide and ethambutol HCl. However, since ethambutol HCl shows no significant ultraviolet absorption as opposed to rifampicin, isoniazid and pyrazinamide, an alternative method of detection had to be developed for the ethambutol HCl.

Walubo et al. (1994), demonstrated a method for an HPLC assay for rifampicin, isoniazid and pyrazinamide using UV detection. The main disadvantage of this method is that it utilizes two separate solvent systems. In contrast, in this study, a single solvent HPLC method utilising UV detection was developed for the simultaneous detection of rifampicin, isoniazid and pyrazinamide.

For the assay of ethambutol HCl, an HPLC assay method utilizing electrochemical detection was developed. HPLC methods using electrochemical detection has been shown by Riggin et al. (1977) to be two to three orders of magnitude more sensitive than for ultraviolet detection and can be more selective for compounds that can be ionised. In addition Jane et al. (1985) have shown that the HPLC analysis of basic drugs using non-aqueous ionic eluents can be detected electrochemically. In this study, water, ammonium perchlorate and methanol, as the non-aqueous ionic eluent was used to develop an electrochemical method for the assay of ethambutol HCl. Ammonium perchlorate was

chosen amongst others as the ionic modifier since the perchlorate ion is resistant to oxidation at the potentials used, thus limiting the background current. Aliphatic amines like ethambutol HCl are only oxidisable when present in the non-protonated form. Increasing the pH above 6.7 produces a higher response for oxidisable amines since the non-protonated form is favoured, giving more oxidisable molecules at the electrode. In this study it was found that the optimum pH is 9.3. Using this method, it was possible to detect rifampicin, isoniazid and ethambutol HCl but not pyrazinamide which was present in the injection solution. By varying the pH as well as the water-methanol ratio in the buffer did not make it possible to detect the pyrazinamide present. Regardless, of whether pyrazinamide, rifampicin, isoniazid and ethambutol HCl are dissolved in the non aqueous, non ionic buffer or methanol, it was still possible to assay the combination by either UV or electrochemical detection.

The different compounds that have been reported by Walubo et al. (1994) and Jane et al (1985), to be utilised as internal standards were not applicable in either of the two methods developed in this study. Therefore, an external standard method of calibration was used for each of the two assay methods.

Of primary concern to the formulator of a combination dosage formulation is the occurrence of drug-drug interactions. The FT-IR studies demonstrated that it was unlikely that any drug interactions occurred during the physical mixing process. Furthermore, during the assay of the drug combination no apparent degradation products

were observed in the chromatograms. Therefore, the FT-IR results, together with observations from the HPLC analysis confirm that no major drug-drug interactions are likely to occur.

These studies therefore suggest that it is feasible for an anti-tuberculosis drug combination sachet formulation to be developed.

5.0 CONCLUSION

The above results demonstrate that the HPLC methods developed for the analysis of the isoniazid, pyrazinamide, rifampicin and ethambutol HCl are precise, accurate and rapid. The direct injection of sample solutions allows the analyst to avoid the drawbacks of laborious and time-consuming procedures in the pretreatment of samples. The two methods allow for the simultaneous assay of three anti-tuberculosis drugs in combination. These results suggested that these methods, with some modifications, may be adapted for the analysis in plasma of anti-tuberculosis drugs when used in combination. Furthermore, Fourier transform infrared spectroscopy studies reveal that a combination dosage form of rifampicin, isoniazid, ethambutol HCl and pyrazinamide are stable in combination and could be formulated as a sachet dosage form.

FUTURE OBJECTIVES

1.To modify the electrochemical detection HPLC assay so that pyrazinamide may be detected together with rifampicin, isoniazid, and ethambutol HCl in one HPLC system.

2. To undertake studies to monitor therapeutic outcomes.

3.To evaluate compliance in tuberculosis patients with the combination dosage form.

