Chapter 3

VALIDATION OF HPLC METHOD FOR DETERMINATION OF RIFAMPICIN AND ISONIAZID

3.1 Introduction

To ensure that the analytical method is precise and reliable the validation method for the determination of rifampicin and isoniazid was performed prior to formulation and development of dry powder inhalers. High performance liquid chromatography (HPLC) was employed as a tool for drug analysis in this study.

3.2 Materials

Acetonitrile (J.T.Baker, NJ, USA)

Dimethylsulfoxide (Lab-scan, Bangkok, Thailand)

Isonicotinic acid hydrazide (Sigma chemical company, St. Louis, MO, USA)

Methanol (J.T. Baker, NJ, USA)

Polyamide membrane 0.45 μm (Sartorius, Germany)

Rifampicin (Fluka, Switzerland)

Sodium dihydrogen phosphate (Riedel-de Haën, Germany)

3.3 Equipment

Chromatographic experiments were performed using HPLC system consisting of a Waters™ 600 controller and Waters™ 600 pump with a Waters™ 717 plus autosampler equipped with a Waters™ 486 tunable absorbance diode-array detector connected to Waters™ 746 data module (Milford, MA, USA). The microbondapak C18 column (Phenomenex, USA) (250×4 mm i.d., 5 μm) was used in this study.

3.4 Chromatographic Conditions

The mobile phases consisted of 0.05 M sodium dihydrogen phosphate and acetonitrile (55 : 45, v/v for rifampicin and 97 : 3, v/v for isoniazid). These mobile phases were set at a flow rate of 1 ml/min at ambient temperature. The UV detector was operated at 254 nm. The injection volume was 50 μl (Calleri *et al.*, 2002 and Reverchon *et al.*, 2002).

3.5 Methods

3.5.1 Stability indicating assay

During the development of pharmaceutical products, a stability indicating assay for the dry substance is generally required. This method must separate and measure all peaks that are 0.05% - 0.1% of the parent peak. To ensure that the method is acceptable and to check for degradation products, the standard preparations were analyzed against a freshly prepared standard. The reference solutions of rifampicin and isoniazid were analyzed immediately after the preparation and stored at 4°C in the dark, and analyzed after 1 day, 3 days and 1 week after

diluting them to the final concentrations. The solutions were considered stable if the variation in the concentrations was less than 2%.

3.5.2 Method validation of rifampicin and isoniazid

A stock solution was prepared by the following method. Ten mg rifampicin were weighed and placed in a 100 ml volumetric flask, dissolved in dimethylsulfoxide and completed to 100 ml. The stock solution of rifampicin was diluted stepwise with a mobile phase to obtain concentrations of 1, 2, 4, 6, 8, 10, 15 and 20 μg/ml.

A stock solution of isoniazid was prepared similarly. Ten mg of isoniazid was placed in a 100 ml volumetric flask, dissolved and diluted to volume with water to obtain drug concentrations of 2, 4, 6, 8, 10 and 12 μg/ml.

The intra-day precision was determined by performing five repeated analyses of a preparation on the same day. The inter-day precision was determined by analyzing freshly prepared samples for three days over a period of 1 week. The precision was determined by calculating the analyte peak area over the mean peak area presented as a percentage of the relative standard deviation (%RSD). This value should be less than 2%.

The accuracy of an analysis was determined by the systemic error involved. It was determined by calculating the recovery of the analyte by a standard addition method at concentration between 1-20 μg/ml for rifampicin and at concentration between 2-12 μg/ml for isoniazid. Recovery was evaluated by comparing the theoretical and measured concentration of a spike analyte.

The linearity responses for rifampicin and isoniazid were determined by analyzing the corresponding reference standards three times for each concentration in the range of 1-20 μg/ml for rifampicin and the range 2-12 μg/ml for isoniazid. The linearity was determined by calculating the correlation coefficient value (r^2), generated by plotting the analyte peak area versus the concentration of the drug. Linearity was confirmed if the %RSD values of the slope and the intercept were less than 3%.

3.5.3 Calibration curve for standard rifampicin and isoniazid

Stock solutions of rifampicin and isoniazid were prepared by dissolving 10 mg of each drug separately in dimethylsulfoxide and water, respectively. Then, volume was completed to 100 ml with the same solvent. The stock solutions were kept at 4°C protected from light until use.

