CHAPTER 2

DEVELOPMENT OF ANALYTICAL METHODS

2.1 INTRODUCTION

A number of analytical methods have been reported for 5FU, MTX and cyclophosphamide in the literature. However, there is still ample scope to develop newer methods of analysis which are simple, sensitive, selective and more economical.

2.11 Colorimetric Methods :

A variation of the colour of a system with change in the concentration of some component forms the basis of colorimetric analysis. The absorption in the visible region occurs due to excitation of bonding electrons. The absorbing species may include transitions involving :

a) $\pi - \pi^*$ or $n-\pi^*$ where π^*= non bonding orbital).

b) d and f electrons.

c) charge transfer electrons.

A detailed analysis of the chemical structure of 5FU, MTX and cyclophosphamide has revealed that there are a number of functional groups in these molecules which may undergo reaction to give coloured products which can then be quantified. This formed the basis for the development of a number of new colorimetric methods for these three drugs.

2.12 Fluorimetric Method :

Fluorescence is caused by absorption of radiant energy and the re-emission of some of this energy in the form of visible light. In true fluorescence the absorption and emission takes place in a short but measurable time of order 10^{-12} to 10^{-9} sec.

The fluorescence intensity is directly proportional to concentration of fluorescent substance by the equation $F=K'C$ where F=intensity of fluorescent radiant energy, K'=constant, and C=concentration of the fluorescing substance.

2.13 HPLC Method :

HPLC is characterised by use of online detection system, controlled eluent flow rates and definite eluent composition under the influence of high pressure. The aim is to increase, compared to column or thin layer chromatography, the efficiency of separation and reproducibility and accuracy of retentions and peak areas in order to achieve quantitative and qualitative separations. Because a wide range of separation modes can be used, HPLC is an extremely versatile technique and can be used to determine virtually any non-gaseous analyte as long as it is soluble in an organic or inorganic solvent.

A selective and a sensitive method was developed for estimation of 5FU from dosage forms and body fluids.

2.2 COLORIMETRIC METHODS FOR 5FU

2.21 Materials :

The following reagents and chemicals were used for development of various colorimetric methods for 5FU.

5FU (Biochem Industries, India), copper acetate, cobalt acetate, ammonium metavanadate, manganese chloride, ferric chloride, nickel chloride (Qualigens, India), sodium hydroxide pellets, sodium acetate anhydrous, sodium nitrite,

sulfanilic acid, sulfanilamide (E. Merck, India), o-nitroaniline, p-nitroaniline, paraaminobenzoic acid, anthranilic acid, p-aminophenol, o-phenylene diamine, p-toluidine, p-anisidine (National Chemicals, India), sodium carbonate, dithizone, diphenylcarbazone, (E. Merck, India), potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium hydrogen phthalate, boric acid, mercuric sulphate, p-dimethyl amino benzaldehyde, ascorbic acid, anhydrous sodium sulphate (Qualigens, India), diethylamine, isopropylamine, bromine, (s.d. fine chemicals, India) chloroform, ethylacetate, isopropanel, methanol, solvent ether, glycerol, hydrochloric acid (concentrated), glacial acetic acid, (Qualigens, India), purfied water I.P.

All the reagents and chemicals used were of A.R. grade and were used as such without further purification. All spectral measurements were made on Systronics-108, UV-visible spectrophotometer.

2.22 Complexation of 5FU with Metals of First Transition Series :

The published literature did not reveal any analytical method involving metal complexation for the estimation of 5FU. The compounds having -CONHCO-, -CONHCS- groups (uracil, barbiturates) which are structurally similar to 5FU have been reported to react with divalent metal ions like copper and cobalt to give coloured complexes in nonaqueous alkaline medium (1). So attempts were made to develop colorimetric methods for estimation of 5FU on similar lines.

2.221 Reagents :

The following reagents were prepared :

a) Solvent mixture : A 3:2 mixture of chloroform : methanol ($CHCl_3$ - MeOH) treated with anhydrous sodium sulphate was used as the solvent mixture.

b) Stock solution of 5FU : 100mg of 5FU was accurately weighed into a clean, dry 100ml volumetric flask, dissolved and made upto volume with solvent mixture.

c) Metal solutions : 50mg of the metal salts viz. copper acetate, cobalt acetate, ammonium meta-vanadate, manganese chloride, ferric chloride, nickel chloride were accurately weighed and transferred into separate 100ml volumetric flasks. 5ml of methanol was added to dissolve the metal salt and volume was made up to 100ml with the solvent system.

d) Diethylamine solution (o.5% v/v) (DEA) : 0.5ml of DEA was pipetted out into a clean dry 100ml volumetric flask and made upto volume with the solvent mixture.

e) Isopropylamine solution (20% v/v) : 20ml of isopropylamine was pipetted out into clean dry 100ml volumetric flask and made upto volume with the solvent mixture.

2.222 Experimental procedures :

Two sets of experiments were performed. 1ml of stock solution of 5FU was accurately transferred to separate test tubes in each set. To each of these test tubes, 2ml of different metal solutions were added. To the first set of test tubes, 1ml of DEA solution was added while to the second

set 1ml of isopropylamine solution was added. The contents of each test tube were mixed thoroughly and the colour formed if any was compared with the corresponding reagent blank.

Chromogenic reactions were observed only in cases of copper and cobalt metals. This indicates that 5FU gives coloured complexes with copper acetate (in DEA medium) and cobalt acetate (in isopropylamine medium). Hence an attempt was made to quantify these reactions in order to develop colorimetric methods for estimation of 5FU.

2.223 Development of colorimetric method for 5FU with copper acetate :

a) Scanning of the spectrum :

To a suitable aliquot (0.1ml) of drug solution in 10ml volumetric flask, 1ml of metal solution and 1ml of DEA solution were added and made to volume with the solvent mixture. The absorption spectrum of yellowish green coloured complex was scanned between 400-800nm against the reagent blank. The spectrum scan is shown in Fig. 2.1.

b) Optimisation of DEA concentration :

A suitable aliquot (0.1ml) of drug solution and 1ml of metal solution were transferred into different 10ml volumetric flasks, 1ml of varying concentrations (0.1, 0.2,...., 0.5% v/v) of DEA solution were added and made to volume with the solvent mixture. Absorbance of each of the solutions was measured at 350nm against the appropriate reagent blank. Observations are recorded in Table 2.1.

c) Optimisation of metal concentration :

To a suitable aliquot (0.1ml) of drug solution in different 10ml volumetric flasks, 1ml of varying concentrations (50µg, 100µg....750µg) of copper acetate solution were added followed by 1ml of DEA solution. The volume was made up with the solvent system and the absorbance of each of the solutions was measured at 350nm against the reagent blank. Observations are recorded in Table 2.2.

d) Preparation of calibration curve :

Aliquots of drug solution equivalent to 4µg, 8µg....110µg per ml were accurately transferred into clean, dry, separate 10ml volumetric flasks. 1ml of each of copper acetate solution and 0.5%v/v of DEA solution were added and made to volume with the solvent mixture. The absorbance of the yellowish green coloured solution in each flask was measured at 350nm against the reagent blank and the observations are recorded in Table 2.3. The calibration curve is shown in Fig. 2.2.

e) Estimation of 5FU from injection :

5FU is available as injection in usual strength of 500mg in 10ml, pH adjusted to 9 with sodium hydroxide solution (Biochem Industries, India). A volume of injection equivalent to 100mg of the drug was transferred to a 100ml volumetric flask, 10ml of water was added, the pH of the solution was adjusted to 7 with glacial acetic acid and made to volume with water. A 5ml aliquot was

pipetted out and extracted successively with five quantities each of 5ml of ethylacetate:isopropanol (7:3) mixture. The combined organic extracts were evaporated under vacuum and the residue was dissolved in 25ml of $CHCl_3$: MeOH (3:2) mixture and the colour was developed with a suitable aliquot (0.5ml) of the drug solution as described under the preparation of calibration curve.

f) Estimation of 5FU from cream :

5FU was estimated from the 5% cream prepared in polyethyleneglycol base. A portion of the cream equivalent to 50mg of 5FU was accurately weighed into a 500ml volumetric flask. The drug was extracted from the cream base as per the USP method, 400ml of pH 4.7 acetate buffer was added and the contents of the flask was shaken for 5 minutes, made upto volume with the buffer and filtered. 20ml of the clear filtrate was extracted successively with five 10ml portions of solvent ether and four 20ml portions of chloroform. The organic extracts were discarded. The pH of the aqueous solution was adjusted to 7 with 0.1N sodium hydroxide solution. Further operations were carried out as described in the estimation of 5FU from injection starting with the words "A 5ml aliquot was pipetted out and extracted....".

The results of estimation of 5FU from injection and cream are recorded in Table 2.4 and the percentage recoveries were compared with I.P and U.S.P. method respectively.

g) Results and discussion :

5FU gave a yellowish green coloured complex with copper acetate in anhydrous $CHCl_3$-MeOH medium made alkaline with DEA. The absorption spectrum of the complex shows a maxima at 350nm (Fig.2.1). The reagent blank did not show any interference at the analytical wavelength. DEA solution was added to make the medium alkaline for formation of coloured metal complex with drug. From Table 2.1 it may be observed that 1ml of 0.5%v/v solution of DEA was optimum for colour development. The optimum metal concentration (Table 2.2) was found to be 500µg/ml. The reaction was carried out at room temperature and the colour was found to be stable for more than four hours.

From the data of the mean absorbance values (Table 2.3 and Fig 2.2) it may be observed that the calibration curve was rectilinear between 0.4-11.0µg/ml. The senstivity obtained was 0.4 µg/ml. The a and b values for the line of regression (y=a+bx) were calculated and found to be a=0.085 and b=0.031. The correlation coefficient r=0.992. Molar absorptivity was found to be 5.22×10^3 $lmol^{-1}$ cm^{-1}.

The percentage recoveries of 5FU from injection and cream (Table 2.4) were obtained between 98.95% to 99.98% and were comparable with I.P and U.S.P. method respectively.

On the basis of analysis of the data for estimation of the drug from formulation it may be concluded that the

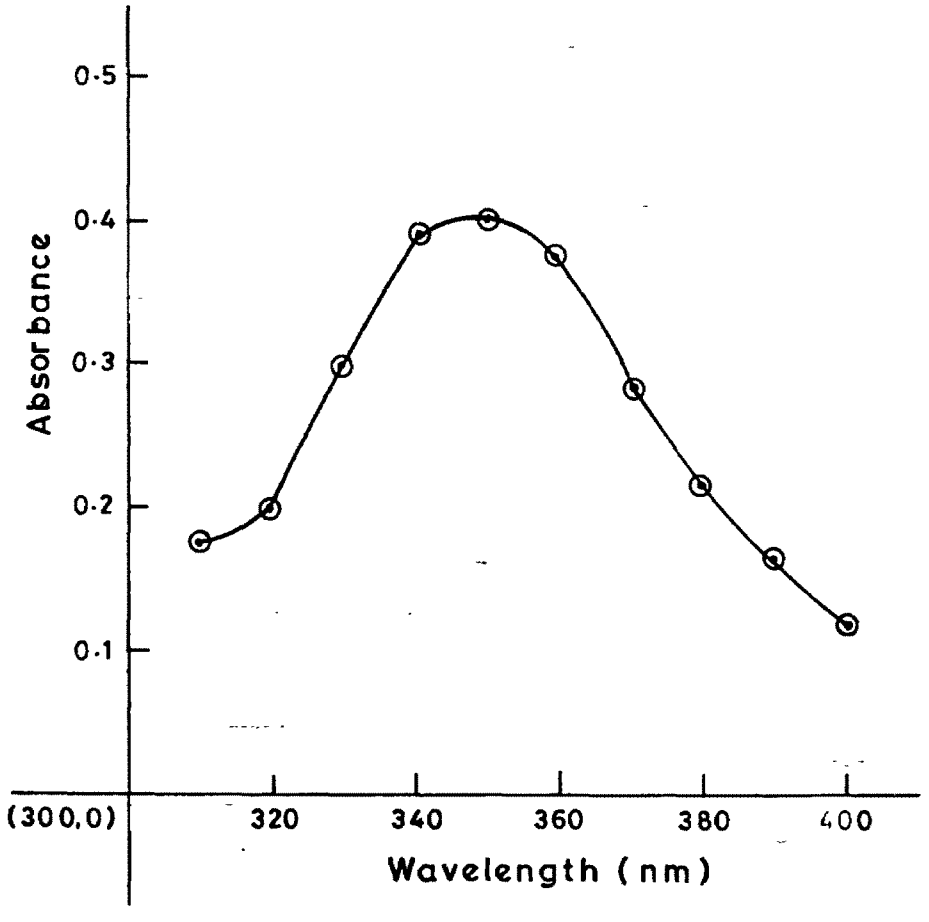


FIG. 2.1: WAVELENGTH SCAN OF 5 FU-COPPER COMPLEX

TABLE 2.1

OPTIMISATION OF DEA CONCENTRATION

Drug concentration - 10µg/ml.

λmax - 350nm

DEA concentration % v/v	Absorbance
0.1	0.053
0.2	0.100
0.3	0.275
0.5	0.401
1.0	0.401
5.0	Precipitation occurs

TABLE 2.2

OPTIMISATION OF COPPER ACETATE CONCENTRATION

Drug concentration - 10µg/ml

λmax - 350nm

Concentration of metal µg/ml	Absorbance
50	0.051
100	0.115
250	0.209
500	0.401
600	0.400
750	Interference of blank solution

TABLE 2.3

CALIBRATION CURVE OF 5FU - COPPER COMPLEX

λmax = 350nm

Concentration of 5FU µg/ml	Mean absorbance (±S.D.)
0.4	0.087 (0.016)
0.8	0.098 (0.008)
1.0	0.128 (0.015)
1.6	0.136 (0.006)
3.2	0.157 (0.006)
6.4	0.262 (0.013)
8.0	0.320 (0.021)
10.0	0.401 (0.032)
11.0	0.448 (0.006)

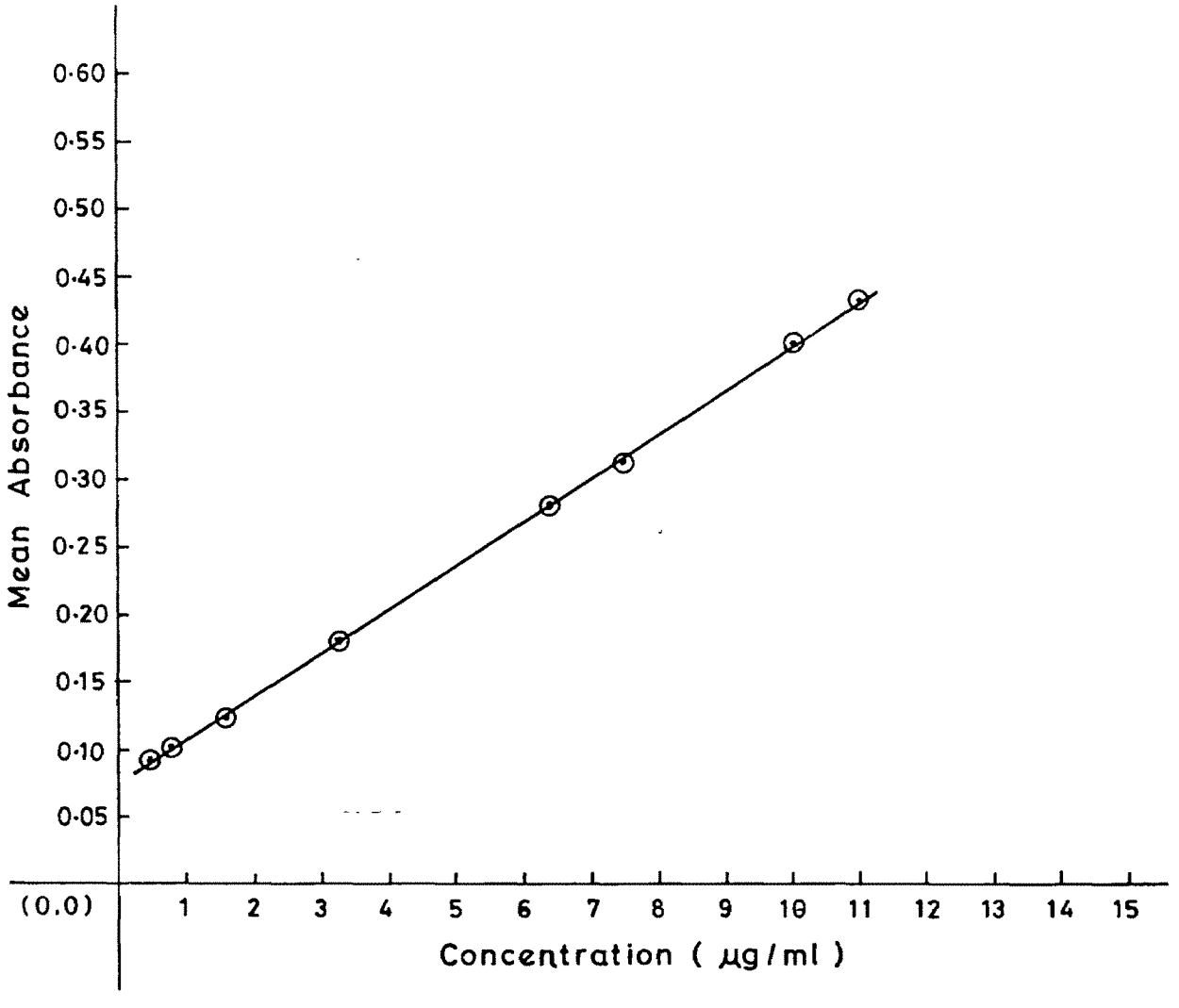


FIG. 2.2: CALIBRATION CURVE OF 5FU-COPPER COMPLEX

TABLE 2.4

ESTIMATION OF 5FU IN INJECTION AND CREAM

Dosage form	Concentration in aliquot µg/ml of 5 FU	% Recovered (± S.D.)	
		UV spectrophotometric method λmax = 266nm	5 FU-copper complex λmax = 350nm
Injection (50mg/ml)	10	100.38 (0.543)	99.98 (0.970)
Cream (5%w/w)	10	99.31 (0.796)	98.95 (0.751)

method is reproducible and comparable with the pharmacoepial methods.

