CHAPTER 4 : *HPLC Analysis*

CHAPTER 4

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

This chapter describes the details regarding HPLC instrumentation, chromatographic conditions, procedure for calibration graph, formulation analysis and results and discussion of the HPLC methods developed.

4.1 EXPERIMENTAL

Instrument:

The analysis were performed on a Shimadzu HPLC system LC-6A equipped with a variable wavelength detector SPD-6AV, a single channel recorder CR-3A, Zorbax ODS column or Rexochrome ODS column of 5μ packing (25cm x 4.6 mm i.d.) and a Rheodyne injector (Model 7125) with 40 μl loop.

Reagents:

All reagents used were of analytical grade except acetonitrile, methanol and water which were of HPLC grade.

Mobile Phase:

For the analysis of DFS a mobile phase consisting of methanol and 0.046 M monobasic potassium phosphate in the ratio of 7:3 was used. For DLZ analysis a mobile phase consisting of acetonitrile, methanol and 0.05 M monobasic potassium phosphate in the ratio of 25:20:55 was used. For the analysis of FMD a mobile phase consisting of methanol and 0.02 M sodium acetate (pH adjusted to 4.5 with acetic acid) in the ratio of 1:4 was used. All mobile phases were filtered through 0.45 μ Nylon filter and degassed under vacuum before use.

Standard stock solutions:

Standard stock solution of DFS was prepared by dissolving 25 mg of the drug in 50 ml of water. For DLZ analysis 25 mg of the drug was dissolved in 50 ml of methanol. For the analysis of FMD a stock solution was prepared by dissolving 25 mg of the drug in 10 ml methanol and further diluting it to 50 ml with water.

Internal standard:

An internal standard solution containing 25 mg of cyproheptadine hydrochloride in 50 ml of methanol was used in the analysis of diltiazem hydrochloride.

4.2 HPLC method for the determination of DFS (Method A-17)

HPLC analysis of DFS was performed on a Zorbax ODS column with mobile phase flow rate of one ml per min and UV detector set at 254 nm.

4.2.1 Procedure for calibration graph:

Into a series of 50 ml volumetric flasks varying amounts of the drug solution (1-10 ml, 0.5 $mg.ml^{-1}$) were pipetted out and the volume was made upto volume with mobile phase. The solutions were injected in triplicate and the average peak area was calculated.

Formulation analysis:

Weight of powdered tablets or volume of injectable equivalent to 50 mg of diclofenac sodium was transferred into a 100 ml flask. The drug was dissolved in 50 ml water and diluted to 100 ml. The solution was filtered and five ml of the filtrate was further diluted to 50 ml with water. The resulting solution was injected in triplicate and the average peak area was calculated. The amount of the drug

corresponding to peak area was found out from the calibration graph.

Specificity of the method:

A stock solution containing 0.5 $mg.ml^{-1}$ of DFS was prepared in water. Five ml of stock solution was mixed with either 5 ml of 1 N sodium hydroxide or 5 ml of 1 N hydrochloric acid or one ml of 3 % hydrogen peroxide in 50 ml volumetric flask. The flasks were kept in boiling water bath for 30 min. After cooling the pH was adjusted to 7.0 with 1 N HCl or 1 N NaOH. The volume was made upto the mark with water and analysed.

4.2.2 RESULTS AND DISCUSSION

Typical chromatograms recorded by injecting standard DFS sample and DFS solution treated with hydrochloric acid, sodium hydroxide and hydrogen peroxide at 100°C are given in Fig 4.1. No degradation was observed in boiled aqueous solution and in presence of 0.1 N NaOH. DFS exhibits instability in acidic medium when heated. The drug is also susceptible to oxidative degradation. One or more additional peaks appeared on the chromatograms due to the formation of degradation products which were not identified. However, the peaks were well separated and hence the method is applicable to the selective determination of DFS.

The practicality of the method was demonstrated by the analysis of DFS formulations (tablets and injectables). For this purpose a calibration graph was constructed by plotting peak area versus concentration of DFS. The plot was linear in the range of 10-100 $\mu g.ml^{-1}$ with an intercept of 0.0206. A correlation coefficient of 0.9996 and percent RSD of 0.72% were obtained. By using this calibration graph and the dilution factor the content of DFS in the formulation was calculated. The results are given in Table 4.1

