CHAPTER 3 : *Flow Injection Analysis*

CHAPTER 3

FLOW INJECTION ANALYSIS

This chapter describes the details regarding apparatus, reagent preparation, analytical manifold used, procedure for calibration, results and discussion of the FIA methods.

3.1 EXPERIMENTAL

Reagents:

1. DCNP solution: A 0.02% w/v solution of 2,4-dichloro-6-nitrophenol (DCNP) was prepared by dissolving 100 mg of reagent (Fluka) in 500 ml of methanol. This reagent should be protected from light and used within 2 days.

2. Mercuric thiocyanate solution: A stock solution of 1.3×10^{-4} M mercuric thiocyanate was prepared by dissolving 0.416 g of reagent (Aldrich) in 100 ml of methanol and filtering after 3 hour.

3. Ferric nitrate solution: A stock solution of 0.272 M ferric ions was prepared by dissolving 11.0 g of $Fe(NO_3)_3.9H_2O$ in 30 ml water and 6 ml of concentrated nitric acid and diluting to 100 ml with water.

4. Mixed mercuric reagent: Prepared by mixing mercuric thiocyanate stock solution, ferric nitrate stock solution and 0.4 M nitric acid solution in ratio of 1:1:2.

5. Cupric acetate solution: A 0.025 M solution was prepared by dissolving 1.25 g of cupric acetate monohydrate in 250 ml water.

6. Acetate buffer (0.2 M, pH = 5.0): Prepared by dissolving 13.6 g of sodium acetate trihydrate and 6 ml of glacial acetic acid in 500 ml water.

Standard drug solutions:

Stock solutions of DFS (0.5 $mg.ml^{-1}$), FMD(0.5 $mg.ml^{-1}$) and KTR (1 $mg.ml^{-1}$) were prepared in methanol. Aliquots of stock solutions were suitably diluted with methanol to get the concentration in the range of 5 to 50 $\mu g.ml^{-1}$ for DFS, 10 to 50 $\mu g.ml^{-1}$ for FMD and 10 to 120 $\mu g.ml^{-1}$ for KTR and used in the method using DCNP. Stock solution of DLZ was prepared by dissolving 50 mg of the drug in 100 ml of water. In the determination of FMD using cupric acetate, a 1 $mg.ml^{-1}$ stock solution was prepared by dissolving 100 mg of FMD in 100 ml of acetate buffer of pH 5.0.

3.2 Flow injection analysis of DFS, FMD and KTR using DCNP (Methods A-16, C -9, D-5)

The proposed FIA method for the determination of DFS, FMD and KTR is based on the reaction of these drugs with DCNP in methanol to form charge transfer complexes which was measured with a spectrophotometric detector set at 450 nm. Under optimized conditions sampling rate of 40 per hour was achieved with RSD less than 1.6%.

3.2.1 Apparatus

FIA systems employed in the proposed methods were assembled using following components: Plunger pumps (Model LC-6A), UV-vis detector (Model SPD-6AV) and computing integrator (Model C-R3A) from Shimadzu Corporation, Japan and six bore injector (Model 7125) from Rheodyne Inc., USA. The reaction coil and back pressure coil and connecting tubes used were made of stainless steel. (Simple peristaltic pumps instead of the sophisticated HPLC pumps can also be used).

3.2.2 PROCEDURE

FIA system employed in this study is shown in Fig.3.1. The carrier solvent, methanol was delivered with the plunger pump (A) at a flow rate of 0.6 $ml.min^{-1}$ and mixed with the DCNP reagent delivered through the second plunger pump (B) at a flow rate of 0.8 $ml.min^{-1}$. Sample solution (40 µl) was injected manually into the carrier stream. The sample and the DCNP reagent were allowed to mix in the reaction coil (2 m, i.d. 0.8 mm) and the absorbance was measured at 450 nm. The area of the absorbance peaks were recorded by using the computing integrator.

The calibration graph for each drug was constructed by injecting 40 µl of the standard solutions in triplicate at a rate of one injection per 90 sec. The output (peak) of the spectrophotometer was recorded on the chart recorder. When the baseline of the peak was reached, the next sample was injected. The areas of the absorbance peaks were used for quantification (The peak heights can also be used for the purpose).