2.224 Development of colorimetric method for 5FU with cobalt acetate :

a) Scanning of the spectrum :

To 0.5ml of the drug solution in a 5ml volumetric flask, 1ml of metal solution and 1ml of isopropylamine solution were added and made to volume with solvent mixture. The absorption spectrum of purple coloured complex was scanned between 400-800nm against the reagent blank. The wavelength scan is shown in Fig.2.3.

b) Optimisation of isopropylamine concerntration :

To 0.5ml of the drug solution and 1ml of metal solution in different 5ml volumetric flasks, 1ml of varying concentrations (5,10,...30,50%v/v) of isopropylamine were added and made up to volume with the solvent mixture. The absorbance of each of the solution was measured at 570nm against the reagent blank. The observations are recorded in Table 2.5.

c) Optimisation of metal concentration :

To 0.5ml of drug solution in different 5ml volumetric flasks, 1ml of varying concentrations (0.15, 0.20,....0.4%w/v) of cobalt acetate solution were added followed by 1ml of isopropylamine solution. The volume was made up with the solvent and the absorbance of the solution was measured at 570nm against the reagent blank. Observations are recorded in Table 2.6.

d) Preparation of calibration curve :

Aliquots of drug solution equivalent to 100µg, 200µg...1000µg/ml of 5FU were accurately transferred into clean, dry, separate 5ml volumetric flasks. 1ml of 0.3%w/v cobalt solution and 1ml of 20%v/v of isopropylamine solution were added and made up to volume with the solvent mixture. The absorbance of the purple coloured complex formed in each flask was measured at 570nm against the reagent blank and the observations are recorded in Table 2.7. The calibration curve is shown in Fig.2.4.

e) Estimation of 5FU from injection :

A volume of injection equivalent to 100mg of the drug was transferred into a 100ml volumetric flask, 10ml of water was added and the pH was adjusted to 7 with a few drops of glacial acetic acid and made to volume with water. From 5ml of the aliquot, drug was extracted as per the procedure described under copper acetate method (section 2.223e.). The residue of the drug was dissolved in 50ml of $CHCl_3$-MeOH mixture and colour was developed with a suitable aliquot (0.5ml) of the drug solution as described under preparation of calibration curve.

f) Estimation of 5FU from cream :

A portion of the cream equivalent to 500mg of 5FU was accurately weighed and the drug was extracted by the U.S.P. method as described under copper acetate method (section 2.223f). Further operations were carried out as

described in the injection starting with the words "The residue of the drug was dissolved in 50ml of $CHCl_3$:MeOH...".

The result of the estimation of 5FU from injection and cream are recorded in Table 2.3 and the percentage recoveries were compared with I.P. and U.S.P. methods respectively.

g) Results and discussion :

5FU gave a purple coloured complex with cobalt acetate in anhydrous $CHCl_3$-MeOH medium made alkaline with isopropylamine.

The absorption spectrum of 5FU cobalt acetate complex showed a maxima at 570nm (Fig 2.3). The reagent blank did not show any interference at the analytical wavelength. Isopropylamine was added to make the pH alkaline and the optimum concentration required was found to be 1ml of 20%v/v solution (Table 2.5). The optimum metal concentration (Table 2.6) for colour development was found to be 1ml of 0.3% w/v solution of cobalt acetate. The reaction was carried out at room temperature and the colour obtained was stable for more than four hours.

The calibration curve was rectilinear within the range of 20-200µg/ml (Table 2.7 and Fig. 2.4). The sensitivity of the method was found to be 10µg/ml. The a and b values for the line of regression were found to be a=0.016 and b=0.0021. Correlation coefficient 'r' and molar absorptivity were found to be 0.991 and 0.60×10^3 $lmol^{-1}cm^{-1}$ respectively.

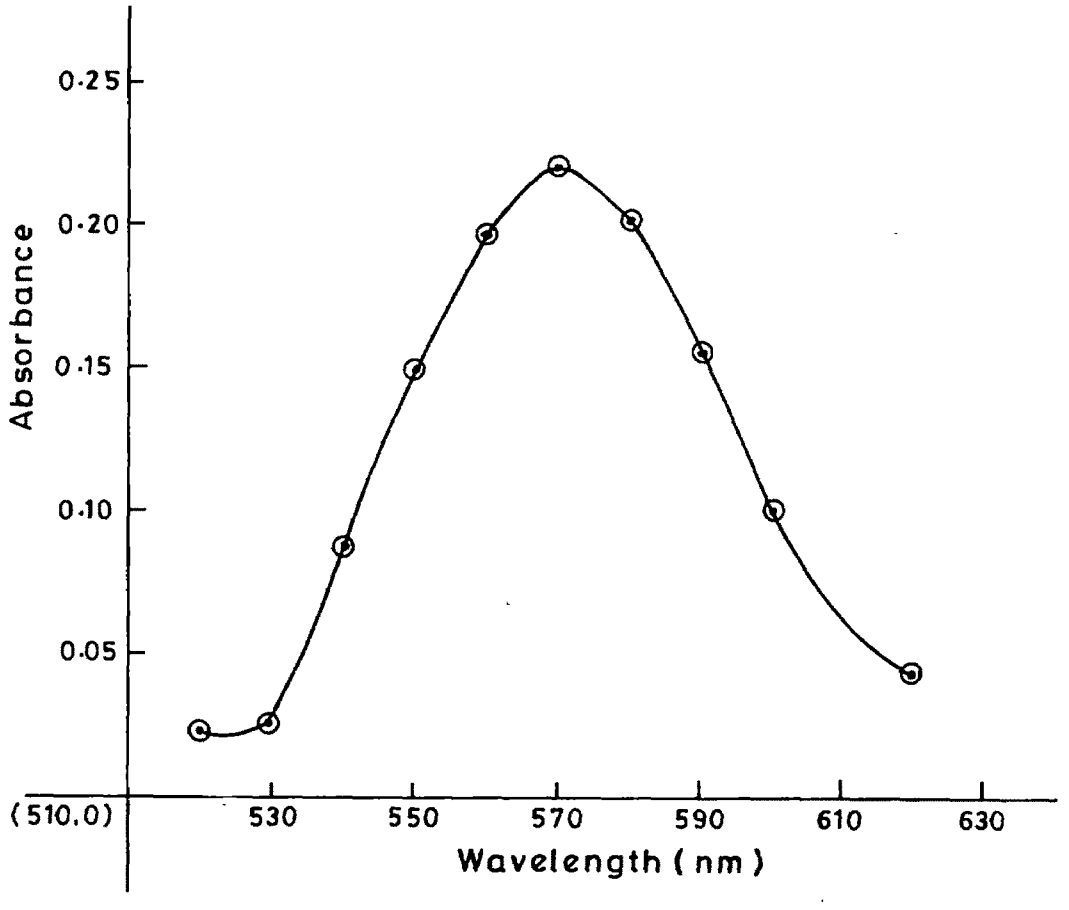


FIG. 2.3: WAVELENGTH SCAN OF 5 FU-COBALT COMPLEX

TABLE 2.5

OPTIMISATION OF ISOPROPYLAMINE CONCENTRATION

Drug concentration - 500µg/ml
λmax - 570nm

Isopropylamine concentration % v/v	Absorbance
5	0.089
10	0.175
15	0.195
20	0.223
25	0.223
30	0.221
50	Precipitation of metal occurs.

TABLE 2.6

OPTIMISATION OF COBALT ACETATE CONCENTRATION

Drug concentration - 50µg/ml
λmax - 570nm

Metal concentration % w/v	Absorbance
0.15	0.185
0.20	0.201
0.25	0.215
0.30	0.223
0.35	0.223
0.4	Interference of metal

TABLE 2.7

CALIBRATION CURVE OF 5FU-COBALT COMPLEX

Concentration µg/ml of 5FU	Mean absorbance (± S.D.)
20	0.082 (0.001)
40	0.165 (0.024)
50	0.223 (0.012)
80	0.325 (0.023)
100	0.410 (0.014)
120	0.501 (0.008)
160	0.684 (0.014)
200	0.917 (0.012)

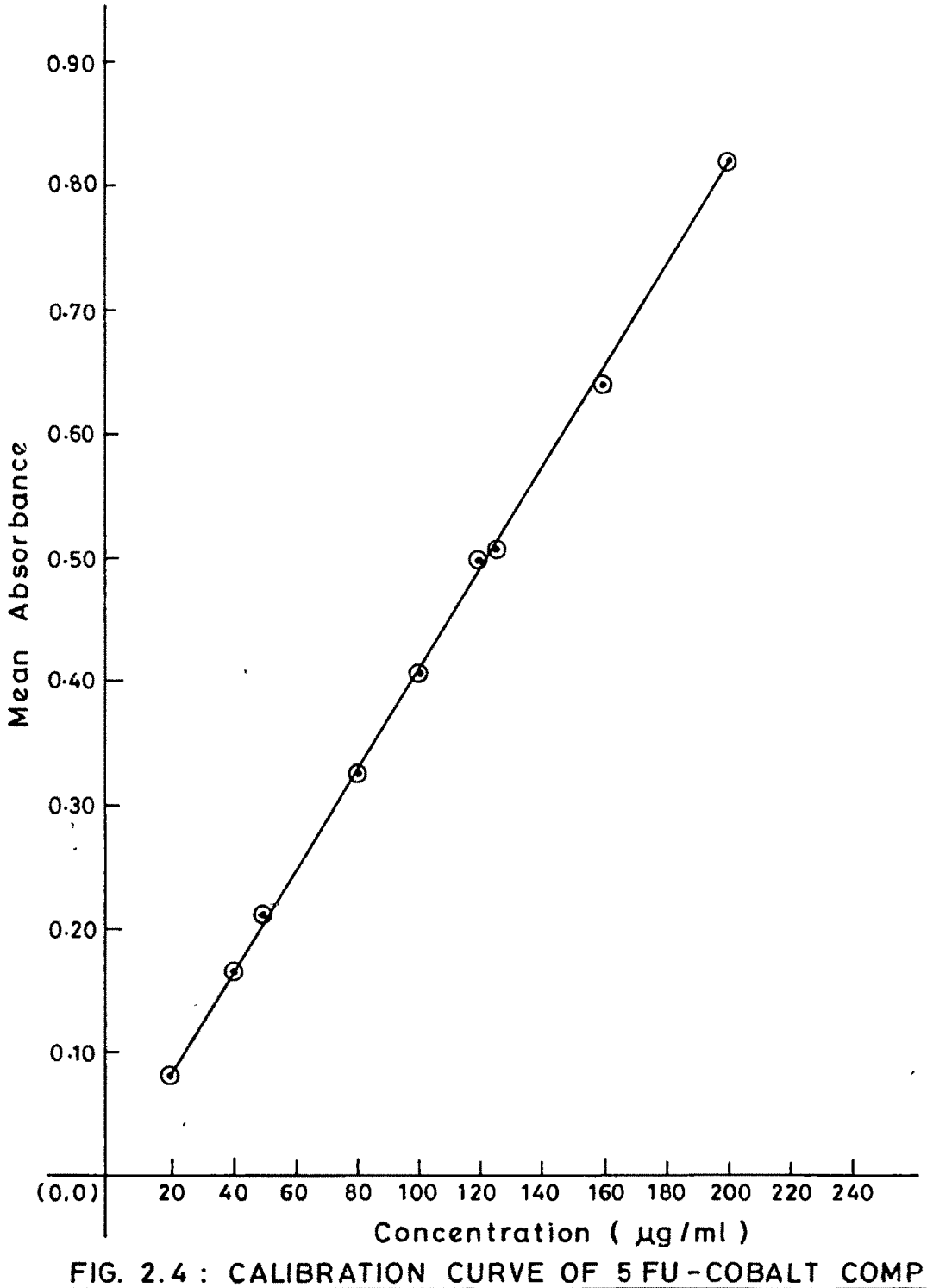


FIG. 2.4 : CALIBRATION CURVE OF 5 FU-COBALT COMPLEX

TABLE 2.8

ESTIMATION OF 5FU IN INJECTION AND CREAM

Dosage form	UV spectrophotometric method λmax = 266nm		5FU-Cobalt Complex λmax = 570nm	
	Conc. of 5FU in aliquot µg/ml	%Recovered (± S.D.)	Conc.of 5FU in aliquot µg/ml	% Recovered (± S.D.)
Injection (50mg/ml)	10	100.38 (0.543)	50	99.38 (0.571)
Cream (5% w/w)	10	99.31 (0.796)	50	98.23 (0.799)

The percentage recoveries of 5FU from injection and cream (Table 2.8) were obtained between 98.23% - 99.38% and were comparable with the I.P. and U.S.P. method respectively.

On the basis of analysis of the data obtained for the estimation of the drug from formulations it may be concluded that the cobalt acetate method is reproducible and comparable with the pharmacoepial methods.

2.23 Coupling with Diazotised Aromatic Primary Amines :

Some of the pyrimidine derivatives (eg. thymine) couple with diazotised sulfanilic acid to give a coloured dye (2). Since no reports are available for estimation of 5FU with diazotised sulfanilic acid, attempts were made to investigate the possibility of coupling between 5FU and diazotised aromatic primary amines.

2.231 Reagents :

a) Sodium hydroxide solution (0.01N) : 0.04g of sodium hydroxide was weighed into a 100ml volumetric flask, dissolved and made upto volume with water.

b) Hydrochloric acid solution (10% v/v) : 10ml of concentrated hydrochloric acid was diluted to 100ml with water.

c) Sodium nitrite solution (0.5% w/v) : 500mg of sodium nitrite was weighed into a 100ml volumetric flask, dissolved and made to volume with water.

d) Solution of primary amines (0.9% w/v) : 900mg of aromatic primary amines viz. sulfanilic acid, sulfanilamide,

aniline, p-aminophenol, para aminobenzoic acid (PABA), anthranilic acid, o-phenylenediamine, o-nitroaniline, p-nitroaniline, p-toluidine, p-anisidine were weighed accurately and transferred into separate 100ml volumetric flasks. The amines were dissolved and made upto volume with 10% hydrochloric acid solution.

e) Diazo reagent : A common procedure was used for the preparation of diazo reagent with all the amines.

6ml of the primary amine solution was mixed with 6ml of sodium nitrite solution. After 10 minutes, 24ml of sodium nitrite solution was added and volume made up with water. The reagent was stored at 0-4^{o}C.

f) Stock solution of the drug : 100mg of 5FU was accurately weighed into a 100ml volumetric flask. The drug was dissolved and made upto volume with 0.01N sodium hydroxide solution.

g) Sodium carbonate solution (1.5% w/v) : 1.5g of sodium carbonate was dissolved and made to 100ml with water.

h) Glycerol - sodium hydroxide mixture (1:1) : 50ml of glycerol and 50ml of 20% w/v solution of sodium hydroxide were freshly mixed before use.

2.232 Experimental procedure :

A common procedure was followed for all primary amines.

Two sets of experiments were performed. 1ml of stock solution of 5FU was accurately transferred to separate test tubes in each set. To the first set, 4ml of sodium carbonate solution and 2ml of diazo reagent were added. The colour formed if any was compared with the reagent blank.

To the second set, 4ml of sodium carbonate solution and 2ml of diazo reagent were added followed by 2ml of glycerol-sodium hydroxide solution. The colour formed if any was compared with the appropriate reagent blank.

In the first set of test tubes a faint red colour was observed which was not stable.

In the second set, a distinct red colour was observed in tubes containing drug and the following diazotised primary amines : diazotised sulfanilic acid, sulfanilamide, PABA, anthranilic acid. A distinct orangish yellow colour was observed in tubes containing drug and diazotised o- and p-nitroanilines. No distinct colour was observed with diazotised aniline, p-aminophenol, o-phenylenediamine, p-toluidine and p-anisidine.

Since the colour formed in the second set was stable and reproducible, the reaction was extended on a quantitative basis for estimation of 5FU from bulk and dosage forms.

2.233 Development of colorimetric method for 5FU with selected diazotised primary amines :

a) Scanning of the spectrum :

To a suitable aliquot (0.5ml) of drug solution, in a 10ml volumetric flask, 4ml of sodium carbonate solution and 2ml of diazo reagent were added. After 10 minutes 2ml of glycerol-sodium hydroxide mixture was added and volume was made upto 10ml with water. The yellowish orange to red colour was scanned between 400-800nm against the appropriate reagent blank. The wavelength scans for various diazotised primary amines are shown in Figs.2.5 and 2.6.

b) Optimisation of volume of sodium carbonate solution :

To 0.5ml of drug solution in separate 10ml volumetric flasks, 4ml of varying concentrations (0.5,1,....5%w/v) of sodium carbonate solution were added followed by 2ml of the diazo reagent. After 10 minutes, 2ml of 1:1 glycerol-sodium hydroxide solution was added and volume made upto 10ml with water. The absorbance of orangish yellow colour formed with diazotised o-and p-nitro aniline were read at 450nm and the absorbance of red colour formed with other diazotised primary amines was read at 520nm against the appropriate reagent blank. The observations are recorded in Table 2.9.

c) Optimisation of volume of diazo reagent :

To 0.5ml of drug solution in 10ml volumetric flasks, 4ml of sodium carbonate solution was added followed by varying volumes of diazo reagent (0.5,1,....5ml). After 10 minutes 2ml of 1:1 glycerol-sodium hydroxide solution was added and volume made upto 10ml with water. The absorbance of orangish yellow colour formed with diazotized o-and p-nitroanilines were read at 450nm and the absorbance of the red colour formed with other diazotised primary amines was read at 520nm against appropriate reagent blank. The observations are recorded in Table 2.10.

d) Optimisation of volume of glycerol-sodium hydroxide mixture :

To 0.5ml of drug solution in 10ml volumetric flasks, 4ml of sodium carbonate solution and 2ml of diazo reagent

were added. After 10 minutes, 2ml of varying ratios of glycerol : sodium hydroxide mixture (0.5:1,1:0:5,....1:2) were added, volume was made upto 10ml with water. The absorbance of orangish yellow colour formed with diazotised o-and p-nitroanilines was read at 450nm and the absorbance of red colour formed with other diazotised amines was read at 520nm against appropriate reagent blank. The observations are recorded in Table 2.11.

e) Preparation of calibration curves :

Aliquots of the drug solution equivalent to 100µg, 200µg... 1000µg/ml were accurately transferred into clean, dry, separate 10ml volumetric flasks, 4ml of 1.5%w/v sodium carbonate solution and 2ml of diazo reagent were added. After 10 minutes, 2ml of 1:1 glycerol-sodium hydroxide mixture was added and volume was made upto 10ml with water. The absorbance of orangish-yellow colour formed with diazotised o-and p-nitroanilines in each flask was read at 450nm and the absorbance of red colour formed in each flask with diazotised sulfanilic acid, diazotised sulfanilamide, diazotised PABA and diazotised reagent blank. The observations are recorded in Table 2.12 and Figs. 2.7-2.12.

f) Estimation of 5FU from injection :

An aliquot of the injection equivalent to 100mg of 5FU was accurately transferred into a 100ml volumetric flask and made to volume with water. A suitable aliquot (0.5ml) of the drug solution was taken and the colour was

developed by the procedure described under the preparation of calibration curve.

g) Estimation of 5FU from cream :

"A portion of the cream equivalent to 500mg of 5FU was weighed and the drug was extracted by the U.S.P. method as described in section 2.223f starting with the words" 400ml of pH 4.7 acetate buffer.... organic extracts were discarded". The pH of the aqueous solution was adjusted to 9 with 0.01N sodium hydroxide solution. A suitable aliquot (0.5ml) of the drug solution was taken and colour was developed by the procedure given in the preparation of calibration curve. The results of the estimation of 5FU from injection and cream are recorded in Table 2.13 and the percentage recoveries were compared with I.P. and U.S.P. methods respectively.

h) Results and Discussion :

When 5FU was reacted with diazotised o-and p-nitroanilines in presence of glycerol-sodium hydroxide mixture, orangish yellow colour was developed with λ max at 450nm (Fig.2.5) while a red colour was developed with diazotised sulfanilic acid, sulfaniliamide, PABA and anthranilic acid with λ max at 520nm (Fig. 2.6) in each case.