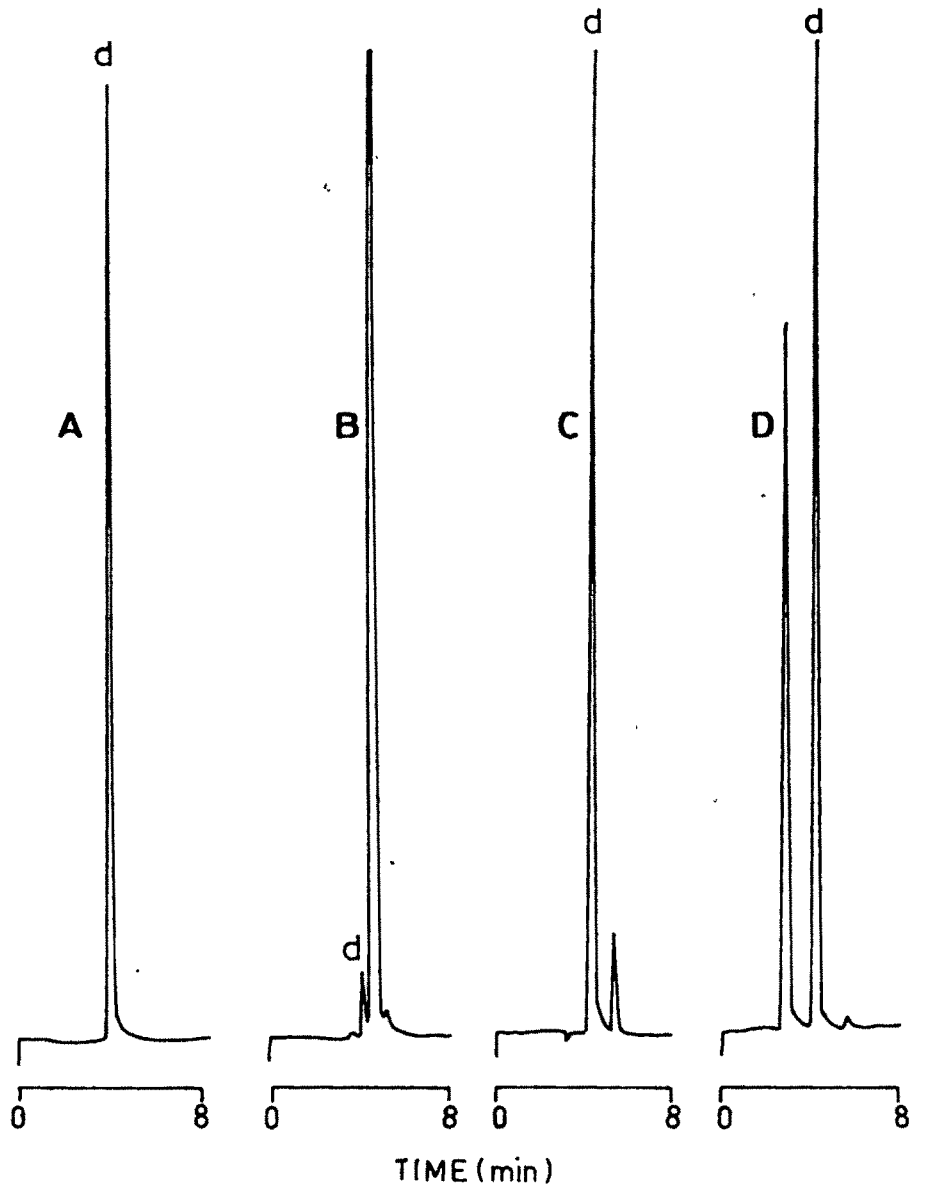

Fig. 4.1a : Typical HPLC chromatogram obtained for DFS solution boiled for 30 min. in (A) Water (B) 0.1N HCl (C) 0.1N NaOH (D) 30% H_2O_2
peak d- diclofenac sodium