Analysis of tablets and injectables:

For DFS tablet analysis an accurately weighed portion of the tablet powder equivalent to 50 mg of the drug was transferred into a 50 ml volumetric flask containing 30 ml methanol. Using a mechanical shaker, the powder was completely disintegrated. The solution was made upto the volume with methanol and filtered through Whatman No. 40 filter paper. Two ml of the filtrate was further diluted to 50 ml with methanol. For DFS injections, volume equivalent to 50 mg of the drug was diluted to 50 ml with methanol. Two ml of this solution was further diluted to 50 ml with the same solvent.

For FMD tablet analysis, weight of powdered tablets equivalent to 30 mg of the drug was transfered into a 50 ml volumetric flask. The contents were shaken with 30 ml of methanol for 5 min, diluted to volume with the same solvent and filtered. Five ml of the filtrate was further diluted to 50 ml with methanol.

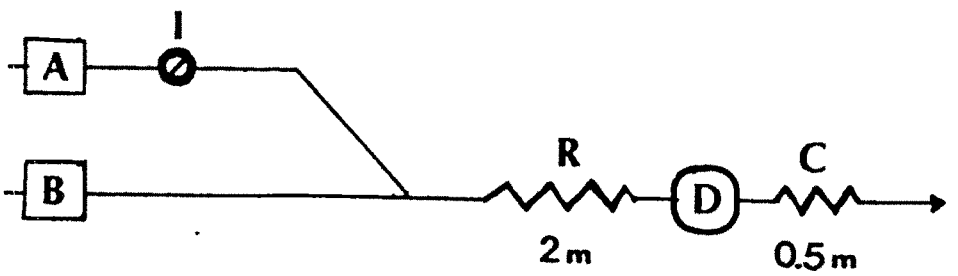


Fig. 3.1a : Analytical manifold used for the flow injection determination of diclofenac sodium, famotidine and ketorolac tromethamine, A and B: pumps, I: Injector, R: reaction coil (0.8 mm i.d.). D: detector (450 nm), C: back pressure coil (0.25 mm i.d.).

For KTR tablet analysis, weight of powdered tablets equivalent to 30 mg of the drug was shaken with 30 ml methanol for 5 min in a 50 ml volumetric flask. The solution was made upto volume with the same solvent and filtered. Five ml of the filtrate was further diluted to 50 ml with methanol.

The sample solutions were injected in triplicate and the amount of the drug corresponding to the peak area was found out from the calibration graph. The amount of the drug in the formulation was calculated using the dilution factor.

3.2.3 RESULTS AND DISCUSSION

Diclofenac sodium, famotidine and ketorolac tromethamine were found to react readily with the reagent DCNP in methanol to yield and intense colour. As mentioned earlier, an electronic absorption additional to the absorption of the components was observed. This may be ascribed to an intermolecular charge transfer from the donor to the acceptor[83]. Due to the presence of a nitro and two chloro substituents, DCNP is expected to act as a π-acceptor. This is supported by the fact that no such CT band is observed in this region when the nitro group in DCNP is replaced by an amino group. The spectral data of the drug-DCNP complexes are given earlier section (Methods A-14, C-7, D-4).

This colour reaction is sensitive, fairly rapid to develop in presence of excess of reagent and utilizes a single solvent and can be adapted for the FIA of these drugs.

Optimization of parameters:

The experimental conditions such as reagent concentration, flow rate and the reaction coil length were optimized for maximum sensitivity, linearity and reproducibility. A concentration of 0.02% (w/v) of DCNP in methanol was found

to be optimum since at higher concentrations of the reagent, the background colour increased and at lower concentrations the intensity of the colour formed was less. Total flow rate was optimized to 1.4 $ml.min^{-1}$ since at higher flow rate sensitivity was less. An increase in the length of reaction coil above 2 m showed a decrease in peak response. Sample sizes above 40 µl showed negative absorption peaks at lower concentrations of the drug and also deviation from linearity. When a 40 µl sample loop was used, the calibration graphs were linear in the range 5-50 $\mu g.ml^{-1}$ for DFS, 10-80 $\mu g.ml^{-1}$ for FMD and 10-120 $\mu g.ml^{-1}$ for KTR.