For each coupling reaction, it was observed that 4ml of 1.5%w/v sodium carbonate solution (Table 2.9) and 2ml of diazo reagent (Table 2.10) were optimum for colour development. The colour was formed at room temperature

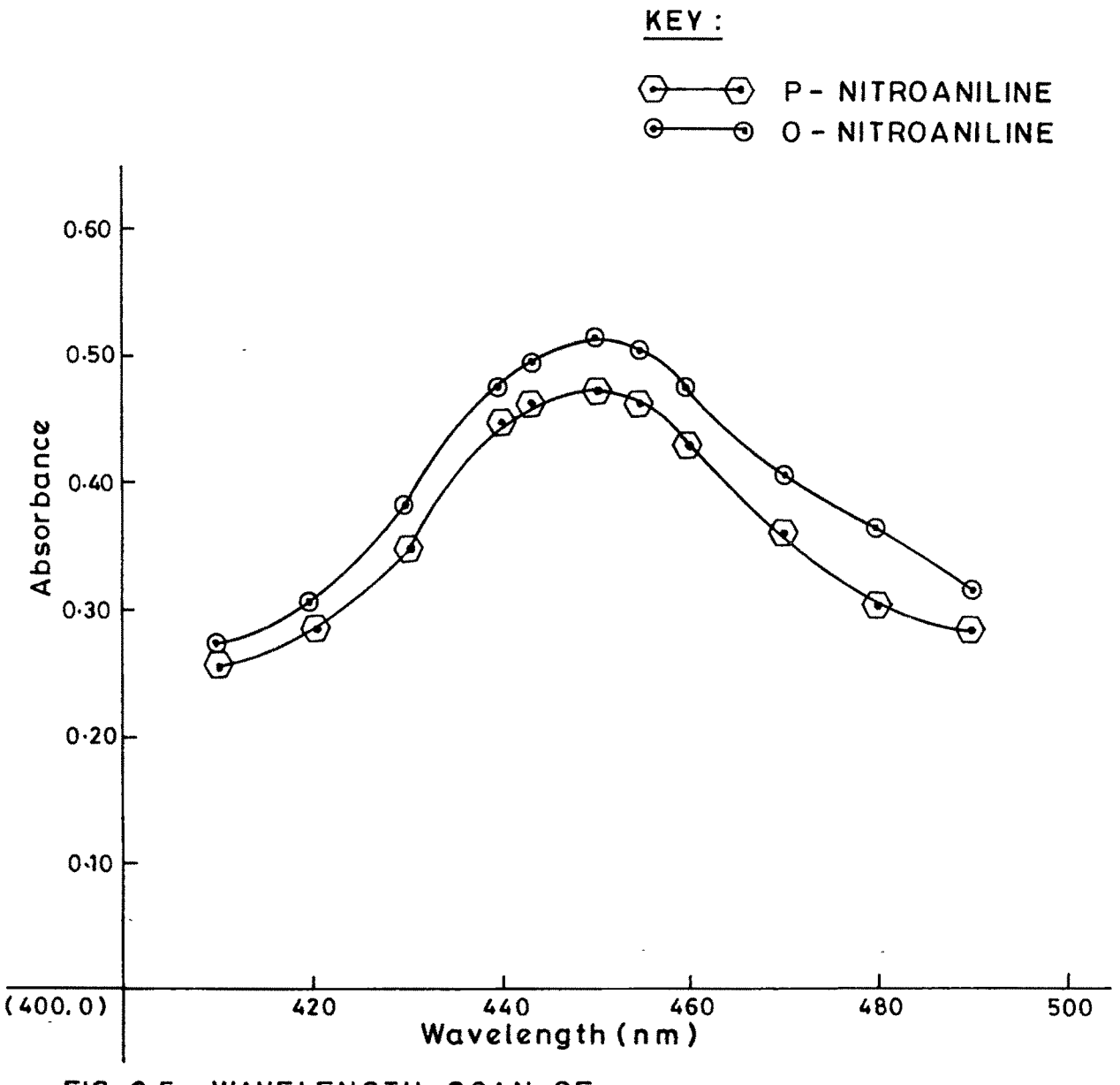


FIG. 2.5: WAVELENGTH SCAN OF 5 FU-DIAZOTISED NITROANILINE

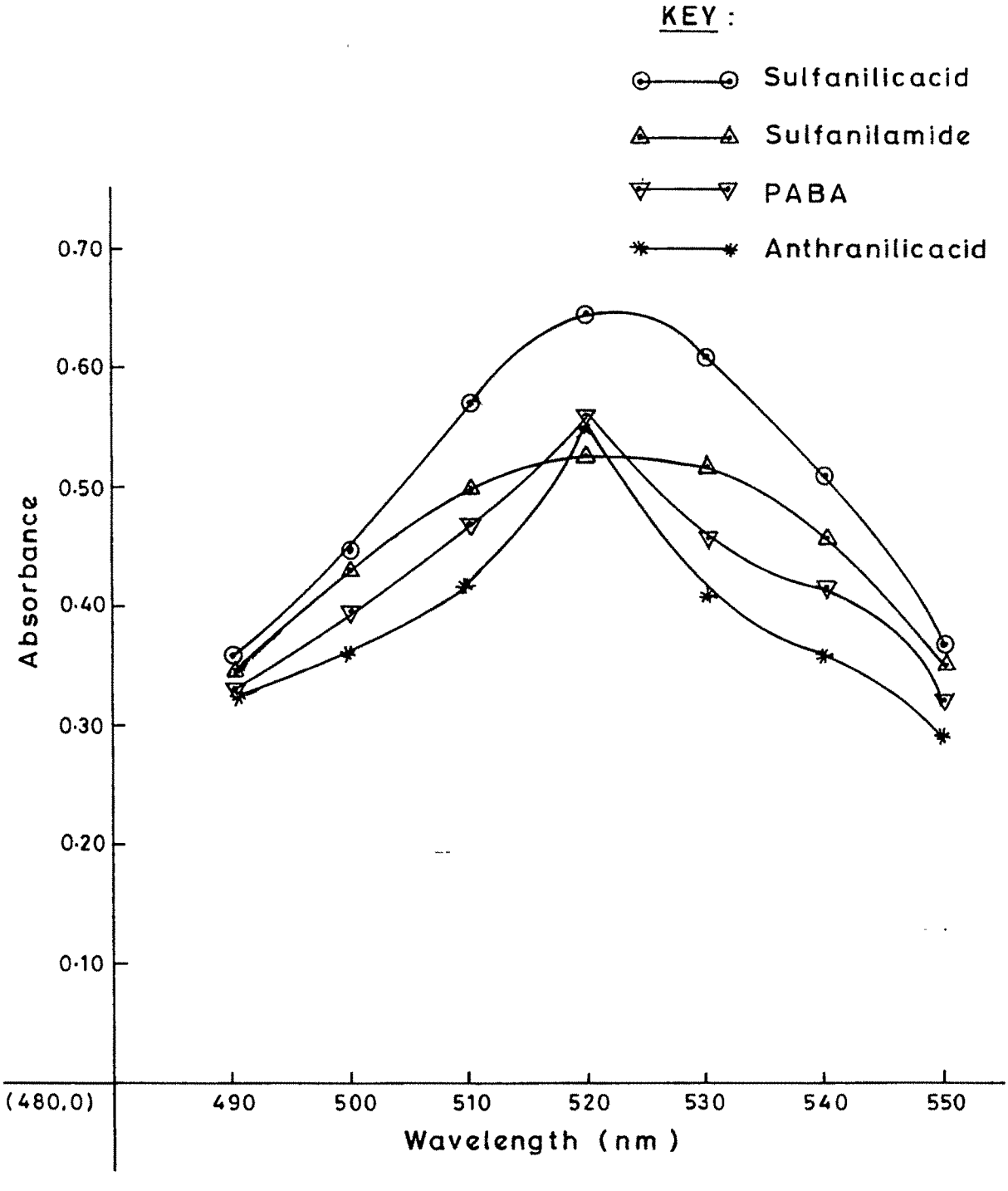


FIG. 2.6 : WAVELENGTH SCAN OF 5 FU - DIAZOTISEDAMINES

TABLE 2.9

OPTIMISATION OF CONCENTRATION OF SODIUM CARBONATE SOLUTION

Drug concentration = 50 µg/ml

Concentration Na_2Co_3 % w/v	5FU+diazotised o-nitro aniline λmax=450nm	5FU+diazotised p-nitro aniline λmax=450nm	5FU+diazotised sulfanilic acid λmax=520nm	5FU+diazotised sulfanilamide λmax=520nm	5FU+diazotised PABA λmax=520nm	5FU+diazotised anthranilic acid λmax=520nm
0.5	0.294	0.245	0.392	0.234	0.294	0.252
1.0	0.423	0.375	0.537	0.395	0.435	0.399
1.5	0.515	0.417	0.645	0.532	0.560	0.551
2.0	0.517	0.470	0.645	0.523	0.562	0.549
2.5	0.514	0.472	0.643	0.521	0.532	0.551
5.0	0.519	0.470	0.642	0.522	0.557	0.551

TABLE 2.10

OPTIMISATION OF CONCENTRATION OF VOLUME OF DIAZO REAGENT

Drug concentration = 50 µg/ml

Volume of diazo -tised reagent	5FU+diazo-tised o-nit -ro aniline λmax=450nm	5FU+diazo-tised p-nit -ro aniline λmax=450nm	5FU+diazo-tised sulfa nilic acid λmax=520nm	5FU+diazo-tised sulfa nilamide λmax=520nm	5FU+diazo-tised PABA λmax=520nm	5FU+diazo-tised anthra nilic acid λmax=520nm
0.5	0.139	0.124	0.177	0.213	0.192	0.173
1.0	0.235	0.201	0.359	0.417	0.417	0.399
2.0	0.515	0.417	0.645	0.532	0.560	0.551
3.0	0.515	0.472	0.645	0.523	0.560	0.547
5.0	0.512	0.459	0.643	0.520	0.557	0.551

TABLE 2.11

OPTIMISATION OF RATIOS OF GLYCEROL - SODIUM HYDROXIDE MIXTURE

Drug concentration = 50 µg/ml

Ratio of glycerol sodium hydro -xide solution	5FU+diazo-tised o-nit -ro aniline λmax=450nm	5FU+diazo-tised p-nit -ro aniline λmax=450nm	5FU+diazo-tised sulfa nilic acid λmax=520nm	5FU+diazo-tised sulfa nilamide λmax=520nm	5FU+diazo-tised PABA λmax=520nm	5FU+diazo-tised anthr. anilic acid λmax=520nm
0.5:1	0.411	0.379	0.432	0.405	0.439	0.392
1:0.5	0.495	0.392	0.427	0.401	0.424	0.399
1:1	0.515	0.471	0.645	0.532	0.560	0.552
2:1	0.515	0.469	0.642	0.533	0.560	0.550
1:2	0.511	0.470	0.644	0.524	0.554	0.529

TABLE 2.12

CALIBRATION CURVES OF 5FU - DIAZOTISED PRIMARY AMINES

Concentration µg/ml	Mean absorbance (± S.D.) 5FU+diazotised o-nitro aniline λmax=450nm	5FU+diazotised p-nitro aniline λmax=450nm	5FU+diazotised sulfanilic acid λmax=520nm	5FU+diazotised sulfanilamide λmax=520nm	5FU+diazotised PABA λmax=520nm	5FU+diazotised anthranilic acid λmax=520nm
10	0.131 (0.007)	0.120 (0.023)	0.258 (0.022)	0.152 (0.059)	0.251 (0.007)	0.232 (0.031)
20	0.211 (0.123)	0.234 (0.079)	0.341 (0.073)	0.252 (0.015)	0.342 (0.012)	0.321 (0.024)
40	0.423 (0.009)	0.382 (0.113)	0.514 (0.004)	0.422 (0.003)	0.470 (0.049)	0.501 (0.105)
50	0.515 (0.014)	0.471 (0.003)	0.645 (0.054)	0.532 (0.122)	0.560 (0.002)	0.552 (0.051)
60	0.601 (0.057)	0.581 (0.101)	0.720 (0.104)	0.592 (0.129)	0.632 (0.027)	0.664 (0.037)
80	0.792 (0.069)	0.740 (0.082)	0.932 (0.017)	0.771 (0.073)	0.814 (0.014)	0.832 (0.004)
100	0.815 (0.012)	0.751 (0.007)	1.093 (0.015)	0.975 (0.011)	0.962 (0.005)	0.997 (0.032)