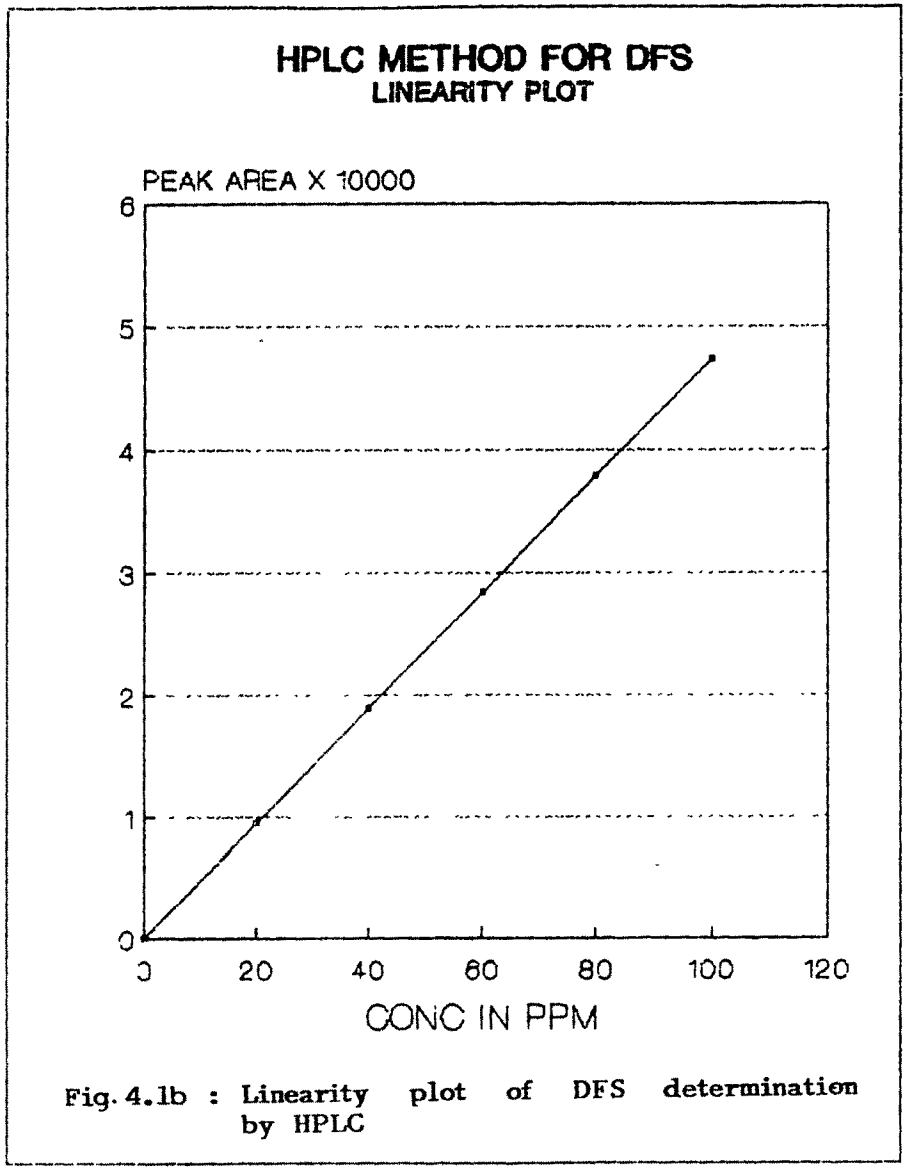

Fig. 4.1b : Linearity plot of DFS determination by HPLC

Common excipients found in tablet and injectable preparations did not interfere. Percent RSD for a single sample was < 1.32%. The method can be applied to formulation containing paracetamol in combination with DFS.

Table 4.1: Results of DFS tablet and injectable analysis by HPLC.

Dosage form	Label claim mg	% Found	Standard deviation %
Tablet	50	99.16	0.38
Tablet*	50	97.84	0.42
Injectable	25(per ml)	100.12	0.51

* with paracetamol 325 mg

4.3 HPLC method for the determination of DLZ (Method B-16)

HPLC analysis of DLZ was performed on a Rexochrome ODS column with mobile phase flow rate of 2 ml per min and spectrophotometric detection at 240 nm. Cyproheptadine hydrochloride was used as internal standard.

4.3.1 Procedure for calibration graph :

Into a series of 50 ml volumetric flasks, varying amounts of standard DLZ solution (0.5 mg.ml^{-1}, 1-5 ml) were pipetted out. To each flask 5 ml of cyproheptadine hydrochloride solution (0.5 mg.ml^{-1}) was added and the volume was made upto the mark with the mobile phase. The solutions were injected in triplicate and ratio of peak areas for diltiazem-internal standard were calculated. A calibration graph was prepared by plotting peak ratio against the drug concentration.

Tablet analysis:

Twenty tablets were weighed and powdered. Tablet powder equivalent to 50 mg of the drug was dissolved in 100 ml of methanol. To 5 ml of the filtrate, 5 ml of the internal standard solution was added and the volume was made upto 50 ml with water and analysed. The amount of the drug corresponding to the peak ratio was found from the calibration graph and the content of DLZ in tablet was calculated using the dilution factor. The results are given in Tablet 4.2.

Specificity of the method

A stock solution containing 0.5 $mg.ml^{-1}$ of DLZ was prepared in water. Five ml of stock solution was mixed with either 5 ml of 0.1 N sodium hydroxide, 5 ml of 0.1 N hydrochloric acid or 2 ml of 30% hydrogen peroxide in 50 ml volumetric flasks. The flasks were kept in boiling water bath for 30 min. After cooling the pH was adjusted to 5.5 with 0.1 N HCl or 0.1 N NaOH. To all the flasks 5 ml of the internal standard solution was added and the volume was made upto the mark with water and analysed.