The stock solution of rifampicin was diluted stepwise with a mobile phase to give final drug concentrations of 1, 2, 4, 6, 8 and 10 μg/ml. For isoniazid, water was used for dilution to obtain final drug concentration of 2, 4, 6, 8 and 10 μg/ml. Both standard solutions of rifampicin and isoniazid were determined by HPLC as described before. Calibration curves of standard rifampicin and isoniazid were generated by plotting the corresponding concentrations versus peak area of the drugs.

3.6 Results and Discussion

3.6.1 The stability of drugs

The initial trials with various compositions of mobile phase (acetonitrile, methanol and buffer) resulted in poor resolution of rifampicin or isoniazid and its degraded products peaks. Finally, mobile phase composition were optimized to be acetonitrile-0.05 M sodium phosphate buffer (45 : 55 v/v for rifampicin and 3 : 97 v/v for isoniazid). This analytical method is stability indicating assay. Figure 3.1 (A and B) shows that parent drugs (rifampicin or isoniazid) are clearly separated from degraded products. The major peak seen at 8 minutes (Figure 3.2 A) is due to rifampicin. The minor peaks seen at approximately 8 and 13 minutes are degraded products of rifampicin. For the results of isonizid (Figure 3.2 B), the minor peak (degraded products) seen before major peak (isoniazid) at 8 minutes. The results show that rifampicin and isoniazid undergoes some hydrolysis when dissolved in these mobile phases.

The stability of rifampicin stored at 4°C and protected from light is shown in Figure 3.2. It was found that rifampicin degraded over 60% at 1 week. Dimethylsulfoxide is used as the solvent for the stock solution of rifampicin because rifampicin is very stable (Gallo and Radaelli, 1976). After diluting these stock solutions with mobile phase, which was composed of water up to 55%, rifampicin was hydrolysed to 3-formylrifamycin SV or rifampicin quinone (Bain *et al.*, 1998).

Remaining percentage of isoniazid at 1 week storage decreased 20% because isoniazid (isonicotinic acid hydrazide) dissolves in water as a result it is easy to obtain hydrolysis reaction. However, the degradation of isoniazid was slower than the degradation of rifampicin stored in the same conditions when compared with %drug remaining at each time interval. After storage rifampicin and isoniazid for 3 and 6 days, %drugs remaining decreased from 90 to 80% of isoniazid and 60 to 40%

of rifampicin (Figure 3.2). The USP advises that after preparing rifampicin for analysis, samples should be injected in HPLC within 30-60 seconds in order to prevent the degradation of the rifampicin. Some studies reported that rifampicin solution in water loses 20% of its original antimicrobial activity after 10 hours at room temperature.