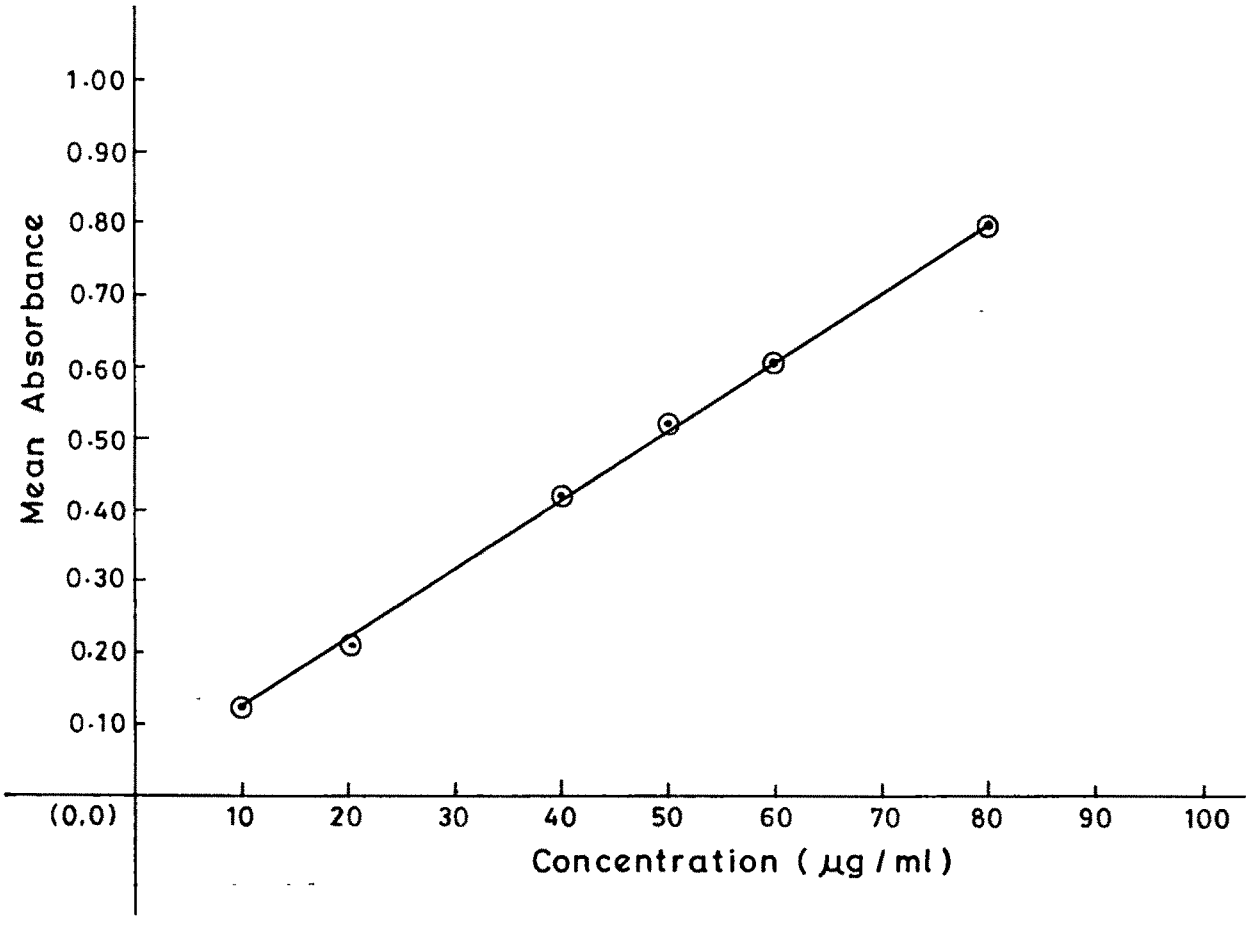


FIG. 2.7: CALIBRATION CURVE OF 5FU-DIAZOTISED O-NITROANILINE

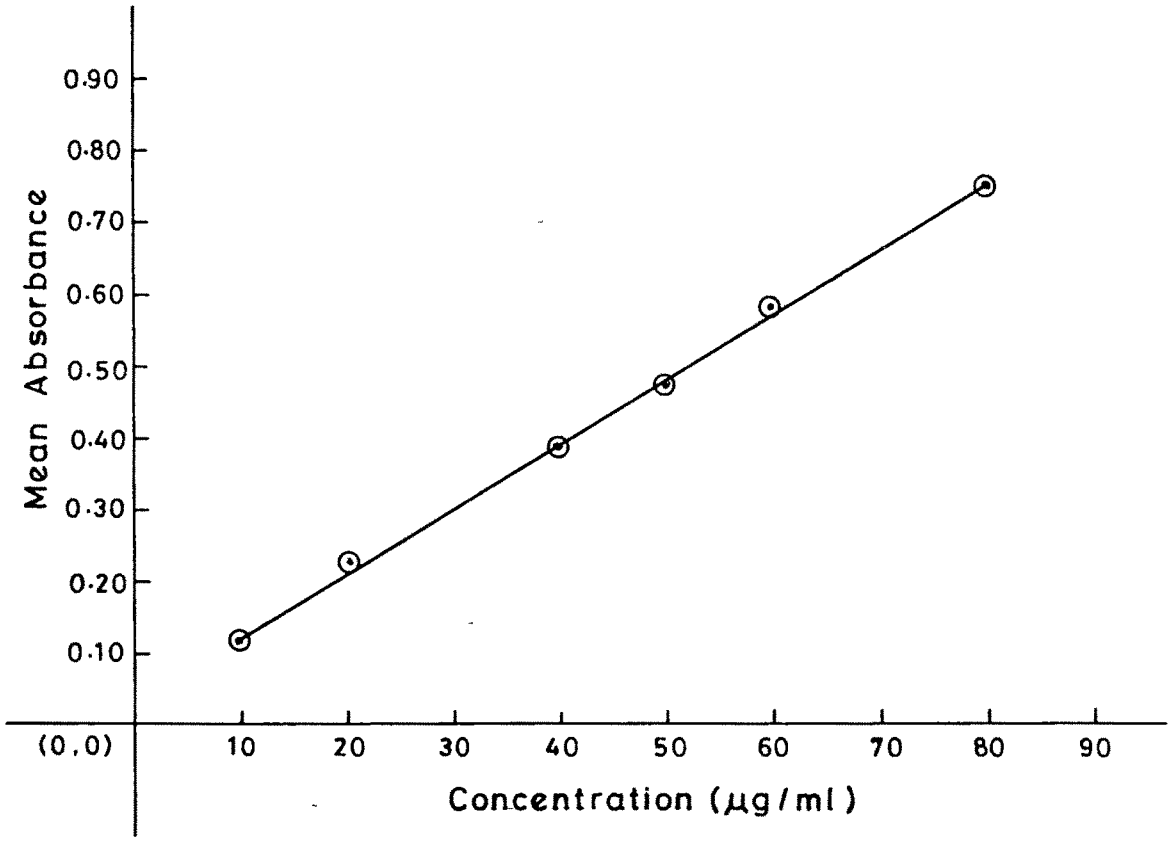


FIG. 2.8 : CALIBRATION CURVE OF 5 FU - DIAZOTISED P-NITROANILINE

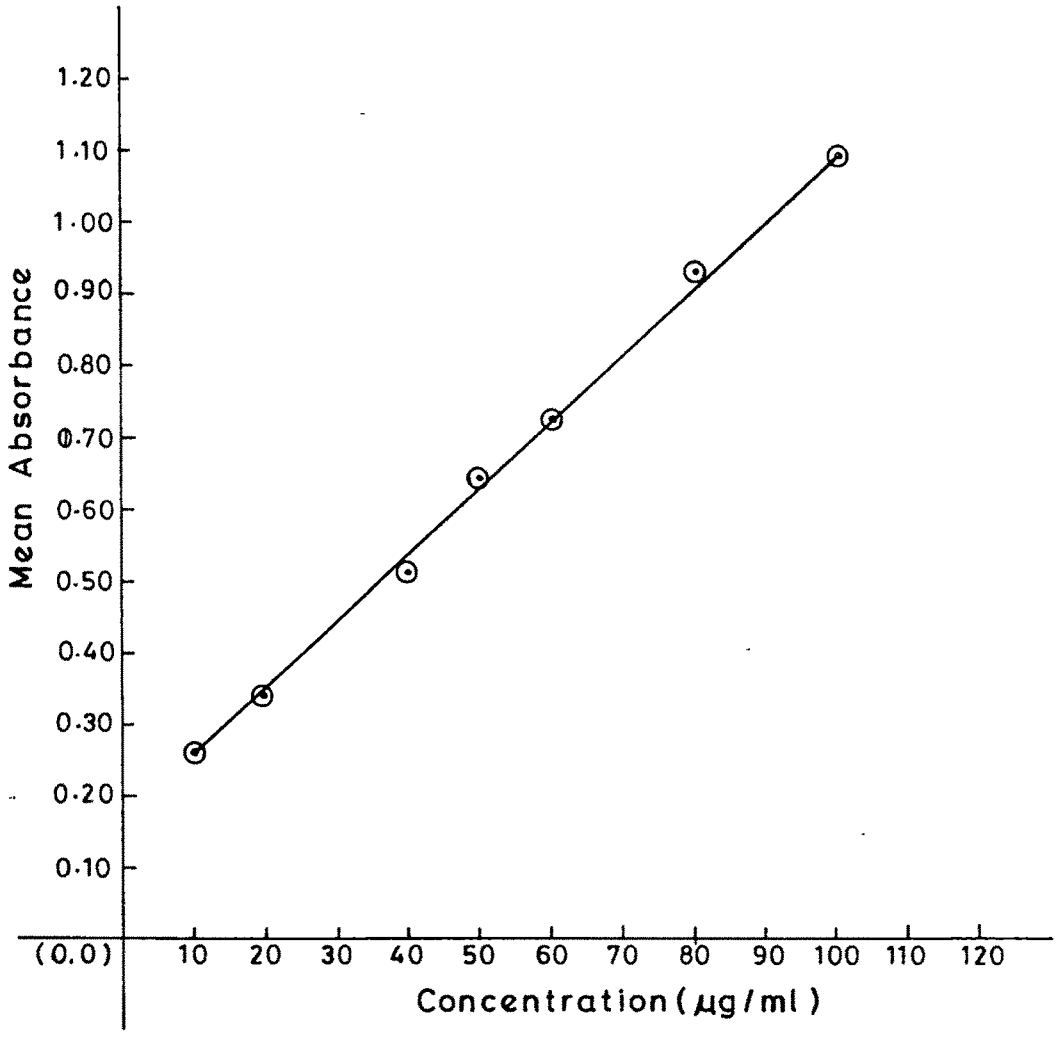


FIG. 2.9 : CALIBRATION CURVE OF 5 FU- DIAZOTISED SULFANILIC ACID

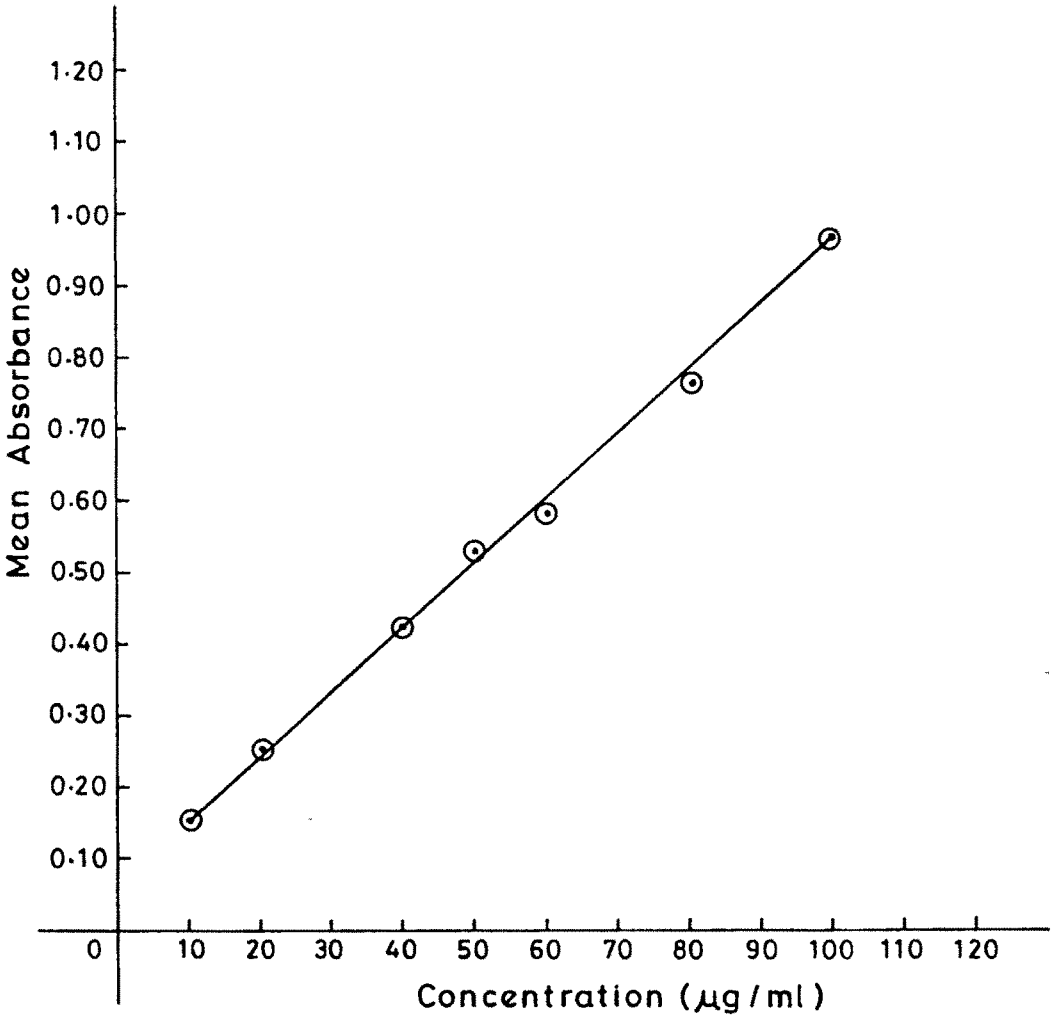


FIG. 2.10 : CALIBRATION CURVE OF 5FU- DIAZOTISED SULFANILAMIDE

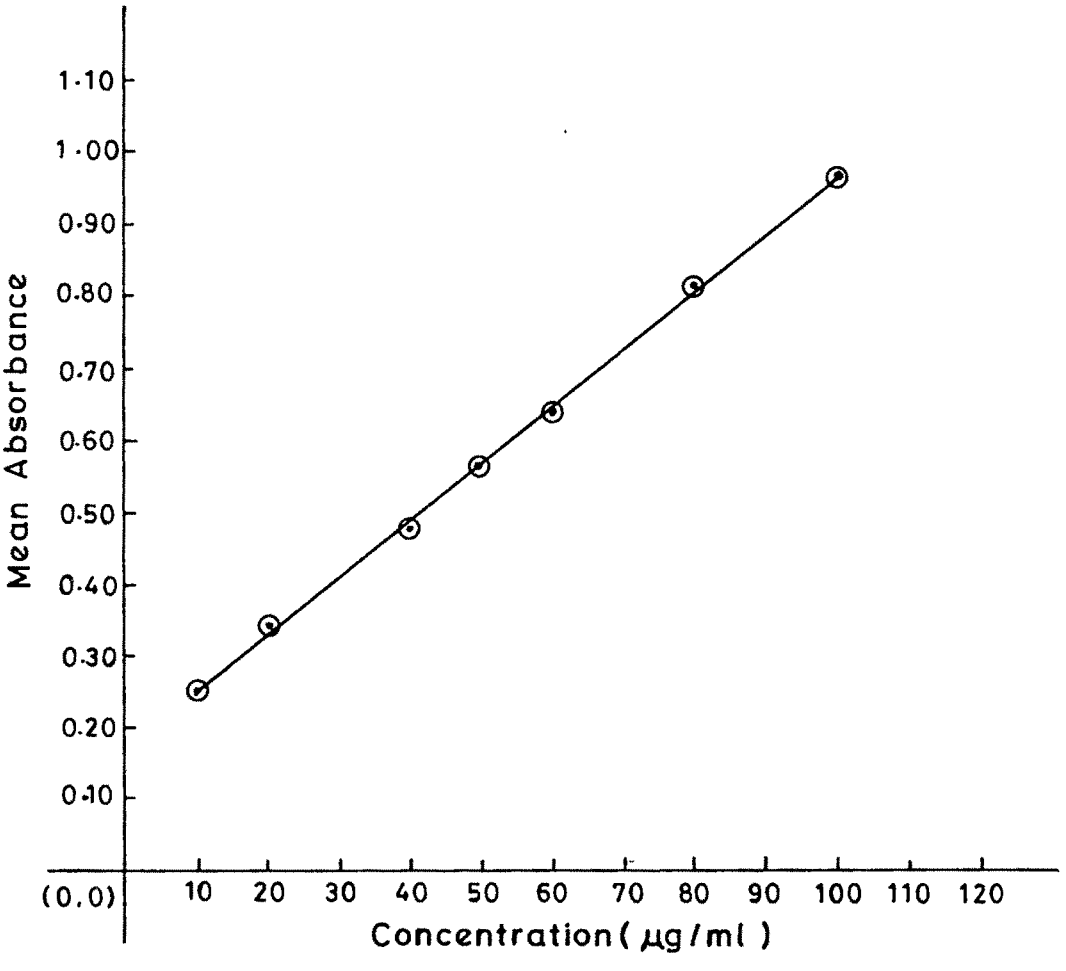


FIG. 2.11 : CALIBRATION CURVE OF 5FU-DIAZOTISED PABA

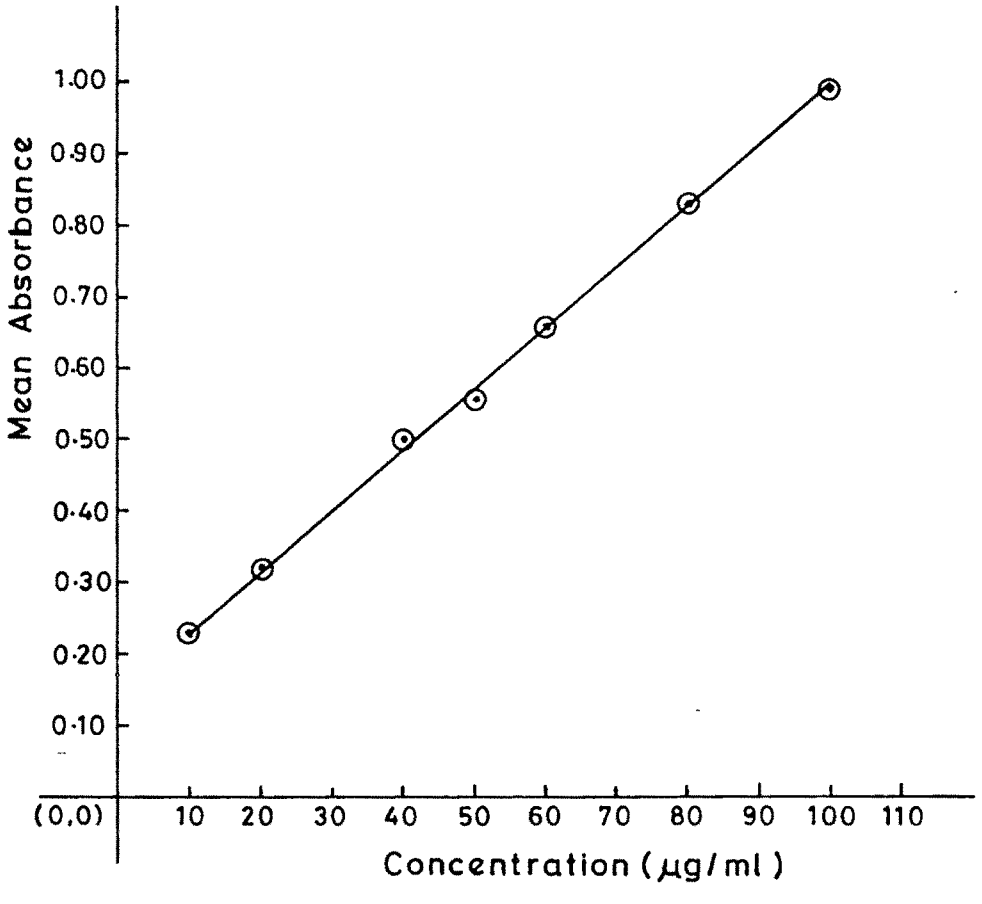


FIG. 2.12 : CALIBRATION CURVE OF 5 FU - DIAZOTISED ANTHRANILIC ACID

TABLE 2.13

ESTIMATION OF 5FU IN INJECTION AND CREAM

Dosage form	Conc.in aliquot µg/ml	% recovered (± S.D.) 5FU - diazotised amines						
		UV spectrophotometric method λmax=266nm	5FU+diazotised o-nitro aniline λmax=450nm	5FU+diazotised p-nitro aniline λmax=450nm	5FU+diazotised sulfanilic acid λmax=520nm	5FU+diazotised sulfanilamide λmax=520nm	5FU+diazotised PABA λmax=520nm	5FU+diazotised anthranilic acid λmax=520nm
Injection (50mg/ml)	20	99.04 (0.516)	99.21 (0.279)	99.02 (0.203)	99.45 (0.618)	99.91 (0.117)	99.79 (0.195)	99.35 (0.139)
Cream (5%w/w)	20	99.04 (0.591)	98.95 (0.379)	99.12 (0.313)	99.33 (0.355)	98.99 (0.412)	99.54 (0.324)	99.09 (0.625)

TABLE 2.14

STATISTICAL PARAMETERS FOR COUPLING OF 5FU WITH DIAZOTISED PRIMARY AMINES

Statistical parameters	5FU+diazotised o-nitro aniline	5FU+diazotised p-nitro aniline	5FU+diazotised sulfanilic acid	5FU+diazotised sulfanilamide	5FU+diazotised PABA	5FU+diazotised anthranilic acid
a	0.033	0.068	0.156	0.185	0.169	0.149
b	0.0095	0.0087	0.0095	0.0070	0.0080	0.0090
molar absorptivity $1mol^{-1}cm^{-1}$	$1.34x10^3$	$1.23x10^3$	$1.68x10^3$	$1.38x10^3$	$1.46x10^3$	$1.44x10^3$
r	0.997	0.997	0.999	0.998	0.992	0.995

and was stable for upto two hours. Glycerol-sodium hydroxide mixture was added to stabilise the colour formed due to the coupling of the drug with the reagent. 2ml of 1:1 ratio of this mixture was found to be optimum for colour development.

Calibration curves (Table 2.12, Figs. 2.7-2.12) were obtained within the range of 10-80µg/ml in case of diazotised o-and p-nitoranilines and within the range of 10-100µg/ml in case of other diazotised amines. The sensitivity was found to be 5µg/ml for all the methods. The a and b values for the line of regression in each case were calculated. The a, b, molar absorptivity values and the correlation co-efficient are recorded in Table 2.14.

The percentage recoveries of 5FU from injection and cream (Table 2.13) were obtained between 98.95% - 99.91% and were comparable with I.P. and U.S.P. methods respectively.

From analysis of the data obtained for the estimation of 5FU in formulations it may be inferred that the colorimetric methods based on the coupling of 5FU with various diazotised primary amines are reproducible and comparable with the pharmacoepial method.

2.24 Estimation of 5FU as Mercuric Complex :

The mercuric complex of compounds having pyrimidinedione nucleus are reported to form coloured compounds with dithizone and diphenylcarbazone solutions(3).

Hence an attempt was made to develop colorimetric methods for 5FU along the same lines.

2.241 Reagents :

a) Stock solution of drug : 100mg of 5FU was accurately weighed into a 100ml volumetric flask dissolved and made upto volume with water.

b) Buffer solutions I.P. : Hydrochloric acid buffer pH 2.0, acid phthalate buffer pH 3.0 and 4.0, acetate buffer pH 5.0, phosphate buffer pH 6.0, 7.0 and 8.0 and borate buffer pH 9.0 and 10.0 were prepared by the procedures given in I.P.

c) Mercuric sulphate solution (0.3%w/v) : 300mg of mercuric sulphate was accurately weighed into a 100ml volumetric flask dissolved and made to volume with water.

d) Dithizone solution (0.02%w/v) : 20mg of dithizone was accurately weighed into a 100ml volumetric flask, dissolved and the volume was made up with chloroform.

e) Diphenylcarbazone solution (0.02%w/v) : 20mg of diphenyl carbazone was accurately weighed into a 100ml volumetric flask dissolved in chloroform, the volume was made up with the same solvent.

2.242 Experimental procedure :

Two sets of experiments were performed.

1ml of stock solution of 5FU was accurately transferred into several separating funnels in each set. To each of the separating funnels in both the sets, 2ml of buffer solutions of pH 2-10 were added followed by 1ml of mercuric sulphate solution. To the first set, 1ml of dithizone solution and to

the second set, 1ml of diphenylcarbazone solution were added. To both the sets 2ml of chloroform was added and the contents were shaken thoroughly. The colour formed if any, in the chloroform layer was compared with the appropriate reagent blank.

Complexation of 5FU with mercury occured at pH8 with formation of a greenish red colour with dithizone in first set and purple colour with diphenylcarbazone in the second set. No colour was observed in other buffer solutions.