4.3.2 RESULTS AND DISCUSSION

The direct measurement of the raw drug for diltiazem hydrochloride and the anticipated breakdown product is shown in Fig. 4.2. The limit of quantitation of diltiazem hydrochloride was 10 $\mu g.ml^{-1}$ under the operating conditions employed. The specificity of the HPLC method was tested with degraded diltiazem samples. No change in diltiazem concentration were seen in the boiled aqueous solution. After 30 min in boiling acid(0.1 N HCl) or base (0.1 N NaOH) diltiazem was partly or completely converted to desacetyl diltiazem as might be expected under these conditions. The drug solution upon boiling for 30 min in 30 % H_2O_2 yielded

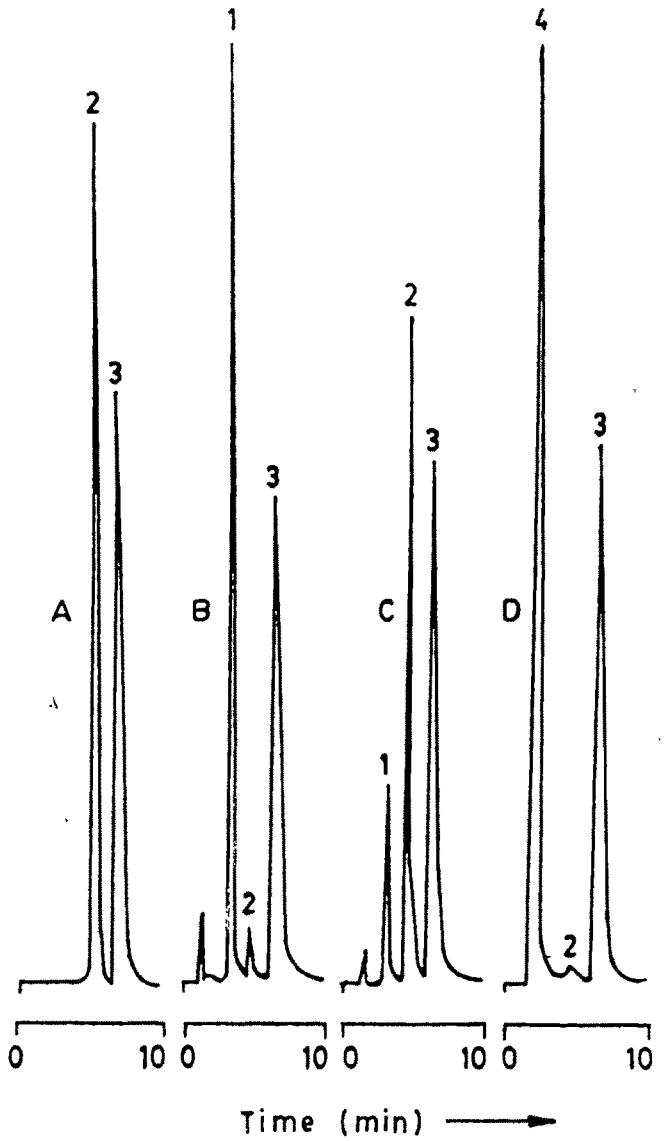

Fig. 4.2a : HPLC chromatograms of diltiazem hydrochloride solution boiled for 30 min in A) water pH 5.5 B) 0.1N NaOH, C) 0.1N HCl D) 30% H_2O_2. Peak 1- Desacetyl diltiazem hydrochloride 2- Diltiazem hydrochloride 3- Internal Standard