APPENDIX A

HPLC INSTRUMENT CONDITIONS-BECKMAN SYSTEM GOLD

Column: L C licrosphere - C_8, 250 x 4mm
Particle size - 5 μm
Particle Shape
End-capped: yes
Column temperature=Ambient
Detector: Beckman System Gold-Detector 168
Mobile Phase: Bottle A= 100% Acetonitrile
Bottle B=KH_2PO_4 buffer
Filter-degas and pH to 3.45
Wavelength: 250nm
Injection Volume: 50μl
Run Time: 15 min.

APPENDIX B

HPLC INSTRUMENT CONDITIONS-HEWLETT PACKARD

Column: L C licrosphere - C_8, 250 x 4mm

Particle size - 5 μm

Particle Shape: spherical

End-capped: yes

Column temperature = Ambient

Detector: Hewlett Packard HP1049A electrochemical detector

Mobile Phase: 200mM Ammonium perchlorate in 75% methanol and 25% water.

Mode: Amperometric

Injection Volume: 50μl

Run Time: 10 min.

APPENDIX C

CERTIFICATE OF ANALYSIS OF RIFAMPICIN

THEMIS CHEMICALS LIMITED

11/12, UDYOG NAGAR
S. V. ROAD, GOREGAON (W),
MUMBAI - 400 104, INDIA.
PHONE : 91-22-8757836(7 LINES)
TELEX : 011-70098
GRAM : 'MEDITHEM' MUMBAI - 400104
FAX : 91-22-8746621/8743643

CERTIFICATE OF ANALYSIS

Qty: 500 Gms.
Date: 8/7/97

Product : RIFAMPICIN BP.
Batch No. : RD 97102
Date of Manufacturing : JULY 1997
Date of Expiry : JUNE 2000
Mfgd. by : THEMIS CHEMICALS LTD.

RESULTS OF ANALYSIS

Sr. No.	Test	Results	Limit IP
1.	Description	Reddish Brown coloured Crystalline Powder	
2.	Solubility	Complies	
3.	Identification	Complies Test A,B & C	
4.	Acidity	5.4	4.5 to 6.5
5.	Heavy Metals	Passes the test	NMT 20 PPM
6.	Related Substance	Complies	
7.	Loss on drying	0.43%	NMT 1%
8.	Sulphates Ash	0.026%	NMT 0.1%
9.	Assay	98.82% on dried basis	97% to 102%

REMARKS : The above sample complies as per B.P., specifications in above respect.

Opinion : In the opinion of the undersigned, the sample is of standard quality/not of standard quality as defined in the drugs and cosmetic Act, 1940 and the rules there under.

Analyst	Checked by	Approved by
R.R. PATEL	P.N. PATEL	Sd/-

THEMIS CHEMICALS LTD.

APPENDIX D

CERTIFICATE OF ANALYSIS OF ISONIAZID

MARSING & CO. LTD. A/S

P.O.BOX 1913 · AVEDØRE-HOLMEN 48 · DK-2650 HVIDOVRE · DENMARK

TELEPHONE +45 36 77 66 33
TELEFAX +45 36 77 10 14
TELEX 19883

COMPANY REG. NO. 61 31 14
IMPORT LICENCE NO. 01-13 000 88
EXPORT LICENCE NO. 01-13 000 00

BANKERS
UNIBANK COPENHAGEN
DEN DANSKE BANK COPENHAGEN

CERTIFICATE OF ANALYSIS

PRODUCT: ISONIAZID
BP 93

CAS NO.: 54-85-3 REF.NO.: 27069 BATCH/LOT NO.: T95083

TESTS:	LIMITS:	RESULTS:
CHARACTERISTICS	WHITE CRYST.POWDER	CONFORMS
SOLUBILITY		CONFORMS
IDENTIFICATION		CONFORMS
MELTING POINT	170 - 174°C	170.5-172.0°C
ACIDITY OR ALKALINITY	6.0 - 8.0	6.9
CLARITY AND COLOUR		CONFORMS
HEAVY METALS	MAX 10 PPM	CONFORMS
HYDRAZINE AND REL.SUBS.		CONFORMS
LOSS ON DRYING	MAX 0.5%	0.0%
SULPHATED ASH	MAX 0.1%	0.0%
ASSAY	99.0 - 101.0%	100.0%
DATE OF MANUFACTURE		JUNE 1995
DATE OF EXPIRY		JUNE 1999