2.243 Development of colorimetric method for 5FU as mercury complex :

A common procedure was followed for estimation of 5FU-mercury complex with dithizone and diphenyl carbazone solutions.

a) Scanning of the spectrum :

Suitable aliquot (0.5ml) of the drug solution was transferred into 60ml separating funnel. 2ml of pH8 buffer and 1ml of mercuric sulphate solution were added followed by 1ml of dithizone solution/diphenyl carbazone solution. The mixture was extracted thrice with 2ml of chloroform. The chloroform extracts were collected, transferred to 10ml flask through a funnel containing anhydrous sodium sulphate and made to volume with chloroform. The absorption spectrum of the resulting coloured solution was scanned between 400-800nm against the reagent blank. The wavelength scans are given in Figs. 2.13 and 2.14.

b) Optimisation of concentration of mercuric sulphate solution :

The above procedure was repeated with 1ml of varying concentrations of mercuric sulphate solution (0.1,0.2....1.0% w/v) and the absorbance of the greenish red colour in case of dithizone solution and the red colour in case of diphenyl carbazone solution was measured at 605nm and 560nm respectively. The results are recorded in Table 2.15.

c) Optimisation of concentration of dithizone solution and diphenyl carbazone solution :

The procedure given under scanning of spectrum was carried out. Here concentrations of dithizone and diphenyl carbazone solutions were varied keeping the other parameters fixed. The absorbance of the greenish red colour in case of dithizone solution and the red colour in case of diphenyl carbazone solution was measured at 605nm and 560nm respectively agaist appropriate reagent blank. The results are recorded in Table 2.16.

d) Preparation of calibration curve :

Aliquots of drug solution equivalent to 50µg, 100µg, 200µg.... 1000µg/ml were accurately transferred into different 60ml separating funnels. 2ml of pH8 buffer and 1ml of 0.3% w/v mercuric sulphate solution were added into each separating funnel followed by 1ml of 0.20% w/v dithizone or diphenyl carbazone solution. The mixture was extracted with 2ml of chloroform thrice. The chloroform extracts were collected, transferred to 10ml

volumetric flask through a funnel containing anhydrous sodium sulphate and made upto volume with chloroform. The absorbances of greenish red colour formed between drug mercury complex-dithizone and the red colour formed between drug mercury complex-diphenyl carbazone were measured at 605nm and 560nm respectively. The observations are recorded in Table 2.17 and the calibration curves are shown in Figs 2.14 and 2.15.

e) Estimation of 5FU from injection :

An aliquot of the injection equivalent to 100mg of 5FU was accurately transferred into a 100ml volumetric flask and made to volume with water. Colour was developed with a suitable aliquot (0.2ml) of the diluted solution by the procedure given under preparation of calibration curve.

f) Estimation of 5FU from cream :

A portion of the cream equivalent to 500mg of 5FU was accurately weighed and the drug was extracted by the U.S.P. method as described in section 2.223f starting with words "400ml of pH 4.7 acetate buffer..... organic extracts were discarded". The pH of the aqueous solution containing the drug was adjusted to 8 with a few drops of 0.01N sodium hydroxide solution. Colour was developed with a suitable aliquot (0.2ml) of the solution as described under preparation of calibration curve.

The results of the estimation of 5FU from injection and cream are recorded in Table 2.18 and the percentage recoveries were compared with I.P. and U.S.P. method respectively.

g) Results and discussions :

The complexation of 5FU with mercury occured in aqueous medium at pH 8 and the complex formed reacted with dithizone and diphenyl carbazone solution in chloroform medium to give a greenish red coloured complex and red coloured complex respectively.

The absorption spectrum of the greenish red colour formed by the reaction between 5FU-mercury complex and dithizone solution showed a λ max at 605nm (Fig 2.13) while the red colour formed in case of 5FU-mercury complex and diphenylcarbazone solution showed a λ max at 560nm (Fig 2.14). The reactions were carried out at room temperature and the colour formed was stable upto 4 hours.

The observations in Table 2.15 and 2.16 reveal that 1ml of 0.3 w/v mercuric sulphate solution and 1ml of 0.02%w/v dithizone/diphenyl carbazone solution were optimum for colour development.

The calibration curves (Table 2.17, Fig. 2.15 and 2.16) were rectilinear in the range of 5-100µg/ml for both the methods. The sensitivity was found to be 5µg/ml. The a and b values for the line of regression for dithizone method and diphenyl carbazone method were found to be a=0.0116, 0.0020 and b=0.009, 0.013 respectively. The correlation co-efficient r and molar absorptivity were found to be 0.997 and 1.17×10^3 $lmol^{-1}cm^{-1}$ for dithizone method, and 0.995 and 1.67×10^3 $lmol^{-1}cm^{-1}$ for diphenyl carbazone method respectively. The percentage

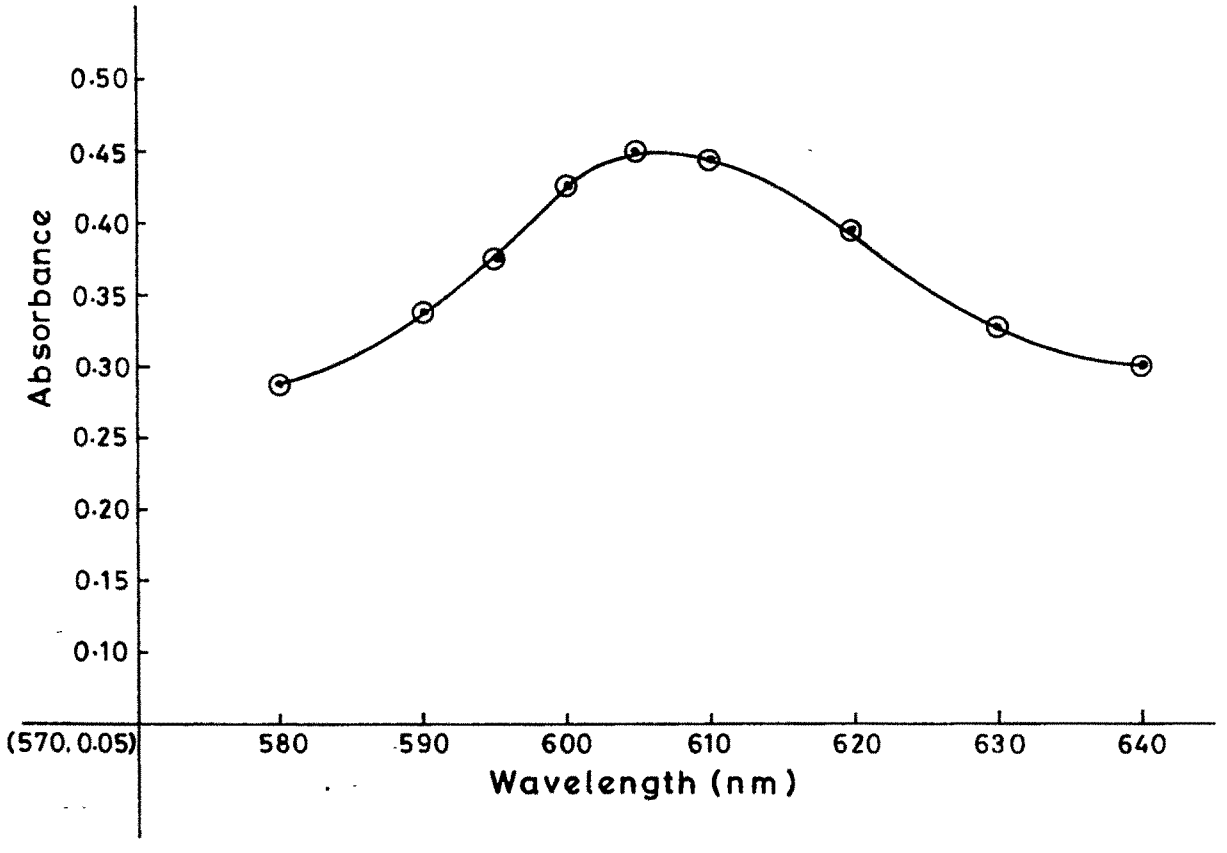


FIG. 2.13 : WAVELENGTH SCAN OF 5 FU-MERCURIC COMPLEX WITH DITHIZONE

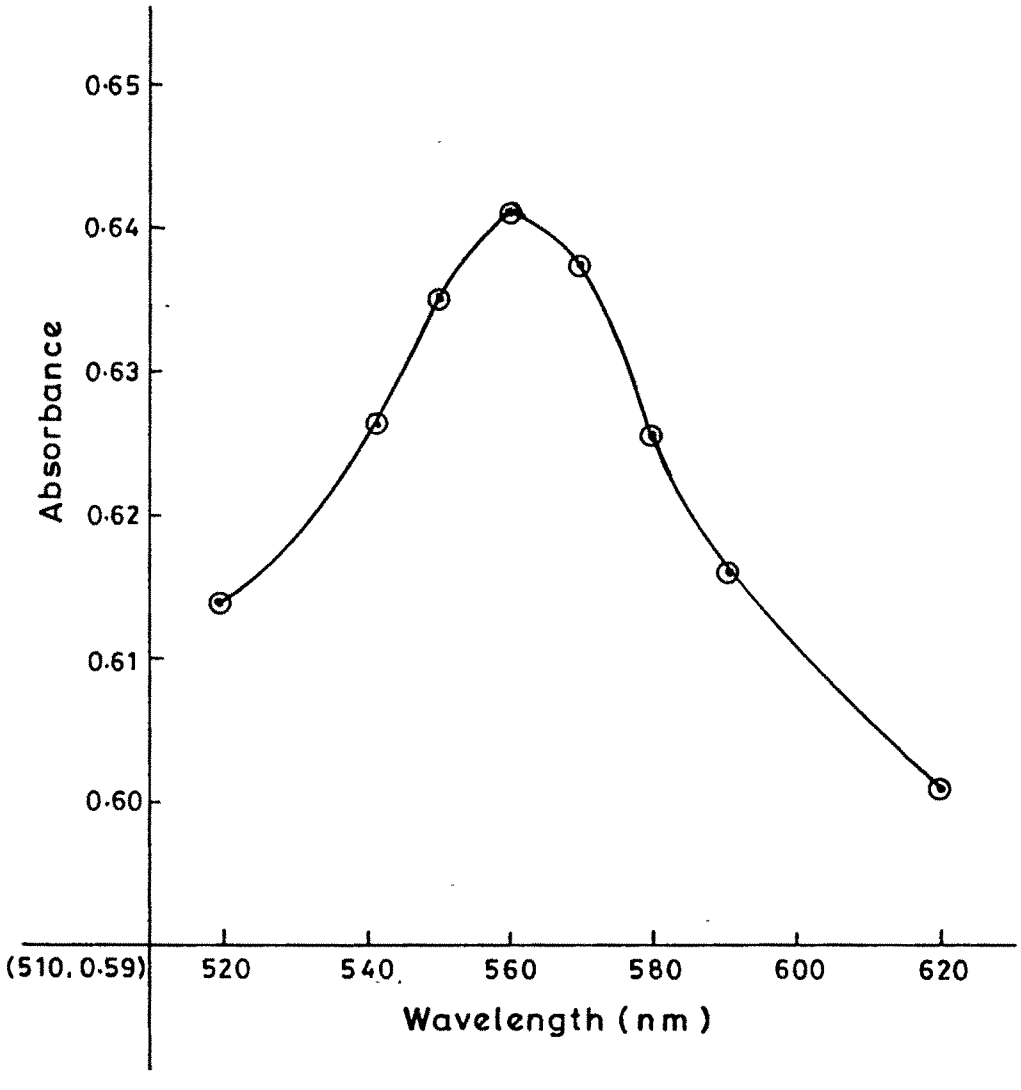


FIG. 2.14: WAVELENGTH SCAN OF 5FU-MERCURIC COMPLEX WITH DIPHENYLCARBAZONE

TABLE 2.15

OPTIMISATION OF CONCENRATION OF MERCURIC SULPHATE SOLUTION

Drug concentration - 50µg/ml

Concentration of mercuric sulphate % w/v	Absorbance	
	5FU - Hg complex + dithizone solution λmax - 605nm	5FU - Hg complex + diphenyl carbazone solution λmax-560nm
0.1	0.275	0.349
0.2	0.352	0.501
0.3	0.450	0.643
0.4	0.451	0.641
1.0	0.450	0.641

TABLE 2.16

OPTIMISATION OF CONCENRATION OF DITHIZONE/DIPHENYL CARBAZONE SOLUTION

Drug concentration - 50µg/ml

Concentration of dithizone/diphenyl carbazone % w/v	Absorbance	
	Dithizone method λmax - 605nm	Diphenyl carbazone method λmax-560nm
0.01	0.391	0.499
0.02	0.450	0.643
0.03	0.451	0.641
0.10	Interference of blank	0.641

TABLE 2.17

CALIBRATION CURVE OF 5FU - MERCURIC COMPLEX WITH DITHIZONE AND DIPHENYL CARBAZONE SOLUTION

Concentration µg/ml	Absorbance (± S.D.)	
	5FU - Hg complex with dithizone λmax - 605nm	5FU - Hg complex with diphenyl carbazone λmax-560nm
5	0.051 (0.011)	0.065 (0.007)
10	0.110 (0.005)	0.129 (0.012)
20	0.175 (0.009)	0.254 (0.011)
40	0.371 (0.011)	0.485 (0.009)
50	0.450 (0.007)	0.643 (0.007)
80	0.711 (0.009)	1.011 (0.011)
100	0.897 (0.011)	1.270 (0.009)

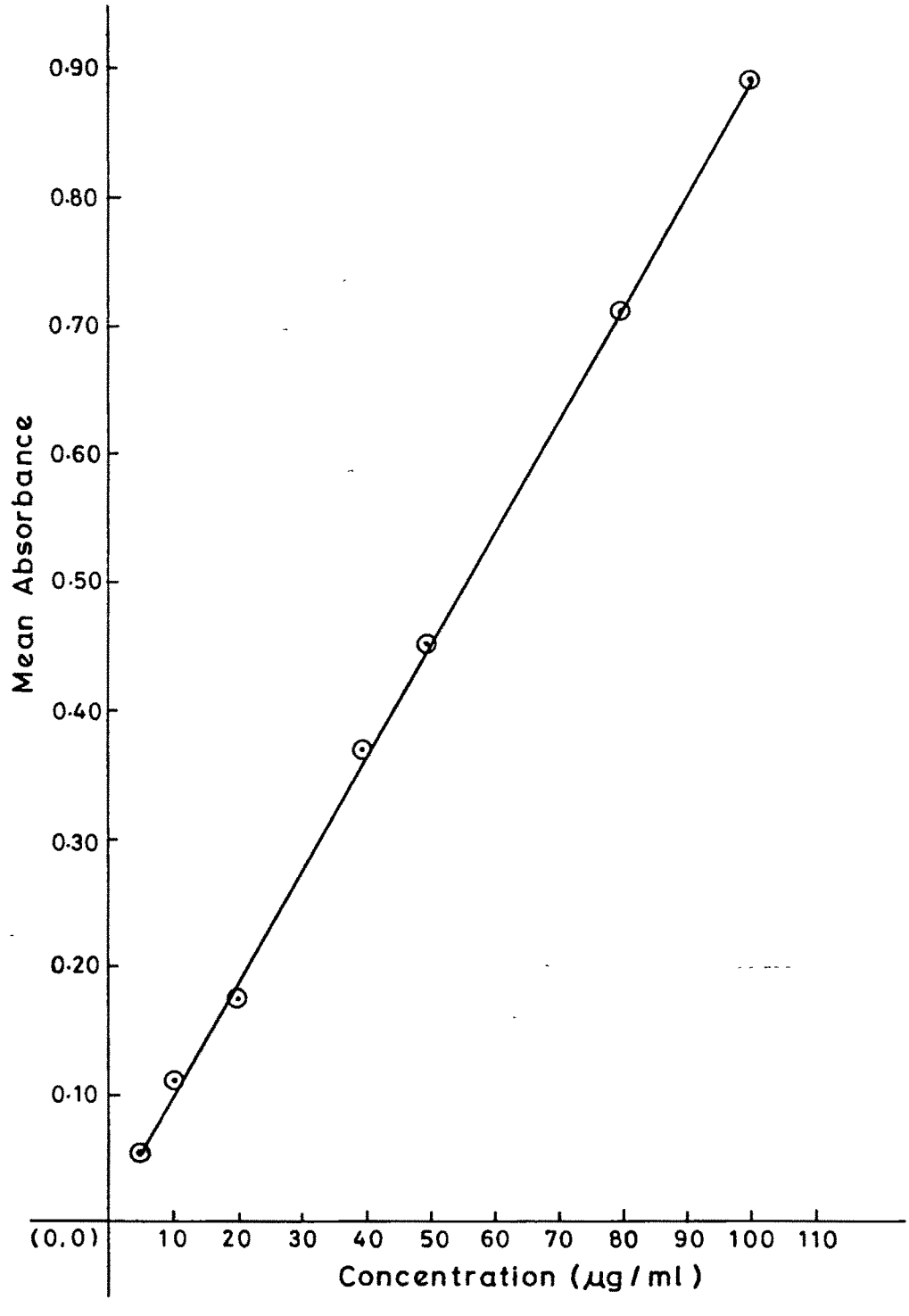


FIG. 2.15 : CALIBRATION CURVE OF 5FU-MERCURIC COMPLEX WITH DITHIZONE

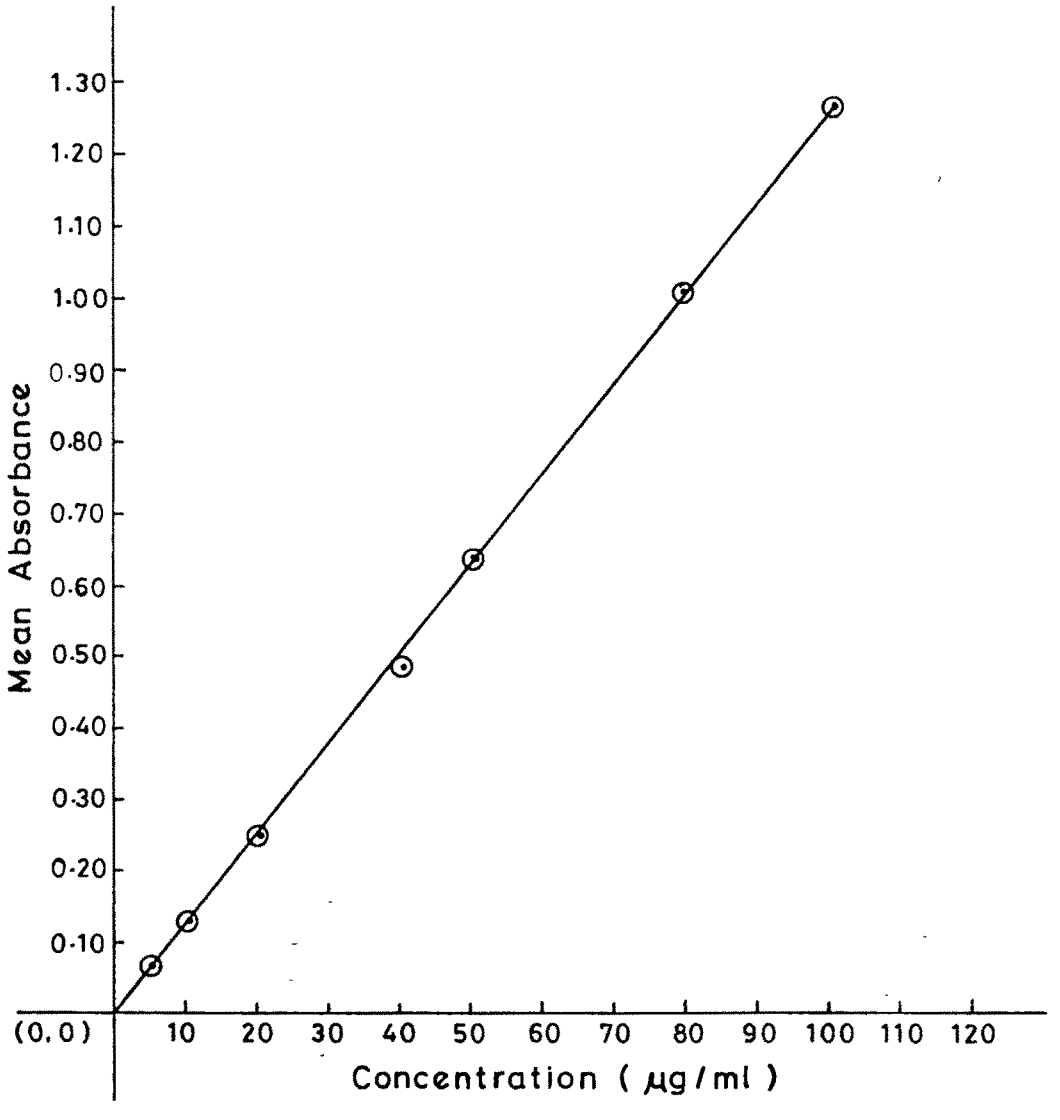


FIG. 2.16: CALIBRATION CURVE OF 5FU-MERCURIC COMPLEX WITH DIPHENYL CARBAZONE

TABLE 2.18

ESTIMATION OF 5FU IN INJECTION AND CREAM

Dosage form	Conc.in aliquot µg/ml	% Recovered (± S.D.) UV spectro photometric method λmax=266nm	5FU-Hg with dithizone λmax=605nm	5FU-Hg with diphenyl carbazone λmax=560nm
Injection (50mg/ml)	20	99.04 (0.516)	99.41 (0.429)	99.72 (0.591)
Cream (5% w/w)	20	99.04 (0.591)	99.86 (0.636)	98.83 (0.742)