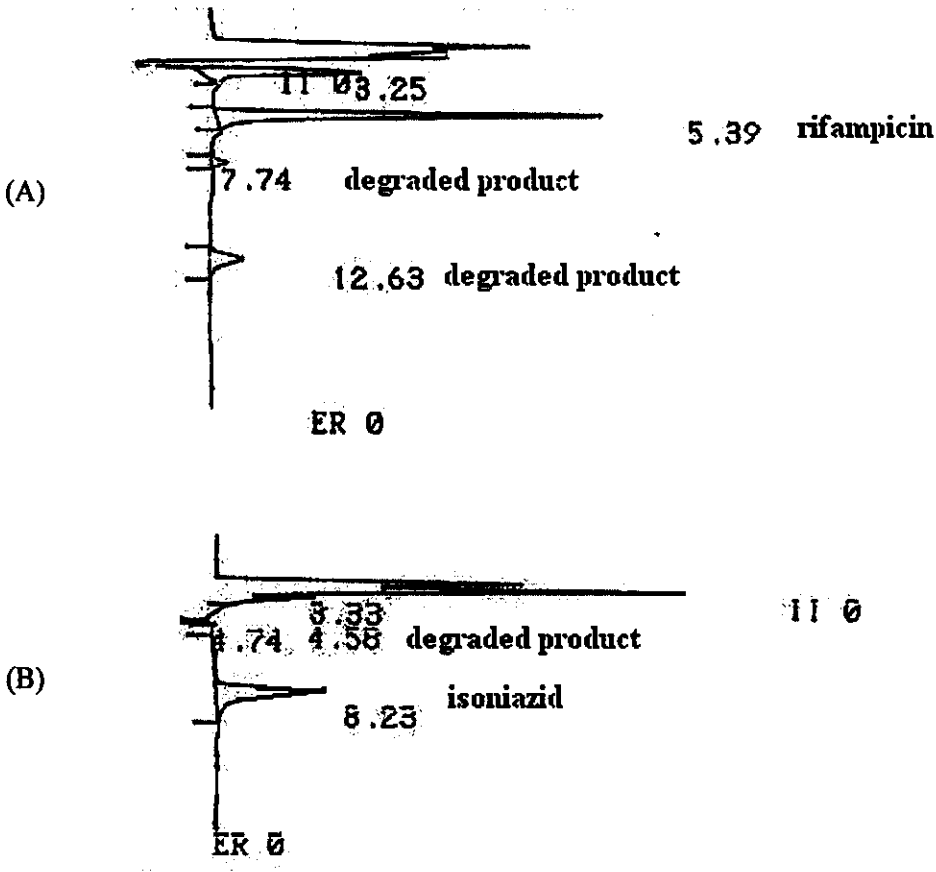

Figure 3.1 Chromatograms obtained from a 2 μg/ml of (A) rifampicin and (B) isoniazid

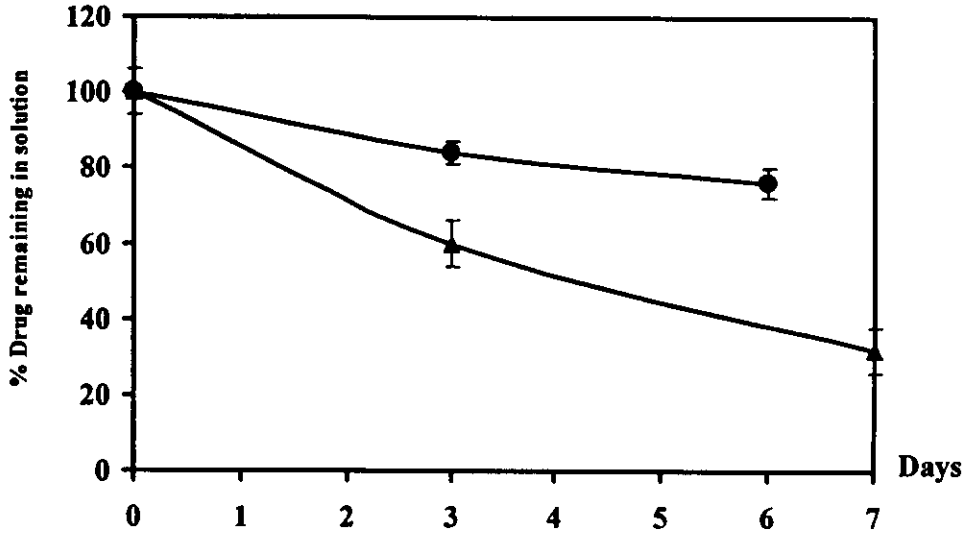

Figure 3.2 Stability of standard rifampicin (▲) and isoniazid (●) (mean ± SD, n = 3)

3.6.2 Method validation of rifampicin and isoniazid

This method validation was followed by guidance for industry : validation of analytical procedure (ICH, 1996). Figures 3.3 and 3.4 show the intra-day and inter-day precision of rifampicin and isoniazid. In Figure 3.3 the %RSD of both rifampicin and isoniazid are not over 2% in all concentrations in this study. The analysis of rifampicin and isoniazid was precise (Figure 3.3) when the same sample was analyzed in the same day (%RSD < 2%). Both rifampicin and isoniazid samples solutions were freshly prepared for inter-day variation. The results show that the %RSD of both rifampicin and isoniazid samples was under 2% when the concentrations were higher than 4 μg/ml (Figure 3.4). However, at the low concentrations (1-4 μg/ml of rifampicin and 2-4 μg/ml of isoniazid), the %RSD was high but still less than 5%. Therefore, the results show that the analytical method was acceptable for analysis of samples on the same day.