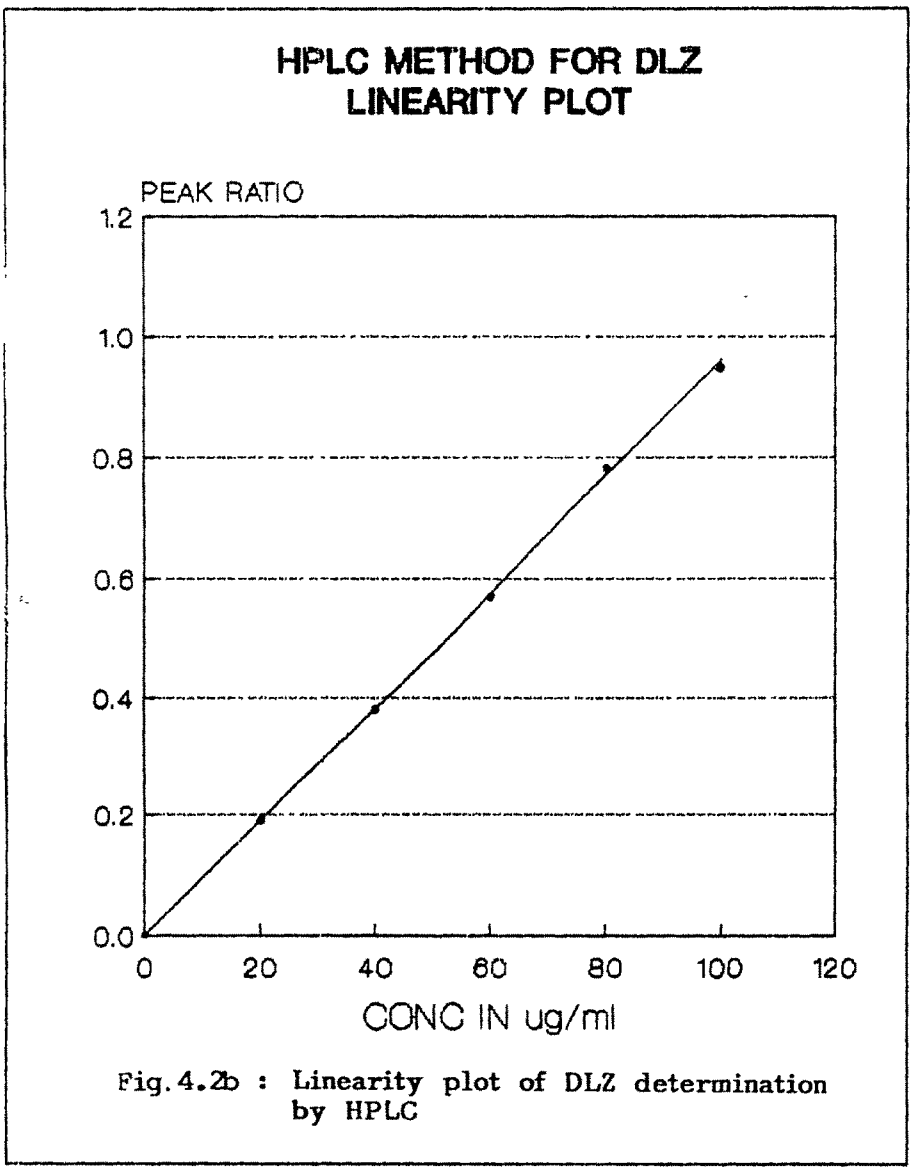

Fig. 4.2b : Linearity plot of DLZ determination by HPLC

several additional unidentified products. In each chromatogram it can be seen that size of diltiazem peak decreases with degradation and that of degradation products increases. The practicality of the method was demonstrated by the analysis of DLZ tablets. A percent RSD of <1.5 % and correlation coefficient of 0.9996 for the calibration graph were obtained. The percent RSD values for a single sample were < 2%.

Table 4.2: Results of diltiazem tablet analysis.

Lot	Labelled amount mg/tablet	Found mg/tablet	%Recovery	RSD %
A	30	29.34	97.80	1.14
B	30	29.26	97.53	1.25
C	60	59.18	98.63	1.46
D	60	58.76	97.93	0.92

In conclusion, the present HPLC method is rapid, precise and accurate for the determination of diltiazem hydrochloride in tablets. In addition the method is stability indicative.

4.4 HPLC method for the determination of FMD (Method C-11)

HPLC analysis of FMD was performed on a Zorbax ODS column with mobile phase flow rate of one ml per min and spectrophotometric detection at 254 nm.

4.4.1 Linearity study:

Aliquots of standard FMD stock solution (0.5 $mg.ml^{-1}$, 0.2-10 ml) were pipetted out into a series of 50 ml volumetric flasks. The volume was made upto the mark with the mobile phase. The solutions were injected in triplicate and the mean peak areas were calculated and used for the construction of the calibration graph.

Specificity of the method:

Five ml of stock solution containing 0.5 $mg.ml^{-1}$ of FMD was mixed with either 5 ml of 2 N hydrochloric acid, 5 ml of 2 N sodium hydroxide or 1 ml of 3% hydrogen peroxide solution in 50 ml volumetric flasks. The flasks were kept in a constant temperature water bath at 65° ± 0.5°C for 30 min. After cooling the pH was adjusted to 5.5 with 0.1 N HCl or 0.1 N NaOH and the volume was made upto the mark with water. Five ml of the solution was further diluted to 25 ml with mobile phase and analysed chromatographically.

Tablet analysis:

Weight of tablet powder equivalent to 40 mg of FMD was shaken with 50 ml methanol in a 100 ml volumetric flask. After diluting the solution to 100 ml with methanol, the solution was filtered. Five ml of the filtrate was further diluted to 50 ml with the mobile phase. The sample solutions were injected in triplicate and the average area was calculated. The drug content was found from the calibration graph.