recoveries of 5FU from injection and cream were found to lie between 98.86% -99.41% for dithizone method and 98.83%-99.72% for diphenyl carbazone method (Table 2.18).

On the basis of analysis of data for estimation of 5FU in injection and cream it may be concluded that the method is reproducible and comparable with the pharmacoepial method.

2.25 Reaction of 5FU with Paradimethylamino Benzaldehyde :

Literature survey has revealed that compounds like uracil and cysteine could be condensed with p-dimethyl amino benzaldehyde and estimated colorimetrically (4). A similar such reaction was attempted with 5FU.

2.251 Reagents :

a) Stock solution of drug : 100mg of 5FU was weighed into a 100ml volumetric flask dissolved and made upto volume with water.

b) Bromine water : 3ml of bromine was shaken occassionally with 100ml of water over a period of 24 hours.

c) Buffer solution I.P. : Buffers of pH 2-8 were prepared as per I.P procedure.

d) Ascorbic acid solution (5%w/v) : 5g of ascorbic acid was weighed into a 100ml volumetric flask dissolved and made to volume with water.

e) Paradimethylamino benzaldehyde solution : 10mg of p-dimethyl amino benzaldehyde was dissolved in 150ml mixture of glacial acetic acid : hydrochloric acid (85:15%v/v).

f) Ferric chloride solution (5%w/v) : 5g of ferric chloride hexahydrate was weighed and dissolved in water to produce 100ml.

2.252 Experimental procedure :

Two sets of experiments were performed. 2ml of stock solution of drug was transferred to separate test tubes in each set followed by 2ml of buffer solution of different (2-8) pH values. To the first set of tubes, 1ml of bromine water was added and the tubes were heated on a boiling water bath for 15 minutes. To the second set of tubes, 1ml of bromine water was added and the tubes were kept at room temperature. To both the sets, 1ml of ascorbic acid solution was added followed by 2ml of each of p-dimethylaminobenzaldehyde solution and ferric chloride solution. Colour formed if any was compared with the appropriate reagent blank.

No chromogenic reaction occured in any of the tubes in both the sets. This indicated that there was no colour reaction between 5FU and p-dimethyl amino benzaldehyde.

2.3 COLORIMETRIC METHODS FOR ESTIMATION OF MTX

2.31 Materials :

Methotrexate (MTX) (Cadila Laboratories, India), diethyl amino ethyl cellulose (DEAE, Sigma, USA), ammonium bicarbonate, nickel chloride, manganese chloride, ferric chloride, copper sulphate, cerric ammonium sulphate, cobalt nitrate, sodium carbonate (Qualigens, India) Folin-Ciocalteau reagent (s.d. fine chemicals, India). starch, lactose,

polyvinyl pyrrolidone, aerosil, dicalcium phosphate, avicel, ethyl cellulose, talc, magnesium stearate (National Chemicals, India), potassium iodide, mercuric chloride, nitric acid (concentrated), ammonia solution (strong), sodium hydroxide pellets, hydroxylamine hydrochloride, hydrochloric acid (concentrated) benzoyl chloride, dioxan (Qualigens, India).

All chemicals were of A.R grade and were used as such without further purification.

The purity of commercially available MTX is about 85%. It is usually contaminated with closely related compounds. Hence, the drug was made chromatographically pure by using the method of Galleli, J.F. and Yokoyama, G.(5). A column chromatographic technique using washed diethyl amino ethyl cellulose (DEAE) column as stationary phase and 0.1M ammonia-ammonium bicarbonate buffer (pH8.3) as moblie phase was used for purification.

2.32 Complexation of MTX with Metals of First Transition Series :

The published literature did not reveal any analytical method involving metal complexation for estimation of MTX. Hence an investigation was carried out to see whether MTX forms coloured complexes with the elements of first transition series.

2.321 Reagents :

a) Stock solution of drug : 100mg of pure MTX was weighed accurately into a 100ml volumetric flask, 25ml of 0.01N

hydrochloric acid was added and diluted to volume with water.

b) Buffer solutions I.P. : Buffers of pH 2-8 mentioned earlier were prepared as per the procedure given in I.P.

c) Stock solution of metals : 500mg of each of the metal salts viz. nickel chloride, manganese chloride, ferric chloride, copper sulphate, cerric ammonium sulphate, cobalt nitrate were weighed into separate 100ml volumetric flasks, dissolved in water and made to volume with the same solvent.

2.322 Experimental procedure :

Two sets of experiments were performed. 1ml of the drug solution was transferred into separate test tubes in each set. To each of these test tubes, 2ml of each of different buffer solutions and different metal solutions were added. The first set of test tubes were heated on a boiling water bath for 2-5 minutes. The second set of test tubes were kept at room temperature. The test tubes were observed for formation of any chromogenic reaction.

No chromogenic reaction was found to take place between MTX and any of the metal solutions at any pH and at both the temperature conditions.

2.33 Reaction of MTX with Folin-Ciocalteau Reagent :

It has been reported that folic acid which is structurally similar to MTX gives chromogenic reaction with Folin-Ciocalteau reagent (F-C. reagent)(6). Hence an attempt was made to develop a colorimetric method for MTX based on similar lines.

2.331 Reagents :

a) Stock solution of drug : 100mg of pure MTX was weighed accurately into a 100ml volumetric flask. The drug was dissolved and diluted to volume with 0.01N sodium hydroxide solution.

b) Sodium carbonate solution (20%w/v) : 20g of sodium carbonate was weighed into a 100ml volumetric flask, dissolved and made to volume with water.

c) F-C reagent : F-C reagent obtained from s.d. fine chemicals was used as such without dilution.

2.332 Experimental procedure :

To 2ml of drug solution in a test tube, 2ml of sodium carbonate solution and 1ml of F-C reagent were added. The contents of the test tube were mixed thoroughly.

A greyish blue colour was formed between MTX and F-C reagent. Hence an attempt was made to quantify this reaction.

2.333 Development of colorimetric method for MTX with F-C reagent :

a) Scanning of the spectrum :

To a suitable aliquot (0.2ml) of drug solution in a 10ml volumetric flask, 2ml of sodium carbonate solution and 1ml of F-C reagent were added, the contents were mixed thoroughly and made to volume with water. The absorption spectrum of greyish blue colour formed was scanned between 400-800nm against the reagent blank. The wavelength scan is shown in Fig. 2.17.

b) Optimisation of concentration of sodium carbonate solution :

To a suitable aliquot (0.2ml) of drug solution in different 10ml volumetric flasks, 2ml of varying concentrations (5,10.....50%w/v) of sodium carbonate solutions were added followed by 1ml of F-C reagent. The contents were mixed thoroughly and made to volume with water. The absorbance was measured at 760nm against the appropriate reagent blank. The observations are recorded in Table 2.19.

c) Optimisation of volume of F-C reagent :

To 0.2ml of drug solution and 2ml of sodium carbonate solution in different 10ml volumetric flasks, varying volumes (0.5, 1....5ml) of F-C reagent were added, the contents were mixed thoroughly and made to volume with water. The absorbance of greyish blue colour formed was measured at 760nm against appropriate reagent blank. The observations are recorded in Table 2.20.

d) Preparation of calibration curve :

Aliquots of drug solution equivalent to 20µg, 40µg.... 500µg/ml were accurately transferred into clean, separate 10ml volumetric flasks. 2ml of 20%w/v sodium carbonate solution and 1ml of F-C reagent were added. The contents of the flask were mixed thoroughly and made to volume with water. The absorbance of greyish blue colour in each flask was measured at 760nm against the reagent blank. The results are recorded in Table 2.21 and the calibration curve is shown in Fig. 2.19.

e) Estimation of MTX from injection :

MTX is available as injection in usual strengths of 25mg in 1ml, 5mg in 2ml, 25mg in 2ml, 50mg in 2ml and 250mg in 10ml (Cadila Laboratories, India). A volume of injection equivalent to 25mg was accurately transferred into a 50ml volumetric flask and made to volume with 0.01N sodium hydroxide solution. Colour was developed with a suitable aliquot (0.4ml) of this solution by the procedure described under preparation of calibration curve.

f) Estimation of MTX from synthetic mixtures :

Synthetic mixtures for MTX tablets were prepared using commonly used excipients to see whether any of these excipients interfere with this method of estimation. The formula of the synthetic mixtures is given in Table 2.22.

A quantity of the mixture equivalent to 25mg of MTX was accurately weighed into a 50ml volumetric flask. 25ml of 0.01N sodium hydroxide solution was added, the mixture was shaken for 15 minutes, diluted to volume and then filtered. Colour was developed with a suitable aliquot (0.4ml) of the clear filtrate as described under preparation of calibration curve.

g) Estimation of MTX in tablets :

MTX tablets of strength 2.5mg/tablet are available (Biddle Swayer, India). 20 tablets were weighed and powdered. A quantity of the powder equivalent to 25mg of MTX was transferred into a 50ml volumetric flask. 25ml

of 0.01N sodium hydroxide solution was added, the mixture was shaken for 15 minutes, diluted to volume with the same and filtered. Colour was developed with a suitable aliquot (0.4ml) of the filtrate as described under preparation of calibration curve.

The result of the estimation of MTX from injection, synthetic mixtures, and tablets are recorded in Table 2.23.

h) Results and discussion :

A greyish blue colour is produced by treating MTX with F-C reagent in alkaline medium.

The benzoyl glutamic acid moiety present in MTX may be responsible for the colour formation. The absorption spectrum (Fig. 2.17) showed a λ max at 760nm. The reagent blank did not show any interference at the analytical wavelength. From Tables 2.19 and 2.20 it may be observed that 2ml of 20%w/v solution of sodium carbonate and 1ml of F-C reagent were optimum for colour development. The colour was formed at room temperature and was stable for several hours.

From the data of mean absorbance values (Table 2.21 and Fig.2.18) it may be observed that the calibration curve is linear between 2-50µg/ml. The sensitivity was found to be 2µg/ml. The a and b values for the line of regression (y=a+bx) were calculated and found to be a=0.186 and b=0.0054. The molar absorptivity was found to be 6.929×10^3 $1mol^{-1}cm^{-1}$ and the correlation coefficient was found to be 0.991.

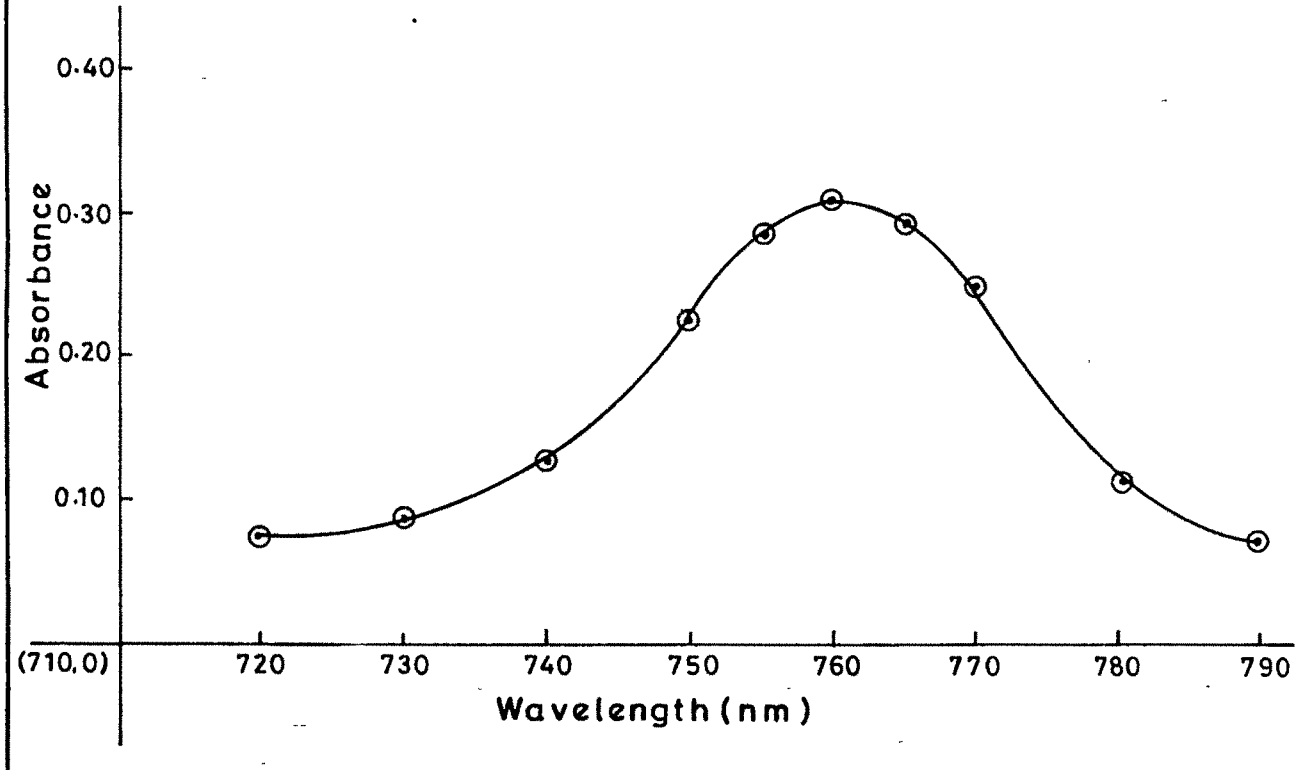


FIG. 2.17: WAVELENGTH SCAN OF MTX - FC REAGENT

TABLE 2.19

OPTIMISATION OF CONCENTRATION OF SODIUM CARBONATE SOLUTION

Drug concentration - 20µg/ml.
λmax - 760nm.

Concentration of sodium carbonate solution % w/v	Absorbance
5	0.197
10	0.256
15	0.287
20	0.305
25	0.305
50	Turbidity was seen.

TABLE 2.20

OPTIMISATION OF VOLUME OF F-C REAGENT

Drug concentration - 10µg/ml.
λmax - 760nm.