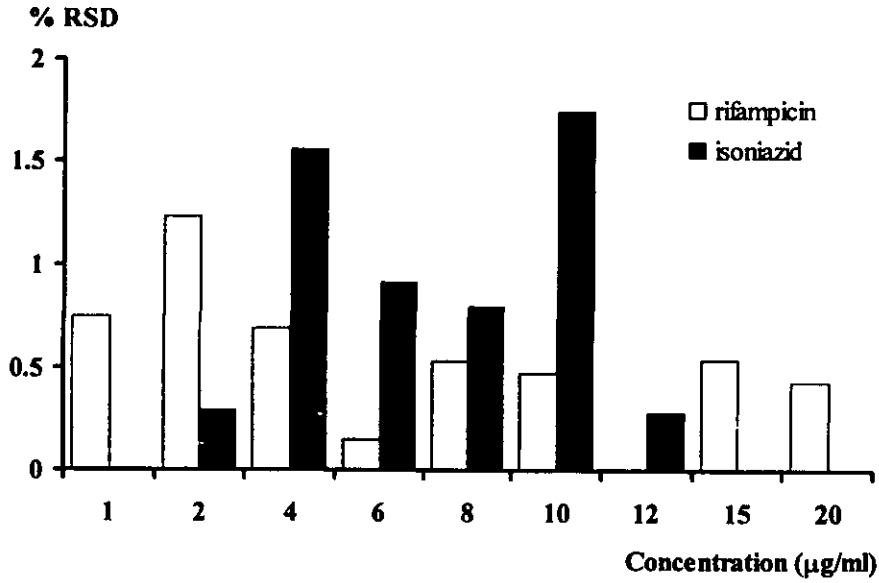

Figure 3.3 Intra-day precision of rifampicin and isoniazid

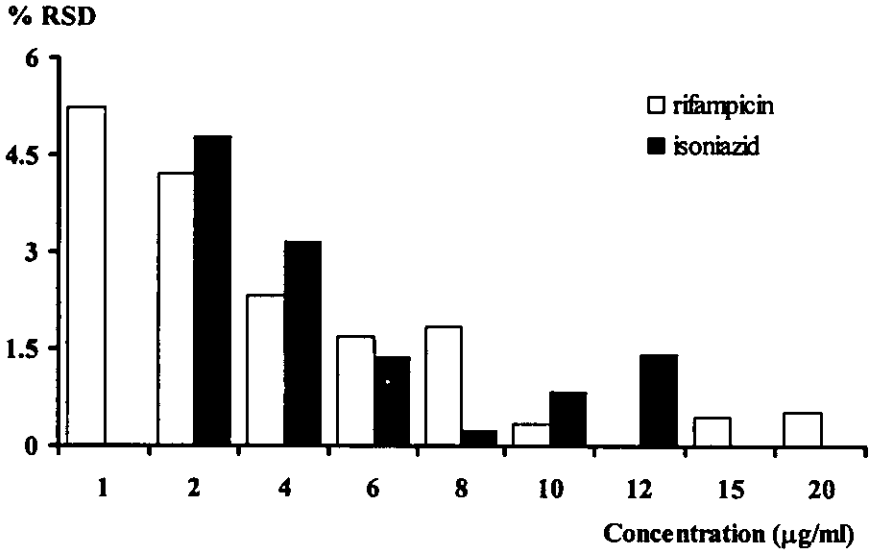

Figure 3.4 Inter-day precision of rifampicin and isoniazid

Tables 3.1 and 3.2 show that both rifampicin and isoniazid determination have good linearity, with a correlation coefficient (r^2 over 0.995) in concentrations between 1-20 μg/ml of rifampicin and 2-12 μg/ml of isoniazid. Therefore, these ranges of concentrations were acceptable for preparing the standard solutions and samples for analysis.

Table 3.1 Linearity of rifampicin standard curve determination by HPLC

No.	intercept	slope	r^2
1	-18761	139089	0.9958
2	-22741	139661	0.9968
3	-20335	139409	0.9966
4	-25852	139963	0.9967
5	-26990	140776	0.9956
%RSD	15.2867	0.4601	

Table 3.2 Linearity of isoniazid standard curve determination by HPLC

No.	intercept	slope	r^2
1	-2934.1	129673	0.9997
2	-8021.1	131058	0.9998
3	20938	127503	0.9989
%RSD	464.65	1.38	

Tables 3.3 and 3.4, show that rifampicin and isoniazid have high accuracy. The %recovery of rifampicin is over than 95 (at concentration 2-22 μg/ml) while the recovery is about 82% at low concentration (at concentration 1 μg/ml). The results suggest that the drug degrad faster at low concentration than that of high concentration. The %recovery of isoniazid is between 95-102%. The results suggest the analytical methods of rifampicin and isoniazid are accurate and able to separate all degraded products. Thus, these methods can be used as stability indicating assay.