Typical absorption peaks obtained for KTR in a linearity study are shown in Fig 3.1. Similar absorption peaks were obtained for the other two drugs. Average peak areas were used for the construction of calibration graphs in all the three cases. The linearity range and correlation coefficient values are shown in Table 3.1.

To establish the practicality of the method, marketed products of DFS (tablets and injectables), FMD (tablets) and KTR (tablets) were analysed by the proposed method and also by the respective HPLC methods[30,10,56]. The results obtained from the proposed method are comparable with those obtained by the respective HPLC methods, as shown in Table 3.2.

Table 3.1: Results of linearity study in FIA determination of DFS, FMD and KTR

Drug	Linearity range $\mu g.ml^{-1}$	Intercept	Correlation coefficient
DFS	5-50	0.0412	0.9994
FMD	10-80	0.0364	0.9992
KTR	10-120	0.0324	0.9996

For recovery studies known amount of the drugs were added to the respective sample solutions which had been analysed earlier. The recovery of the drug was

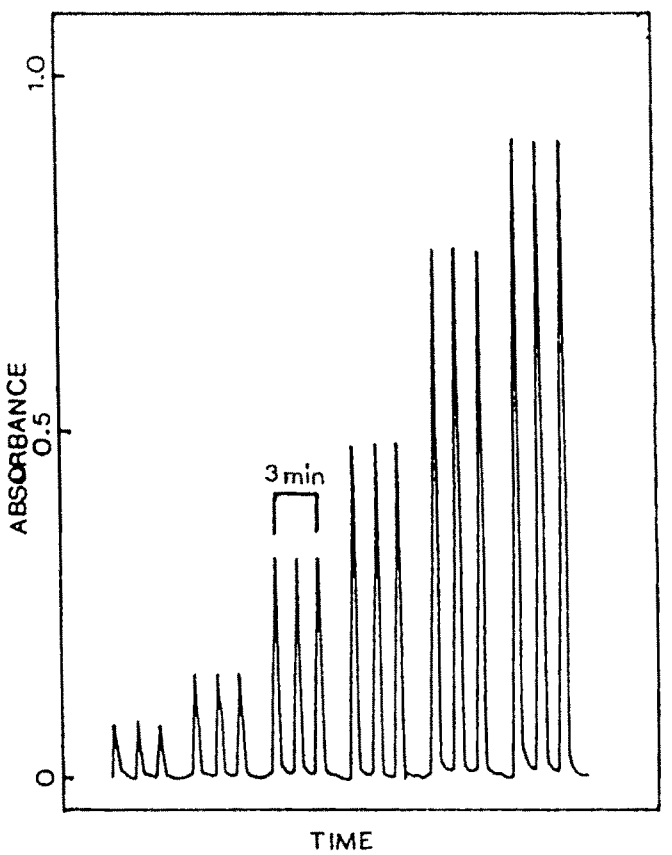


Fig. 3.1b : Typical absorption peaks obtained for KTR in linearity study of FIA

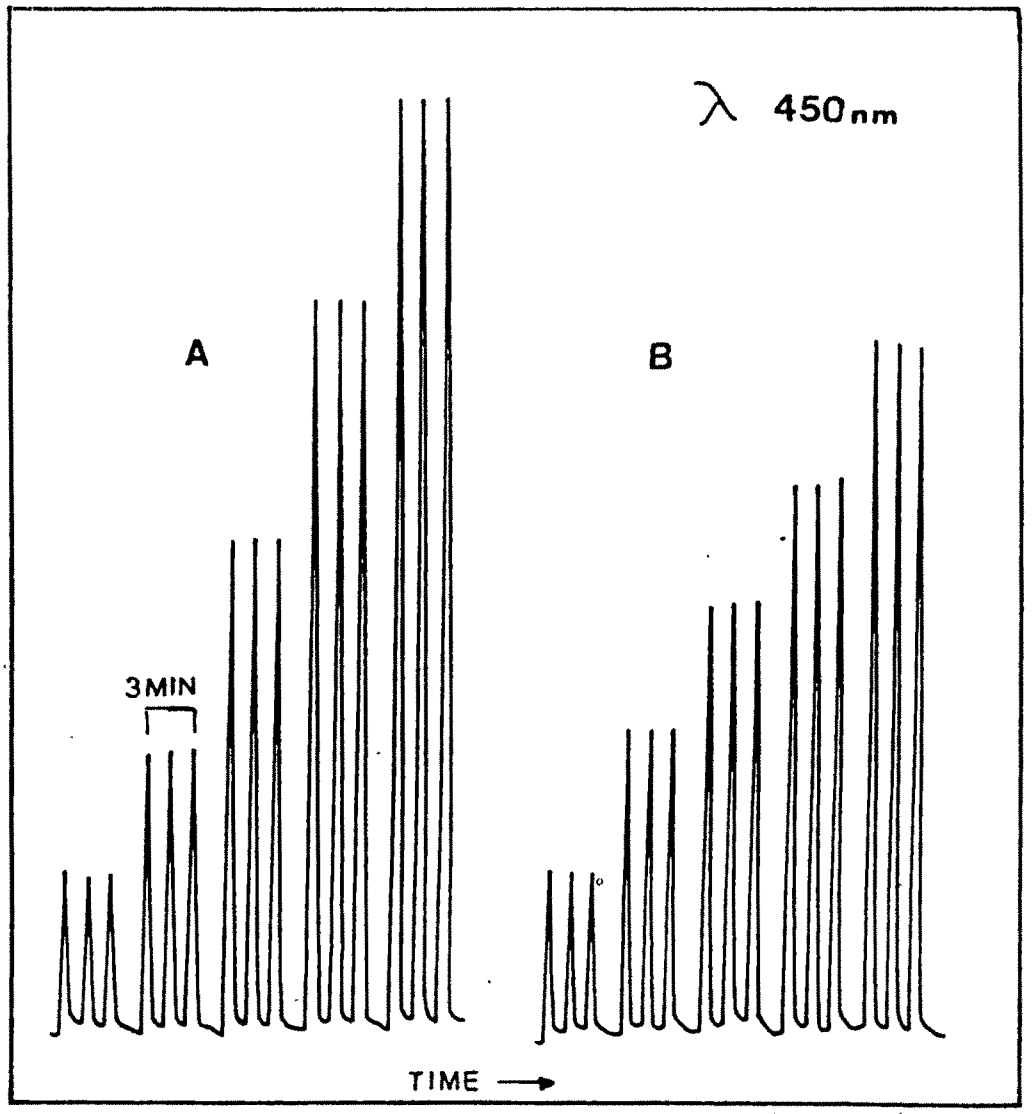


Fig. 3.1c : Typical FIA absorbance peaks used for calibration graph of (A) Famotidine and (B) Diclofenac sodium