Volume of F-C reagent (ml)	Absorbance
0.5	0.275
1	0.305
2	0.305
5	Interference of the blank

TABLE 2.21

CALIBRATION CURVE OF MTX - F.C. REAGENT

λmax = 760nm

Concentration µg/ml of MTX	Mean absorbance (± S.D.)
2	0.084 (0.027)
4	0.110 (0.005)
5	0.122 (0.002)
8	0.156 (0.009)
10	0.184 (0.032)
20	0.305 (0.002)
25	0.386 (0.004)
40	0.550 (0.007)
50	0.678 (0.011)

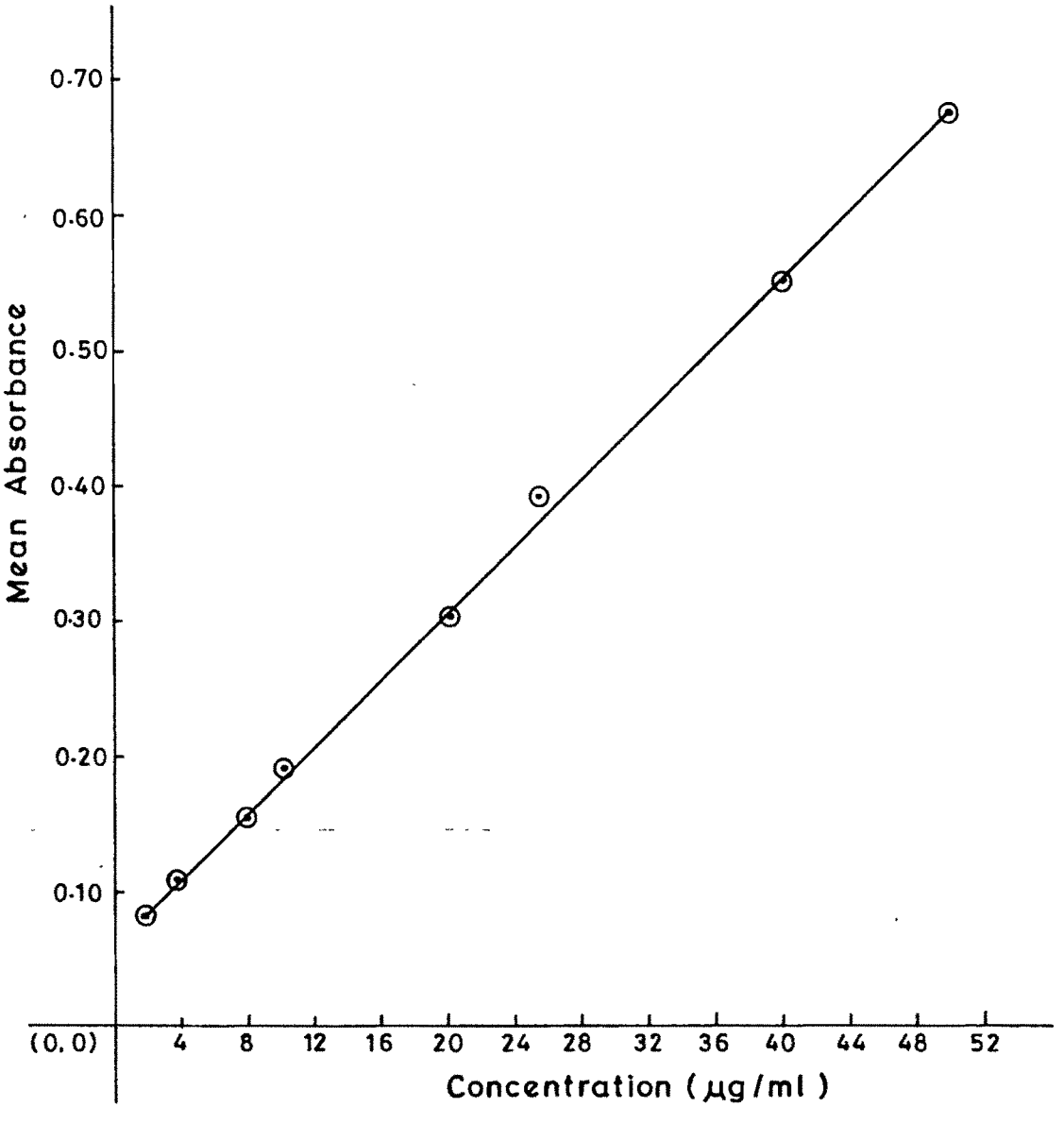


FIG. 2.18 : CALIBRATION CURVE OF MTX-FC REAGENT

TABLE 2.22

FORMULAE FOR SYNTHETIC MIXTURES OF MTX

Ingredients	Quantity/100 tablets	
	Formula I (mg)	Formula II (mg)
MTX	250	250
Starch	4000	-
Lactose	4500	-
Polyvinyl pyrro lidone	1000	-
Dicalcium phosphate	-	4000
Avicel	-	4500
Ethyl cellulose	-	1000
Aerosil	50	50
Talc	100	100
Magnesium stearate	100	100

TABLE 2.23

ESTIMATION OF MTX IN INJECTION, SYNTHETIC MIXTURES AND TABLETS

Dosage form	Conc. in aliquot µg/ml	% Recovered (± S.D.) MTX + F-C Reagent λmax = 760nm
Injection	20	99.73 (0.208)
Synthetic mixture		
I	20	99.76 (0.180)
II	20	99.37 (0.339)
Tablets (2.5mg/tab)	20	99.69 (0.222)

The percentage recoveries of MTX from injection, synthetic mixtures and tablets were obtained between 99.37% - 99.76% which lie within pharmacoepial limits (90-110%). The results of estimation of MTX from synthetic mixtures (Table 2.23) reveal that the commonly used excipients do not interfere in method of analysis of MTX with F-C reagent.

On the basis of analysis of the data for estimation of drug from formulations, it may be concluded that the method is simple, reproducible and does not involve any reduction or oxidative cleavage. It may therefore be used for routine analysis of MTX in bulk and dosage forms.

2.34 Reaction of MTX with Nessler's Reagent :

Since many nitrogenous compounds are reported to give colour reaction with Nessler's reagent, an attempt was made to develop a simple colorimetric method for MTX using this reagent.

2.341 Reagents :

a) Stock solution of drug : 50mg of pure MTX was weighed into a 100ml volumetric flask. The drug was dissolved and made to volume with 0.01N sodium hydroxide solution.

b) Nessler's reagent : It was prepared as per the procedure given in I.P. 1.25g of mercuric chloride and 3.5g of potassium iodide were dissolved in 80ml of water. To this solution, a cold saturated aqueous solution of mercuric chloride was added with constant stirring until

a slight red precipitate remained. 12g of sodium hydroxide and a little more of cold saturated aqueous solution of mercuric chloride were added to the above solution and volume was made upto 100ml with water. The solution was allowed to stand and clear liquid was decanted and used.

2.342 Experimental procedure :

To 2ml of the drug solution, 1ml of Nessler's reagent was added. The solution was thoroughly mixed and observed for colour formation.

An intense yellowish orange colour was observed. So an attempt was made to quantify this reaction.

2.343 Development of colorimetric method for estimation of MTX with Nessler's reagent :

a) Scanning of the spectrum :

To an aliquot (0.2ml) of drug solution in a 10ml volumetric flask, 1ml of Nessler's reagent was added, mixed and made to volume with water. The absorbance of yellowish orange colour was scanned between 350-800nm against the reagent blank. The wavelength scan is shown in Fig. 2.19.

b) Optimisation of volume of Nessler's reagent :

To 0.2ml of drug solution in different 10ml volumetric flasks, varying volumes (0.5, 1.0...5.0ml) of Nessler's reagent were added and made to volume with water. The absorbance of the yellowish orange colour was measured at 430nm against the appropriate reagent blank. The observations are recorded in Table 2.24.

c) Calibration curve for MTX with Nessler's reagent :

Aliquots of drug solution equivalent to 50µg, 100µg, 200µg....1000µg/ml were accurately transferred into clean 10ml volumetric flasks. 1ml of Nessler's reagent was added, the contents were mixed and made to volume with water. The absorbance of yellowish orange colour formed in each flask was measured at 430nm against the reagent blank. The observations are recorded in Table 2.25. The calibration curve is shown in Fig. 2.20.

d) Estimation of MTX from injection :

An aliquot of injection equivalent to 25mg of MTX was accurately transferred into 50ml volumetric flask and diluted to volume with 0.01N sodium hydroxide solution, colour was developed with a suitable aliquot (0.2ml) of the solution by the procedure described under preparation of calibration curve.

e) Estimation of MTX from synthetic mixtures :

Two synthetic mixtures were prepared as per the formula given in Table 2.22. A quantity of the powder equivalent of 25mg of MTX was accurately weighed into 50ml volumetric flask, 25ml of 0.01N sodium hydroxide solution was added, the mixture shaken for 15minutes, made upto volume with the 0.01N NaOH and filtered. A suitable aliquot (0.2ml) of the clear filtrate was taken and colour was developed by the procedure given under preparation of calibration curve.

f) Estimation of MTX from tablets :

20 tablets were weighed and powdered. A quantity of the powdered mixture equivalent to 25mg of MTX was transferred into a 50ml volumetric flask. 25ml of 0.01N sodium hydroxide solution was added and the mixture was shaken for 15 minutes, made upto volume with the same and filtered. Colour was developed with a suitable (0.2ml) aliquot of the filtrate as described under preparation of calibration curve.

The results of estimation from injection, synthetic mixtures and tablets are recorded in Table 2.31.

g) Results and discussion :

When MTX was reacted with Nessler's reagent, an intense yellow colour was formed which showed an absorption maxima at 430nm (Fig. 2.19). The colour formed at room temperature was stable for more than 4 hours. 1ml of Nessler's reagent was optimum for colour development (Table 2.24).

The calibration curve was linear in the concentration range of 5-100µg/ml. (Table 2.25 and Fig. 2.20). The sensitivity was found to be 4µg/ml. The a and b values for line of regression were calculated and are found to be a=0.507, b=0.0063. The molar absorptivity was found to be 8.150×10^3 $ltmol^{-1}cm^{-1}$. The correlation co-efficient r was found to be 0.999.

From Table 2.26 it may be observed that the percentage recoveries were obtained between 99.82% - 99.91% in case of injection and tablets which lie within

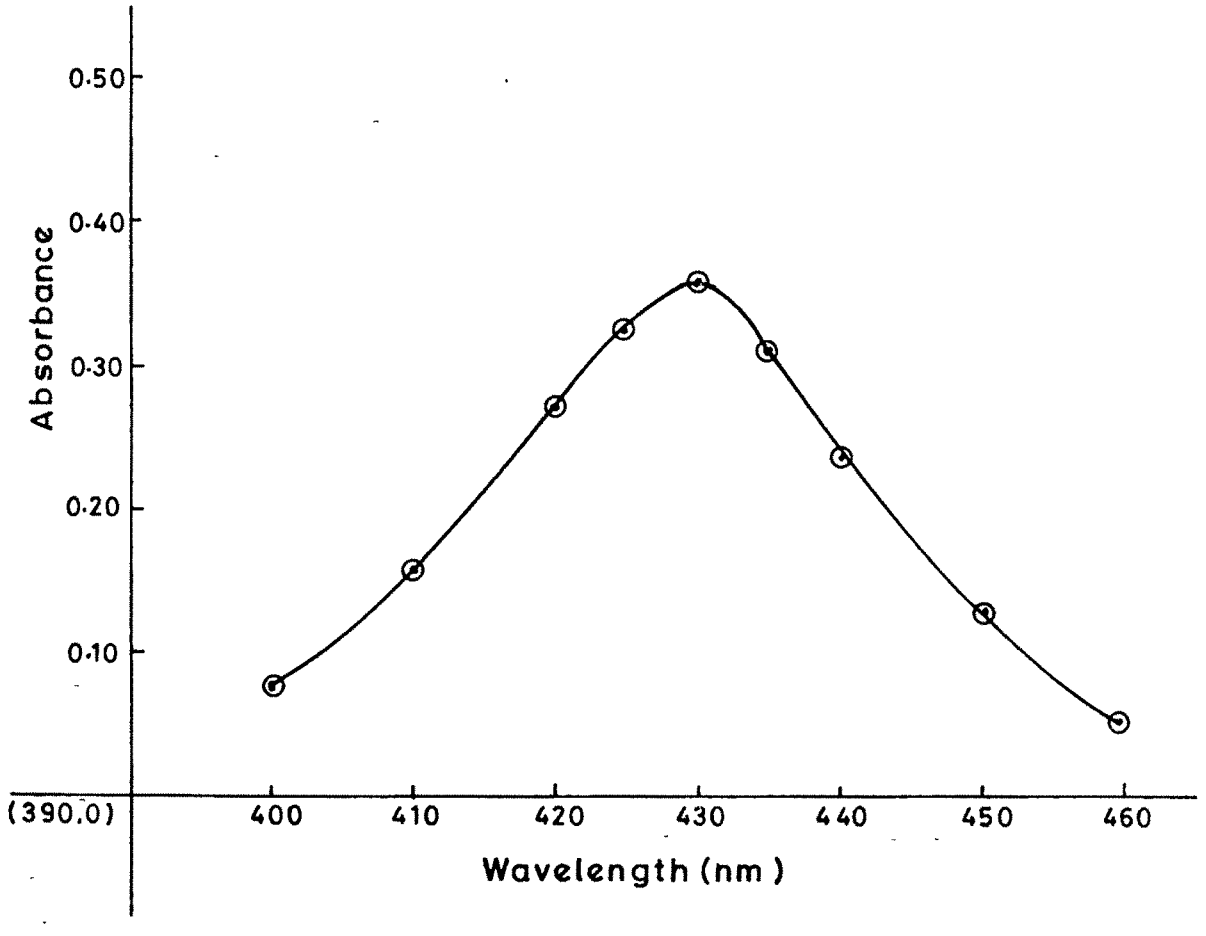


FIG. 2.19: WAVELENGTH SCAN OF MTX-NESSLER'S REAGENT

TABLE 2.24

OPTIMISATION OF VOLUME OF NESSLER'S REAGENT

Drug concentration - 20µg/ml
λmax - 430nm

Volume of Nessler's reagent (ml)	Absorbance
0.5	0.312
1.0	0.359
2.0	0.359
5.0	Turbidity was seen.

TABLE 2.25

CALIBRATION CURVE OF MTX - NESSLER'S REAGENT

λmax = 430nm

Concentration µg/ml	Mean absorbance (± S.D.)
5	0.075 (0.002)
10	0.164 (0.003)
20	0.359 (0.014)
40	0.671 (0.012)
50	0.885 (0.024)
60	1.062 (0.017)
80	1.405 (0.011)
100	1.723 (0.102)

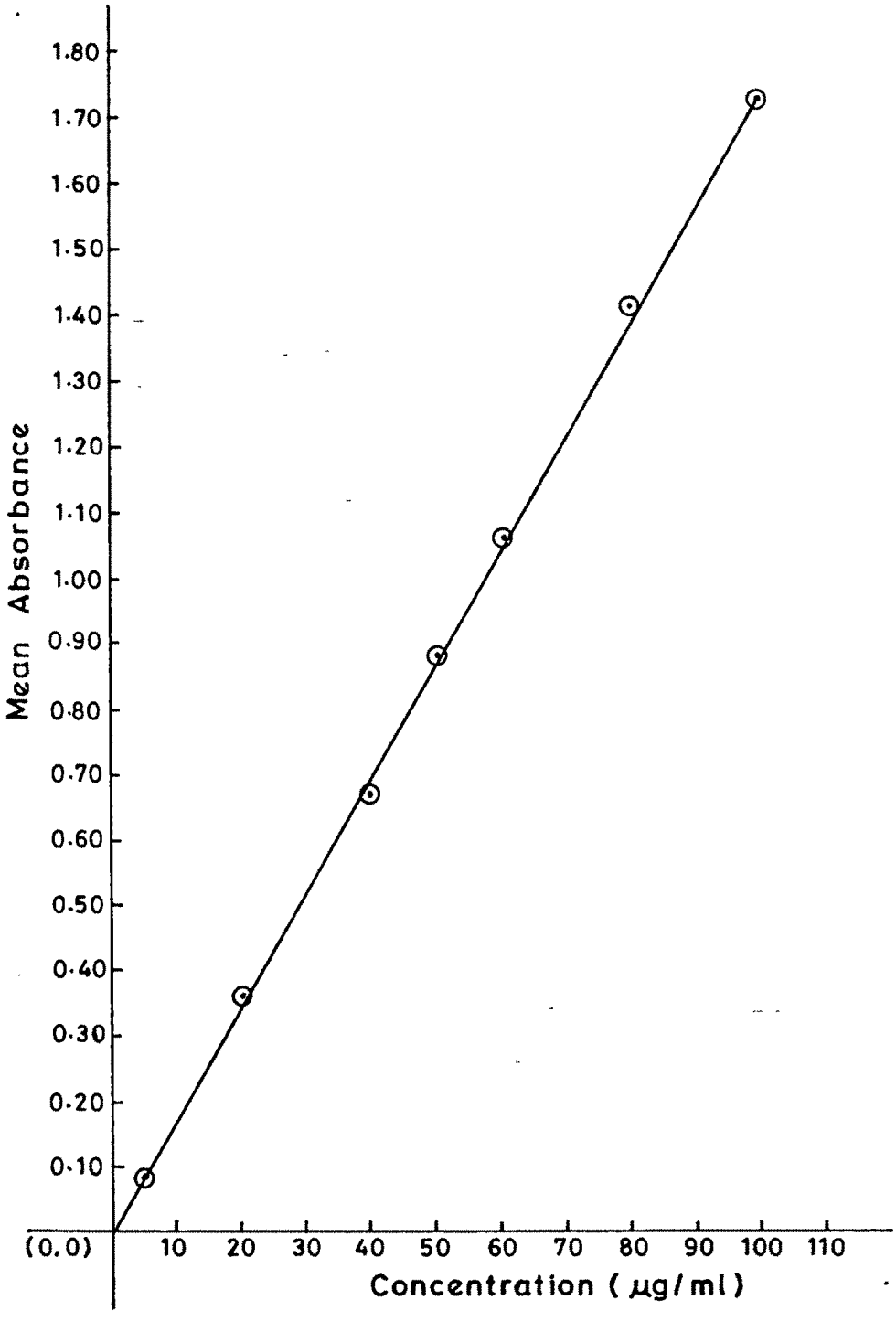


FIG. 2.20: CALIBRATION CURVE OF MTX-NESSLER'S REAGENT

TABLE 2.26

ESTIMATION OF MTX IN INJECTION, SYNTHETIC MIXTURES AND TABLETS

Dosage form	Conc. in aliquot µg/ml	% Recovered (± S.D.) MTX + Nessler's reagent λmax = 430nm
Injection	20	99.904 (0.079)
Synthetic Mixture		
I	20	99.906 (0.073)
II	20	99.908 (0.105)
Tablets (2.5mg/tab)	20	99.816 (0.186)

pharmacoepial limits (90% - 110%). The results of percentage recoveries of MTX from synthetic mixtures reveal that the commonly used excipients do not interfere with the method of analysis of MTX with Nessler's reagent.

On the basis of analysis of the data for estimation of drug from formulations, it may be concluded that the method is simple, reproducible and does not involve any reduction or oxidative cleavage. It may therefore be used for routine analysis from bulk and dosage forms.

2.35 Reaction of MTX with Nitric Acid :

Some compounds structurally related to MTX like folic acid have been estimated by nitration method (7). So attempts were made to develop colorimetric method for estimation of MTX on similar lines.

2.351 Reagents :

a) Nitric acid solution (50% v/v) : 50% v/v aqueous solution of nitric acid was prepared.

b) Stock solution of drug : 25mg of pure MTX was accurately weighed into a 50ml volumetric flask, dissolved and diluted to volume with nitric acid solution.

2.352 Experimental procedure :

Two sets of experiments were performed. A 2ml aliquot of the drug solution was transferred into each set. 2ml of nitric acid solution was added in all the tubes in both the sets.

The first set of tubes were placed on a boiling water bath for 5 minutes. The solutions were then cooled to room temperature. The second set of tubes were kept at room temperature. To both the sets 1ml of strong solution of ammonia was added. Colour formed if any, was compared with that of the reagent blank solution.

An intense yellow colour was noted in first set of tubes. No chromogenic reaction was observed at room temperature.