Method employed for the preliminary kinetic study:

Five ml of the stock solution (0.5 $mg.ml^{-1}$) was mixed with either NaOH or HCl of appropriate concentration in 50 ml volumetric flasks. The flasks were kept in a constant temperature water bath at 45, 55 or 65 (± 0.5)°C. Five ml solutions were sampled at appropriate time intervals. The reaction was quenched by cooling and neutralising with calculated amount of 0.1 N HCl or 0.1 N NaOH in 25 ml volumetric flasks. The volume was made upto the mark with the mobile phase and the change in concentration with the time were monitored by the HPLC method.

4.4.2 RESULTS AND DISCUSSION

Typical HPLC chromatograms recorded by injecting standard FMD sample and FMD solution treated with hydrochloric acid, sodium hydroxide and hydrogen peroxide at 65°C are given in Fig. 4.3. From these it is clear that FMD exhibits instability in both acidic and alkaline media. This drug is also susceptible to oxidative degradation. One or more additional peaks appeared on the chromatograms due to the formation of degradation products which were not identified. However, the peaks were well separated and hence the method is applicable to the selective determination of FMD.

Tablet analysis:

The practicality of the method was demonstrated by the analysis of tablet formulations. For this purpose, a calibration graph was constructed by plotting peak area versus concentration of FMD. The plot was linear in the concentrating range 2-100 $\mu g.ml^{-1}$ with an intercept 0.0508. A correlaiton coefficient of 0.9993 and percent RSD of 0.86% were obtained. The limit of quantitation of FMD was 2 $\mu g.ml^{-1}$ under the operating conditions employed. By using this calibration graph and the dilution factor the content of FMD in tablet was calculated. The results are given in Table 4.3.

Table 4.3: Results of FMD tablet analysis by HPLC.

Tablet	Label claim mg/tablet	Found mg/tablet	%Recovery	RSD %
Lot A	20	19.56	97.80	0.64
Lot B	20	19.73	98.65	0.82
Lot C	40	39.82	99.55	1.14
Lot D	40	39.34	98.35	0.96