in the range of 98.31 to 100.36% for DFS, 98.64 to 100.5% for FMD and 98.23 to 99.86% for KTR. Common excipients found in the tablet and injectable preparations did not interfere. However, interference was observed in the estimation of DFS in the presence of paracetamol.

Table 3.2: Determination of DFS, FMD and KTR in formulations of FIA using DCNP

Dosage form	Label claim	% Found (± SD)*	
		FIA method	HPLC method
Diclofenac sodium tablet	50 mg	98.24(1.54)	98.16(0.32)
Diclofenac sodium injection	25 mg (per ml)	97.92(1.48)	97.86(0.46)
Famotidine tablet	40 mg	99.18(1.22)	99.26(0.28)
Ketorolac tromethamine tablet	10 mg	98.36(1.44)	98.32(0.36)

* Mean of six determination.

Conclusion:

The proposed FIA method for the determination of DFS, FMD and KTR is rapid, sensitive and accurate. The method is simple since it involves a one step reaction and utilizes a single solvent. The reagent consumption is low and interference from the common excipients is limited. The proposed method is the

first reported FIA method for quantification of DFS, FMD and KTR in formulation.

3.3 FIA of Famotidine using cupric acetate (Method C-10)

The proposed FIA method is based on the reaction of FMD with cupric acetate in an aqueous flow system, to form a blue coloured complex, which was measured by a spectrophotometric detector set at 314 nm or 630 nm. The samples could be analysed at rates upto 60 per hour with RSD less than 1.4%.