Table 3.3 Accuracy of rifampicin standard determination by HPLC

Theoretical Concentration (μg/ml)	Measured Concentration (μg/ml)	%Recovery
1.12	0.92	82.50
2.24	2.14	95.60
4.48	4.47	99.85
6.72	6.68	99.42
8.96	8.93	99.65
11.20	11.20	100.04
16.80	16.95	100.87
22.40	22.67	101.20

Table 3.4 Accuracy of isoniazid standard determination by HPLC

Theoretical Concentration (μg/ml)	Measured Concentration (μg/ml)	%Recovery
2.18	2.15	98.41
4.36	4.17	95.63
6.54	6.49	99.21
8.72	8.92	102.32
10.90	11.03	101.21
13.08	13.21	100.96

3.6.3 Calibration curve for standard rifampicin and isoniazid

Examples of standard curves of rifampicin and isoniazid are shown in Figures 3.5 and 3.6. The calibration curves were prepared with a freshly prepared sample preparation. In these figures, correlation coefficients are above 0.999 in the range of 2-10 μg/ml.

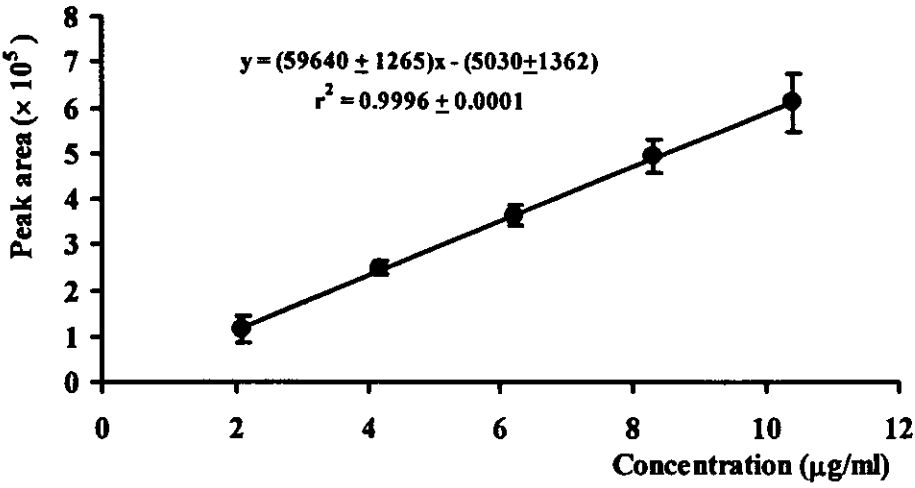

Figure 3.5 Standard curve of rifampicin

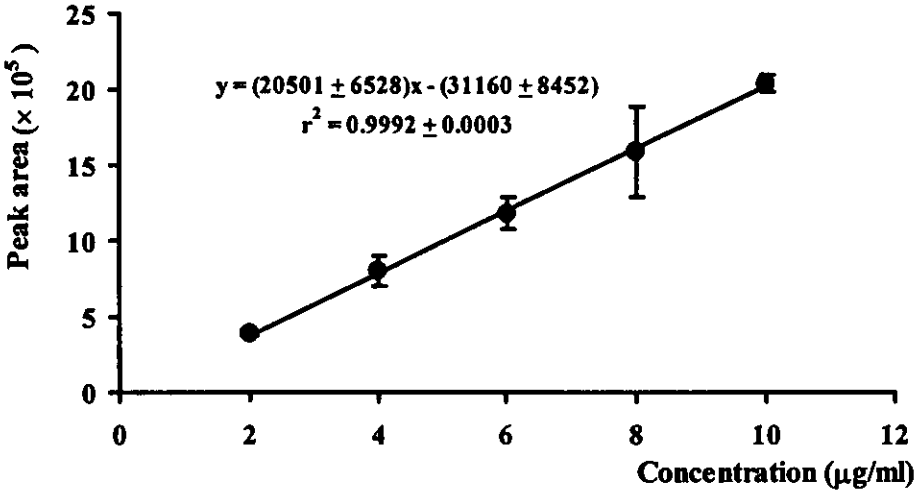

Figure 3.6 Standard curve of isoniazid