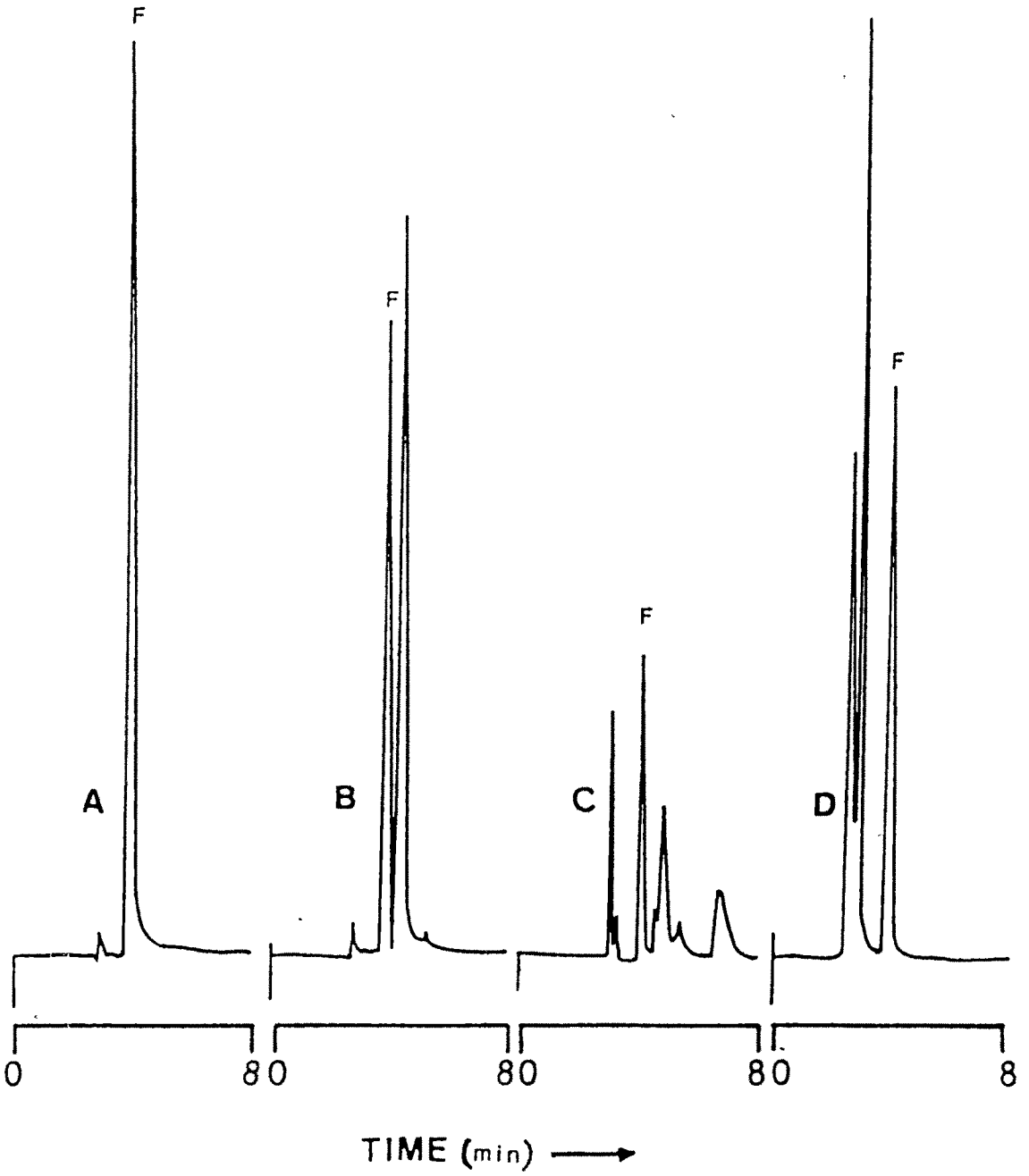

Fig. 4.3a : Chromatograms of Famotidine solution in A. Water B. 0.1N HCl C. 0.1N NaOH D. 0.5% H_2O_2 heated at 65° C for 30 min. (Peak F-Famotidine)