3.3.1 Apparatus and procedure:

A schematic diagram of FIA system employed in determination of FMD is shown in Fig.3.2. The acetate buffer (pH=5.0), which served as carrier solution was delivered with a Shimadzu LC-6A plunger pump(A) at a flow rate of 1.6 $ml.min^{-1}$ and cupric acetate reagent with another plunger pump(B) at a flow rate of 0.8 $ml.min^{-1}$. A sample solution (100 µl) was introduced into the carrier stream by manual injection using Rheodyne six bore injector. The sample solution and cupric acetate solutions were allowed to mix in a 2 m reaction coil. A Shimadzu SPA-6A, UV-visible detector was used for the absorbance measurements at 314 nm and 630 nm. The detector was connected to a single channel Shimadzu CR-3A recorder.

Working standard solutions of FMD in the range 10-50 $\mu g.ml^{-1}$ and 50-500 $\mu g.ml^{-1}$ were prepared by appropriate dilutions of stock solution (1 $mg.ml^{-1}$) with acetate buffer (pH = 5.0). The spectrophotometric detector was set at either 314 nm or 630 nm and the reagents were pumped. The zero absorbance of the detector was set. The standard drug solutions were injected manually in triplicate at intervals of one minute. The output of the spectrophotometer was recorded on the

chart recorder. The area of the absorbance peak was used for the construction of calibration graph. At each wavelength the calibration graph was drawn by plotting the area versus concentration of the drug.

Tablet analysis: Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder equivalent to 40 mg of FMD was transferred into a 100 ml flask. The drug was shaken with 60 ml of acetate buffer solution (pH=5.0) for 5 min. After diluting upto volume with the buffer, the solution was filtered. The filtrate was used for the determination of the drug at 630 nm. Five ml of filtrate was further diluted to 50 ml with the buffer. This solution was used for the determination of the drug at 314 nm.

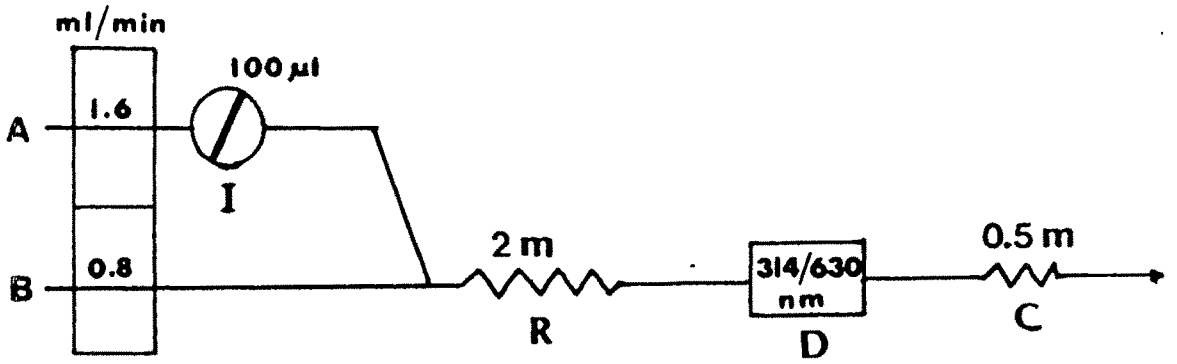
3.3.2 RESULTS AND DISCUSSION

The designed analytical manifold shown in Fig. 3.2a is a compromise of short analysis time, low reagent consumption, good sensitivity and linearity. The effect of cupric acetate concentration on the colour formation was studied. The increase in cupric acetate flow showed negligible effect on the absorbance peaks but the background absorbance of the reagent flow increased. Acetate buffer of pH 5.0 was selected due to the better stability of FMD-cupric acetate complex. Increase in the length of the reaction coil above 2 m showed a decrease in peak response. Injection volume of 100 µl was found to be optimum. At larger injection volumes incomplete reaction was observed. With a total flow of 2.4 ml per min maximum sampling rate and sensitivity were achieved.

Validation of the method:

Typical FIA peaks recorded at 314 nm and 630 nm for generating calibration graph are shown in Fig. 3.2b in increasing concentration order. In each case, a plot of concentration versus peak area is linear in the range shown in Table 3.3.