MARSING & CO. LTD. A/S

APPENDIX E

CERTIFICATE OF ANALYSIS OF PYRAZINAMIDE

THEMIS CHEMICALS LIMITED

11/12, UDYOG NAGAR
S. V. ROAD, GOREGAON (W),
MUMBAI - 400 104, INDIA.
PHONE : 91-22-8757836(7 LINES)
TELEX : 011-70098
GRAM : 'MEDITHEM' MUMBAI - 400104
FAX : 91-22-8746621/8743643

CERTIFICATE OF ANALYSIS

QTY : 500gms
DATE: 24/6/97.

Product : PYRAZINAMIDE B.P.
Batch No. : 239
Date of Manufacturing : JUNE 1997
Date of Expiry : MAY 2002
Mfgd. by : THEMIS CHEMICALS LTD.

RESULTS OF ANALYSIS

Sr. No.	Test	Results	Limit BP 93
1.	Description	A White Crystalline Powder odourless	
2.	Solubility	Complies	
3.	Identification	Complies Test A B & C	
4.	Melting Point	189-190°C	188°C-191° C
5.	Heavy Metals	Passes the Test	NMT 20 ppm
6.	Related Substance	Complies	
7.	Sulphated Ash	0.02%	NMT 0.1%
8.	Water	0.15% w/w	NMT 0.5%
9.	Assay	99.85% on dried basis	NLT 99%

Remarks : The above sample complies as per BP specification in above respect.

Opinion : In the opinion of the undersigned, the sample is of standard quality as defined in the drugs and cosmetic Act 1940 and the rules there under.

Analyst	Checked by	Approved by
R.R. PATEL	P.N. PATEL	Sd/-

THEMIS CHEMICALS LTD.

APPENDIX F

CERTIFICATE OF ANALYSIS OF ETHAMBUTOL HCl

THEMIS CHEMICALS LIMITED

Plot No. 69-A, G. I. D. C. Industrial Estate, VAPI-396 195. Dist. Valsad.

Sr. No. 1308

QUALITY CONTROL LABORATORY Slip No. 1303

Name of Product : Ethambutol Hydrochloride ~~I.P.~~ / B. P. '93 Date 23.11.1996

Mfg. Date : NOVEMBER, 1996 B. No. 5674 Lot of : 400.0Kgs

Exp. Date: OCTOBER, 2001

Manufactured by : Themis Chemicals Ltd. Vapi. Pkg. : 8x50.0Kgs

Results of Analysis	Limit ~~I.P.~~ B.P. '93
Description : A White Crystalline powder	
Solubility : Complies	
Acidity : Complies	PH 3.7 To 4.0
Identification : (A) Complies	
(B) Complies	
(C) Complies	
(D) Complies	
Specific Optical Rotation (+)6.25° at 25°C	(+5.8°to(+)6.6°
Melting Range : 200°C	About 202°C ~~199°C to 204°C~~
(+)-2-Amino butanol : Complies	~~Not more than 1.0%~~
Loss on drying at 105°C 0.23 % when dried to Constant weight	Not more than 0.5%
Heavy metals : Complies	10 Max ~~20~~ ppm
Sulphated Ash : 0.037%	Not more than 0.1%
Assay : 99.56 % of Ethambutol HCl as $C_{10}H_{24}N_2O_2 2HCl$ with reference to the dried substance.	~~Not less than 98.0%~~ 97.0% to 101%

REPORT : The Sample submitted Complies with the prescribed Standard of quality.

UNION BANK OF INDIA ... Princess Street Branch Bombay-2 ... D 285407

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