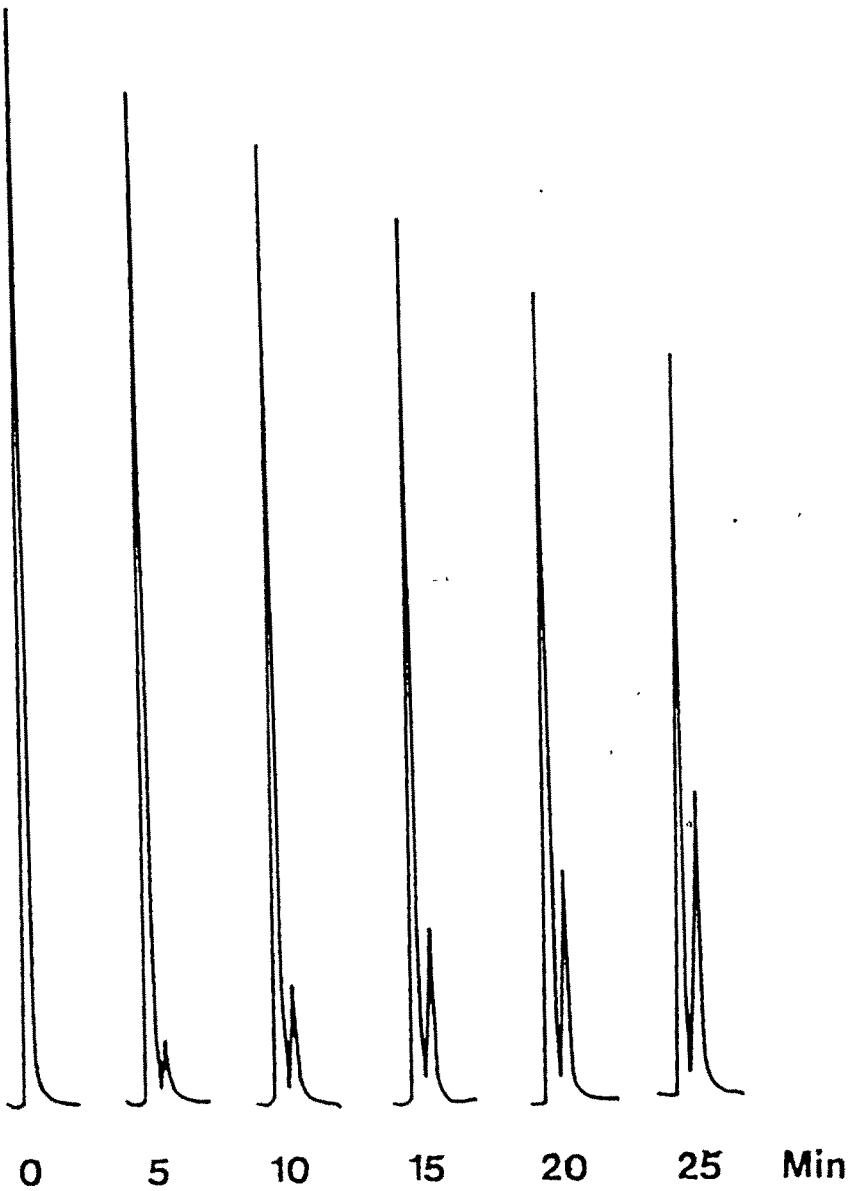

Fig. 4.3b : HPLC chromatograms of degradation of FMD at different time intervals

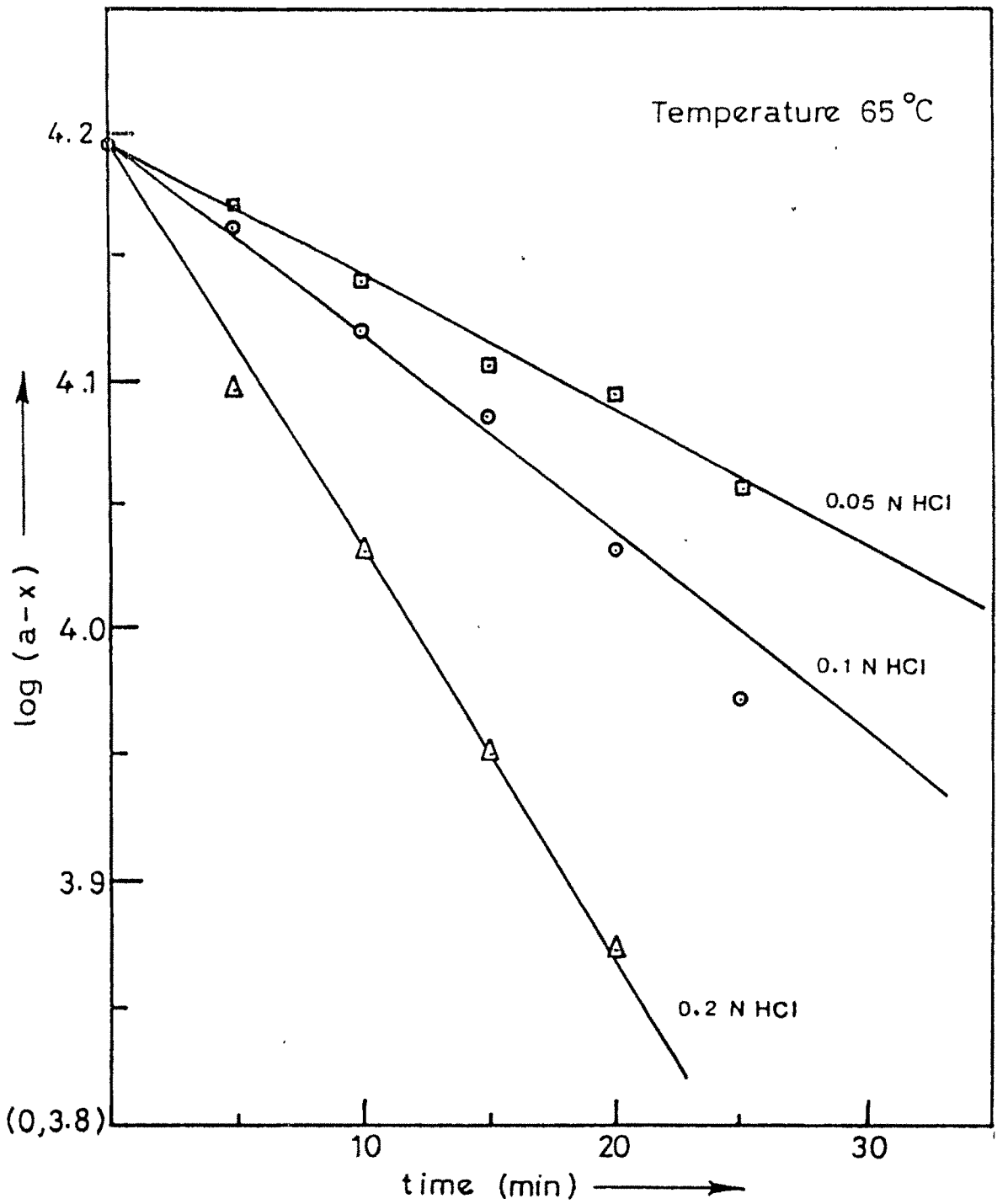

Fig. 4.3c : Pseudo first order kinetic plot of FMD degradation

Common excipients found in the tablet formulation did not interfere. Percent RSD for a single sample was < 1.2%. The present method is thus simple, selective and accurate for the determination of famotidine in tablets.

Degradation kinetics:

The method was found suitable to follow the kinetics of degradation of FMD in acidic solution. Samples withdrawn at various time intervals from the reaction mixture were quenched and analysed. Typical chromotagrams at various time intervals so recorded are shown in Fig 4.3b. Changes in concentration computed from such chromatograms followed pseudo first order kinetics under the conditions employed (Fig 4.3c). Investigation into the effect of temperature on the reaction showed that Arrhenius equation was not strictly obeyed. This is possibly due to the changes in the mechanism of degradation reaction with temperature as evident from the appearance of additional peaks on the chromatograms recorded at different temperatures.