Fig. 3.2a : FIA manifold for the determination of Famotidine A and B : Pumps, I : Injector, R : Reaction Coil (i.d. 0.8 mm) D : Detector, C : Back pressure coil (i.d. 0.25 mm)



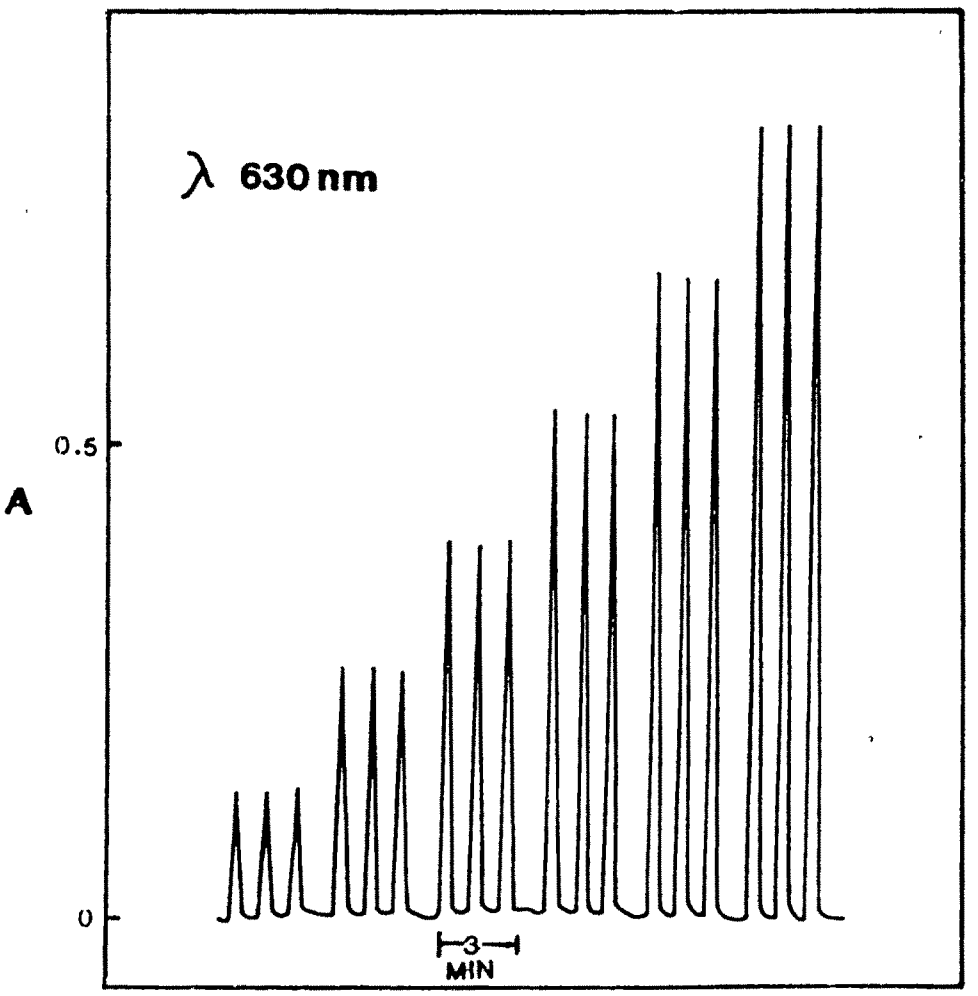


Fig. 3.2b : Typical FIA peaks recorded at 630 nm for FMD- cupric acetate complex

Table 3.3: Results of famotidine determination by FIA (Linearity study)

Detection wavelength nm	Linearity range $\mu g.ml^{-1}$	Intercept	Correlation coefficient
314	10-50	0.0462	0.9995
630	50-500	0.0736	0.9992

The practicality and suitability of the proposed method for quantification of FMD can be judged from the results of tablet analysis given in Table 3.4. The results are comparable with those of the USP method[10].

Table 3.4 : Results of famotidine tablet analysis

Tablet	Label claim (mg)	% Found (± SD)		
		FIA (314 nm)	FIA (630 nm)	USP method
Lot A	20	98.34(0.86)	98.26(1.21)	98.42(0.32)
Lot B	40	99.28(1.04)	99.24(1.32)	99.30(0.46)
Lot C	40	100.86(0.96)	100.82(1.16)	100.85(0.28)

The recovery of the added drug to pre-analysed sample solutions were in the range 98.0-100.6%, which showed the absence of interference from common excipients used in tablet formulation. The reproducibility of the method is satisfactory with RSD less than 1.4%.

The benefits of rapid FIA method were evident in the content of uniformity tests carried out on several marketed products. The results were within the limits

specified by USP. The method is not suitable for the study of dissolution rate under the experimental conditions mentioned in USP, since phosphate buffer used in the dissolution medium was found to interfere in the reaction to give low recovery of the drug.

The present study demonstrates the application of FIA to the determination of FMD in formulations. The proposed method can be used for the routine assays and content of uniformity studies ensuring rapid, accurate and precise results. Absorbance measurement at 314 nm is more sensitive than detection at 630 nm.

3.4 FIA of Diltiazem hydrochloride (Method B-15):

The proposed FIA method for the determination of DLZ is based on the colorimetric mercurothiocyanate estimation of chloride counter ion. Sampling rate of 30 per hour was achieved with RSD less than 1.4%.

3.4.1 Apparatus and procedure: A schematic diagram of FIA system used in the determination of DLZ is shown in Fig. 3.3a. The carrier reagent, 0.4 M nitric acid solution was delivered through a plunger pump (A) at a flow rate of 1.2 $ml.min^{-1}$ and mixed with mercuric thiocyanate reagent delivered through another plunger pump (B) at a flow rate of 1.2 $ml.min^{-1}$. A sample solution (100 µl) was manually injected into the carrier stream. The sample and mercuric thiocyanate reagent were allowed to mix in a 2 m reaction coil and the absorbance was measured at 460 nm.

Calibration curve: Standard drug solution was prepared by dissolving 50 mg of DLZ in 100 ml of water. Aliquots of the drug solution (0.5-2.5 ml, 0.5 $mg.ml^{-1}$) were taken in 25 ml volumetric flasks and diluted to volume with water. The

spectrophotometer was set at 460nm. The reagent solution and carrier solutions were pumped and the zero absorbance was set. The standard solutions were manually injected in triplicate at a frequency of one injection per minute. Average peak areas were used for construction of the calibration graph.

Tablet analysis: Twenty tablets were weighed and finely powdered. An accurately weighed portion of powder equivalent to 30 mg of DLZ was transferred into a 100 ml volumetric flask and diluted with water to volume. Using a mechanical shaker the powder was completely disintegrated and the solution was filtered. Five ml of solution was further diluted to 50ml with water. Sample solutions were injected in triplicate and the content of DLZ was found from the calibration graph. The content of the drug in tablet was calculated using the dilution factor.

Content of uniformity test:

The tablets were dissolved individually in appropriate volume of water as described under tablet analysis. Measurements were made using the calibration graph or for more accurate results, individual standard solution of the drug.

Dissolution studies:

The experimental conditions followed for the dissolution study were as per USP XXII[10]. Water was used as the dissolution medium, with paddle assembly rotating at a speed of 50 rpm. One ml of sample solutions were withdrawn at intervals of 5 min using a syringe and injected into the flow.

3.4.2 RESULTS AND DISCUSSION

The mercurothiocyanate method for the estmiation of chloride ion[88-91] is based on the displacement of thiocyanate from mercuric thiocyanate by chloride in the

presence of ferric ion leading to the formation of a highly coloured ferric thiocyanate complex as shown below:

$$Hg(SCN)_2 + 2Cl^- + 2Fe^{3+} \rightarrow HgCl_2 + 2Fe(SCN)^{2+}$$

The colour of this complex measured at 460 nm is proportional to the orignial chloride ion concentration. This reaction was adapted for the indirect flow injection analysis of DLZ via the estimation of chloride counter ion of the drug.

Optimization of parameters:

The experimental conditions like flow rate, reagent concentration and reaction coil length were optimized for maximum sensitivity, linearity and reproducibility. Using 100 µl sample loop the calibration graph was linear in the range 5-50 $\mu g.ml^{-1}$ of the drug. Increase in the length of the reaction coil above 2 m showed decrease in peak response. The total flow rate was optimized to 2.4 $ml.min^{-1}$ for low reagent consumption. The peak response slightly increased by increasing the flow rates. Under optimized conditions and by manual injection, samples upto 60 per hour can be analysed. Typical absorbance peaks of the standard drug solution used for the construction of calibration graph are shown in Fig. 3.3b. The intercept of the calibration graph was 0.0387 with a correlation coefficient of 0.9996. The relative standard deviation value for triplicate injection was less than 1.4%.

To establish the practicality of the method, marketed products of DLZ tablets were analysed by the proposed methods and also by the USP method. The results obtained by the proposed method compare well with those obtained by the USP method as given in Table.3.5.

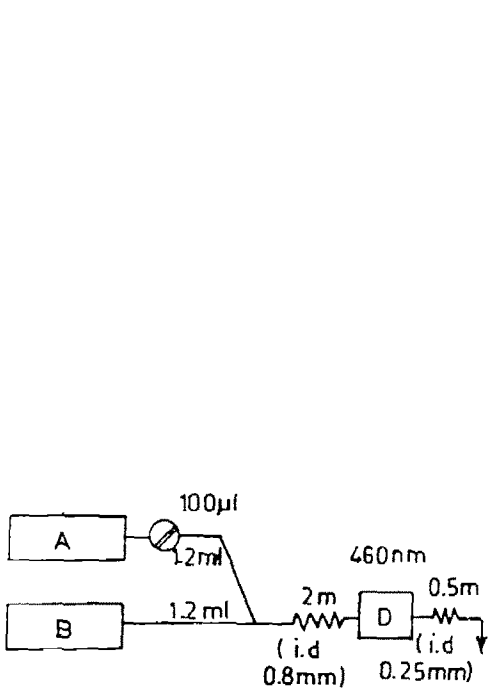


Fig. 3.3a :Analytical manifold for FIA determination of diltiazem hydrochloride.

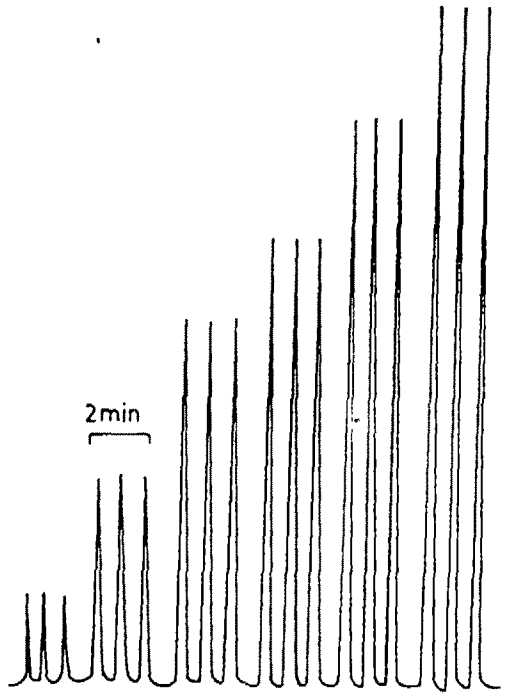


Fig. 3.3b :Typical FIA absorbance peaks used for calibration graph of diltiazem hydrochloride

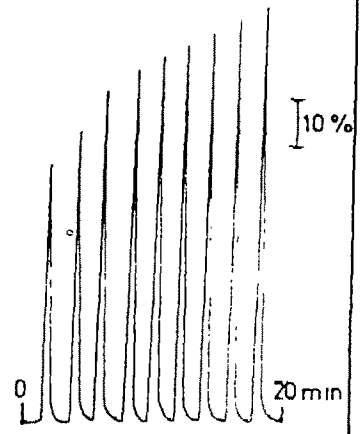


Fig. 3.3c :Typical dissolution profile of diltiazem hydrochloride tablets (30mg)

Table 3.5: Results of DLZ tablet analysis by FIA method compared to USP method.

Tablet	Label claim mg	% Found (± SD)	
		FIA method	USP method
Lot A	30	98.52(1.21)	98.40(0.32)
Lot B	60	99.14(1.38)	99.08(0.46)

Recovery of the added drug to pre-analysed sample solutions was 98.55 to 100.2%. Common excipients found in the tablet formulation did not interfere. However, interference was observed with bromide, iodide and thiocyanate, if present, since they react with the reagent employed.

The benefits of FIA method were established by the content of uniformity tests on several marketed products. The results were well within the limits specified by USP. Suitability of the present method for dissolution studies can be seen from the typical FIA dissolution profile of DLZ tablets (30 mg) given in Fig. 3.3c.

This method is simple, sensitive and rapid. The method is useful in the determination of content of uniformity and in monitoring the dissolution profile of tablets.