2.353 Development of colorimetric method for MTX with nitric acid and ammonia :

a) Scanning of the spectrum :

To an aliquot of the drug solution (0.2ml) in a 10ml volumetric flask, 2ml of nitric acid solution was added. The solution was boiled for 5 minutes, cooled to room temperature and 2ml of ammonia solution was added. The contents were mixed and made up to volume with water. The absorption spectrum of the intense yellow colour produced was scanned between 350-800nm against the reagent blank. The wavelength scan is given in Fig. 2.21.

b) Optimisation of boiling time :

To a suitable aliquot of drug solution (0.2ml) in separate 10ml volumetric flasks, 2ml of nitric acid solution was added; the flasks were placed on a boiling water bath for different periods of time (5,10.....60 minutes). The flasks were cooled, 2ml of ammonia solution was added to each flask, contents were mixed and

made to volume with water. The absorbance was measured at 380nm against the appropriate reagent blank. The observations are recorded in Table 2.27.

c) Optimisation of concentration of nitric acid solution :

Suitable aliquots of drug solution (0.2ml) were taken in different 10ml volumetric flasks, 2ml of varying concentrations of nitric acid solution (10,20...70%v/v) were added, the contents of each flask were boiled for 5 minutes on a waterbath and cooled. 2ml of ammonia solution was added and made to volume with water. The absorbance of the intense yellow colour formed was measured at 380nm against the appropriate reagent blank. The observations are recorded in Table 2.28.

d) Optimisation of volume of ammonia solution :

Suitable aliquot of drug solution and nitric acid solution taken in different 10ml volumetric flasks were boiled for 5 minutes, cooled and varying volumes of ammonia solution were added. The volume was made up with water and the absorbance was measured at 380nm against appropriate reagent blank. The observations are recorded in Table 2.29.

e) Preparation of calibration curve :

Aliquots of drug solution equivalent to 10µg, 20µg...1000µg/ml were accurately transferred into separate 10ml volumetric flasks. 2ml of 50%v/v nitric acid solution was added in each flask and the contents were heated on a boiling water bath for 5 minutes. The solutions were cooled to room temperature, 2ml of ammonia

solution (strong) was added and made upto volume with water. The absorbance of the intense yellow colour produced in each flask was measured at 380nm against the reagent blank. The observations are recorded in Table 2.30. The calibration curve is shown in Fig.2.22.

f) Estimation of MTX in injection :

A volume of injection equivalent to 25mg was accurately transferred into a 50ml volumetric flask, diluted to volume with 0.01N sodium hydroxide solution. Colour was developed with a suitable aliquot (0.2ml) of this solution by the procedure described under preparation of calibration curve.

g) Estimation of MTX from synthetic mixtures :

MTX was estimated in the synthetic mixtures, the formula of which is given in Table 2.22.

A quantity of the powder equivalent to 25mg of MTX, was transferred into 50ml volumetric flask. 25ml of 0.01N sodium hydroxide solution was added, the mixture was shaken for 15 minutes, made to volume and filtered. Colour was developed with a suitable aliquot (0.2ml) of the filtrate by the procedure given under preparation of calibration curve.

h) Estimation of MTX from tablets :

20 tablets were weighed and powdered. A quantity of powder equivalent to 25mg of MTX was transferred into 50ml volumetric flask. 25ml of 0.01N sodium hydroxide solution was added, the mixture was shaken for 15 minutes, made to volume with 0.01N sodium hydroxide

solution and filtered. Colour was developed with a suitable aliquot (0.2ml) of the filtrate by the procedure described under preparation of calibration curve.

The results of the estimation of MTX in injection, tablets and synthetic mixtures are recorded in Table 2.31.

i) Results and discussion :

An intense yellow colour was formed when MTX was boiled with nitric acid and neutralised with ammonia solution. From the absorption spectrum recorded, it may be observed that yellow colour formed showed a λ max at 380nm (Fig. 2.21). The colour was stable for more than 4 hours. Maximum intensity of colour was produced when drug solution was boiled for 5 minutes (Table 2.27). 2ml of 50%v/v of nitric acid solution and 2ml of ammonia solution were optimum for colour development (Tables 2.28 and 2.29).

From Table 2.30 it may be observed that the calibration curve is linear in the concentration range of 1-100µg/ml. The sensitivity was found to be 1µg/ml. The a and b values were calculated for the line of regression and were found to be a=0.292, b=0.009. Molar absorptivity and correlation coefficient were found to be 8.247×10^3 $ltmol^{-1}cm^{-1}$ and 0.997 respectively.

The percentage recoveries (Table 2.31) of MTX from injection and tablets are within the range 99.64%-99.95%. The recoveries lie within the pharmacoepial limit (90%-110%). From the results of analysis of MTX from synthetic

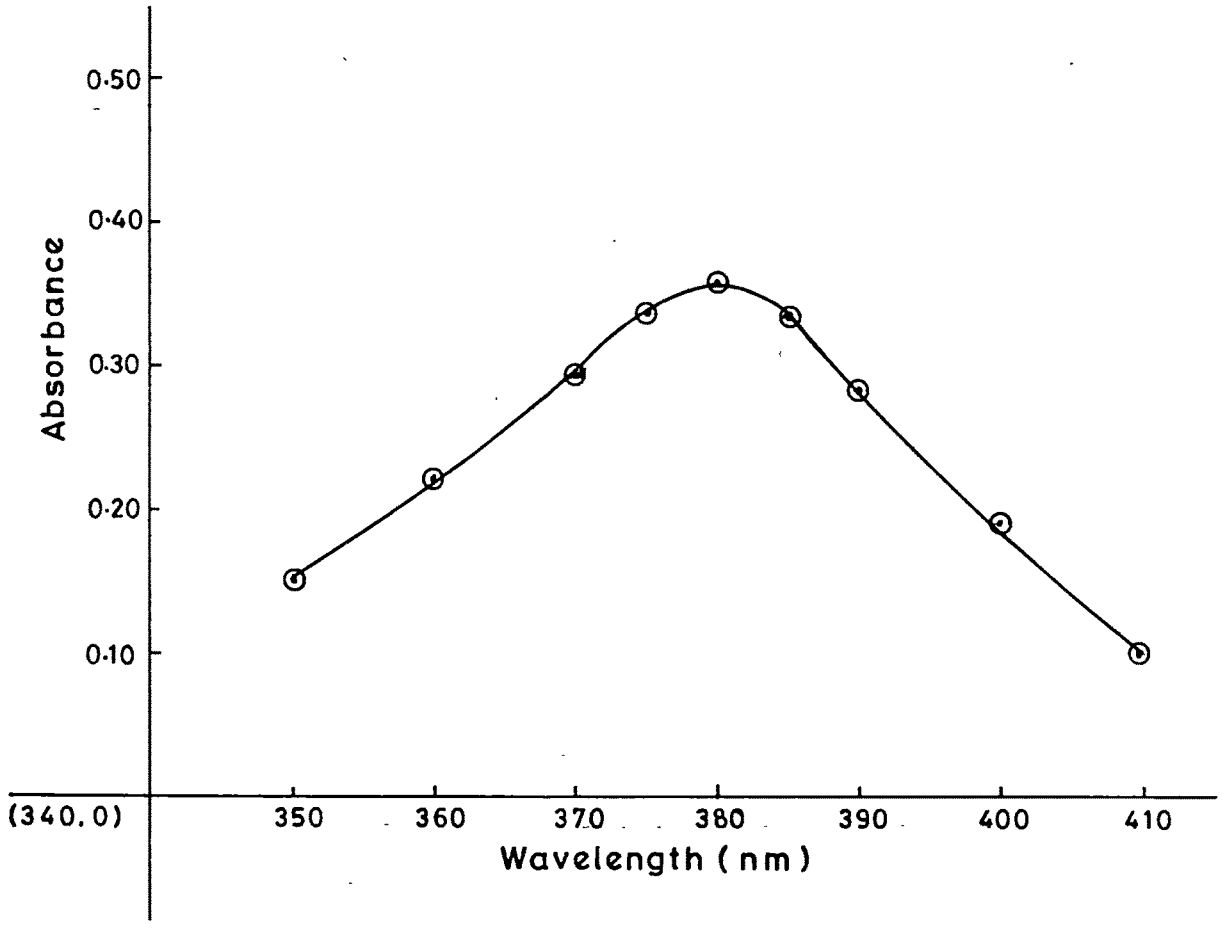


FIG. 2.21: WAVELENGTH SCAN OF MTX-NITRIC ACID SOLUTION

TABLE 2.27

OPTIMISATION OF BOILING TIME OF MTX - NITRIC ACID SOLUTION

Drug concentration - 20µg/ml.
λmax - 380nm.

Time minutes	Absorbance
5	0.363
10	0.363
20	0.362
30	0.362
60	0.362

TABLE 2.28

OPTIMISATION OF CONCENTRATION OF NITRIC ACID SOLUTION

Drug concentration - 20µg/ml
λmax - 380nm

Conc. of nitric acid solution % v/v	Absorbance
10	0.215
20	0.275
40	0.312
50	0.363
60	0.363
70	0.363

TABLE 2.29

OPTIMISATION OF VOLUME OF AMMONIA SOLUTION

Drug concentration - 20µg/ml
λmax - 380nm

Volume of ammonia ml	Absorbance
0.5	0.275
1.0	0.310
2.0	0.363
3.0	0.363
5.0	0.362

TABLE 2.30

CALIBRATION CURVE OF MTX - NITRIC ACID SOLUTION

λmax = 380nm

Concentration µg/ml	Mean absorbance (± S.D.)
1	0.049 (0.002)
2	0.072 (0.003)
5	0.102 (0.001)
10	0.201 (0.092)
20	0.363 (0.005)
40	0.684 (0.012)
50	0.851 (0.007)
80	1.332 (0.004)
100	1.652 (0.002)

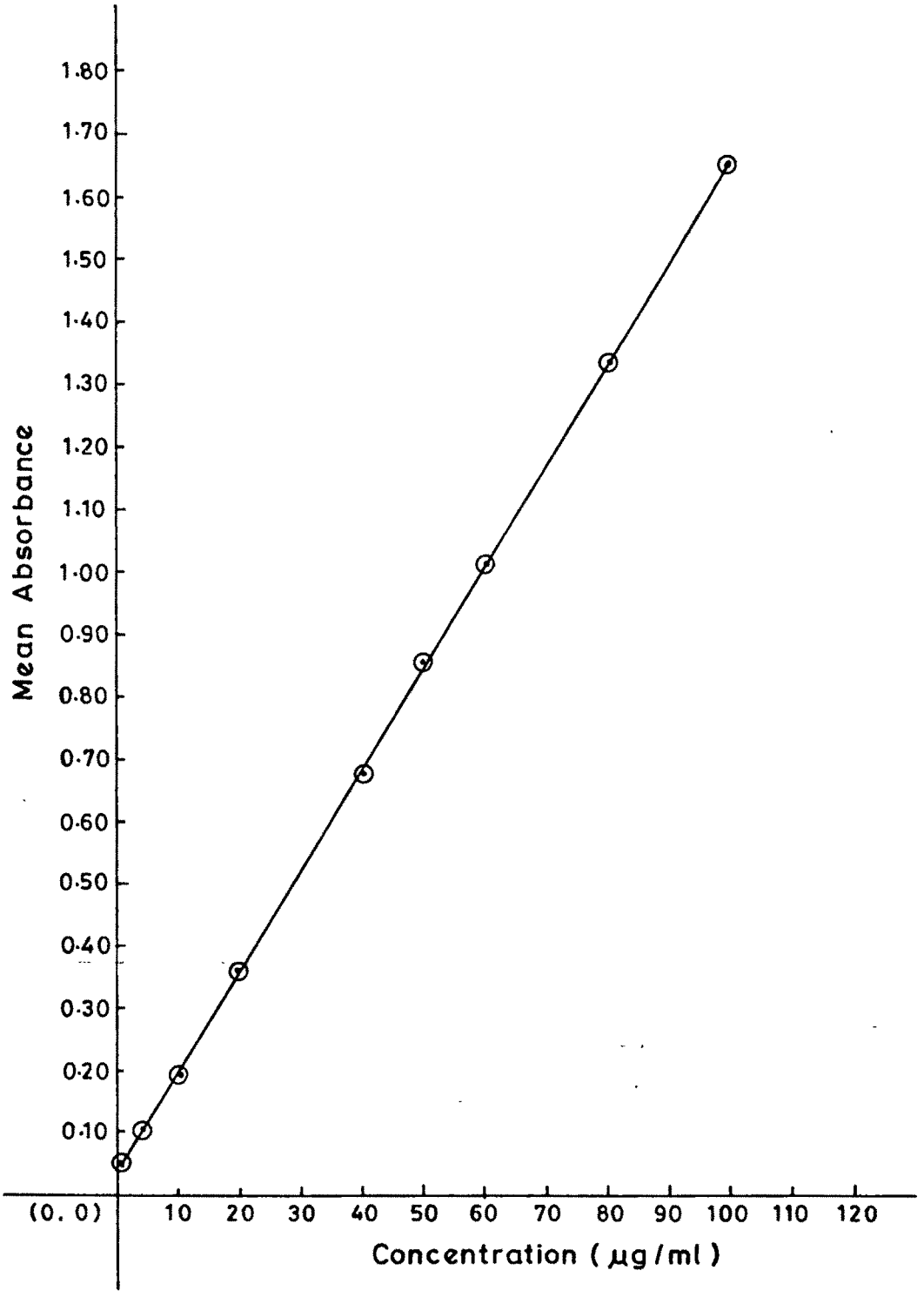


FIG. 2.22: CALIBRATION CURVE OF MTX-NITRIC ACID SOLUTION

TABLE 2.31

ESTIMATION OF MTX IN INJECTION, SYNTHETIC MIXTURES AND TABLETS

λmax - 380nm

Dosage form	Conc. in aliquot µg/ml	% Recovered (± S.D.) MTX + nitric acid solution
Injection	20	99.74 (0.252)
Synthetic Mixture		
I	20	99.64 (0.203)
II	20	99.95 (0.037)
Tablets	20	99.66 (0.256)

mixtures it may be concluded that the commonly used excipients do not interfere with the method of analysis of MTX by nitration.

On the basis of analysis of the data for estimation of drug from formulations it may be concluded that the method is simple and can be used for routine analysis of the drug from formulations.

2.36 Reaction of MTX with Hydroxylamine Hydrochloride and Ferric Chloride :

It is reported that when MTX is boiled with aqueous alkali solution, 4-hydroxy pteridine is obtained as the main product(8). Hence an attempt was made to bezoylate 4-hydroxy pteridine and estimate the ester formed by ferric hydroxamate method.

2.361 Reagents :

a) Sodium hydroxide solution (1N) : 4g of sodium hydroxide was dissolved and diluted to 100ml with water.

b) Sodium hydroxide solution (10%w/v) : 10g of sodium hydroxide was dissolved and diluted to 100ml with water.

c) Hydroxylamine hydrochloride solution (15%w/v) : 15g of hydroxylamine hydrochloride was dissolved and diluted to 100ml with water.

d) Ferric chloride solution (1%w/v) : 1g of ferric chloride was dissolved and diluted to 100ml with water.

e) Hydrochloric acid solution (20%v/v) : 20ml of concentrated hydrochloric acid was diluted and made upto 100ml with water.

2.362 Experimental procedure :

100mg of pure MTX was dissolved in 5ml of 1N sodium hydroxide solution in a 50ml round bottom flask and refluxed for one hour on a boiling water bath. To the resulting solution, 1ml of benzoyl chloride was added and the mixture was shaken vigorously till the odour of benzoyl chloride disappeared. Water was added to destroy excess benzoyl chloride. The mixture was filtered. The precipitated benzoyl ester was dissolved in 5ml of dioxan and diluted to 20ml with water. To a suitable aliquot of the solution, 2ml each of hydroxylamine hydrochloride solution and sodium hydroxide solution (10%w/v) were added. The solution was neutralised with 3ml of hydrochloric acid solution and treated with 1ml of ferric chloride solution.

A red coloured complex was formed. So an attempt was made to quantify this procedure.

2.363 Development of colorimetric method for MTX with hydroxylamine hydrochloride and ferric chloride solution :

a) Scanning of the spectrum :

The absorption spectrum of the red coloured complex formed in the above solution was scanned between 400-800nm against the reagent blank. The wavelength scan is shown in Fig.2.23.

b) Optimisation of heating time :

100mg of MTX was taken in different round bottom flasks of 50ml capacity, 5ml of 1N sodium hydroxide solution was added to each flask and the mixture was

refluxed for different periods of time viz 30 minutes, 1 hour, 2 hours and 3 hours on a boiling water bath. The resulting solution at the end of each heating time was cooled, benzoylated and colour developed by the addition of hydroxylamine hydrochloride solution followed by ferric chloride solution as described earlier. The absorbance of the red coloured complex was measured at 520nm against the reagent blank. Observations are recorded in Table 2.32.

c) Optimisation of concentration of hydroxylamine hydrochloride and ferric chloride solution:

100mg of MTX was taken in different round bottom flasks, 5ml of 1N sodium hydroxide solution was added to each flask and the mixture was refluxed on a boiling water bath for 1 hour. The resulting solutions were cooled and benzoylated with benzoyl chloride. The benzoyl derivative was filtered, washed with water, dissolved in 5ml of dioxan and made upto 20ml with water.

For optimising the hydroxylamine hydrochloride concentration, 0.4ml of benzoylated solution and 1ml ferric chloride solution were transferred into separate 10ml volumetric flasks. 2ml of varying concentrations (5,10....25%w/v) of hydroxylamine hydrochloride solution was added into each flask and made upto volume with water. For optimising the ferric chloride concentration, 0.4ml of benzoylated solution and 2ml of hydroxylamine hydrochloride solution were transferred into separate

10ml volumetric flasks. 1ml of varying concentrations (0.5,10....5.0%w/v) of ferric chloride solution was added into each flask and made upto volume with water.

The absorbance of solution in each case was measured at 520nm against appropriate reagent blank. The observations are recorded in Tables 2.33 and 2.34.

d) Preparation of calibration curve:

100mg of MTX was dissolved in 5ml of 1N sodium hydroxide solution in a round bottom flask and refluxed for 1 hour on a boiling water bath. The resulting solution was benzoylated with benzoyl chloride and the mixture was filtered. The precipitate was dissolved in 5ml of dioxan and diluted to 20ml with water.

Aliquots of this solution equivalent to 1000µg, 2000µg,....5000µg/ml were transferred into separate 10ml volumetric flasks, 2ml of each of hydroxylamine hydrochloride solution (15%w/v) and sodium hydroxide solution (10%w/v) were added followed by 5ml of hydrochloric acid solution (20%v/v) and 1ml of ferric chloride solution (1%w/v). The volume was made up with water and the absorbance of the red coloured solution in each flask was measured at 520nm against the reagent blank. The observations are recorded in Table 2.35 and shown in Fig. 2.24.

e) Estimation of MTX from injection :

A volume of injection equivalent to 100mg of MTX was transferred into a round bottom flask, 5ml of 1N sodium hydroxide solution was added and the other operations

were carried out as described under preparation of calibration curve.

f) Estimation of MTX from synthetic mixture :

Two synthetic mixtures were prepared as per the formula given in Table 2.22.

A quantity of the mixture equivalent to 100mg of MTX was shaken with 10ml of 1N sodium hydroxide solution for 15 minutes and filtered. 5ml of the filtrate was transferred into a round bottom flask and the other operations were carried out as described under preparaion of calibraion curve.

g) Estimation of MTX in tablets :

20 tablets were weighed and powdered and the powdered mixture was shaken with 10ml of 1N sodium hydroxide solution for 15 minutes and filtered. 5ml of the filtrate was transferred into a round bottom flask and the other operations were carried out as described in the preparation of calibration curve. The results of the estimation of MTX from injection, synthetic mixtures and tablets are recorded in Table 2.36.

h) Results and discussion :

The red coloured complex formed between the benzoyl ester (derived from MTX hydrolysis) and its hydroxamate with ferric chloride showed a λ max at 520nm (Fig 2.23).

From Tables 2.32-2.34 it may be observed that 1 hour boiling time, 2ml of 15%w/v solution of hydroxylamine hydrochloride and 1ml of 1%w/v ferric chloride solution were optimum for colour development.

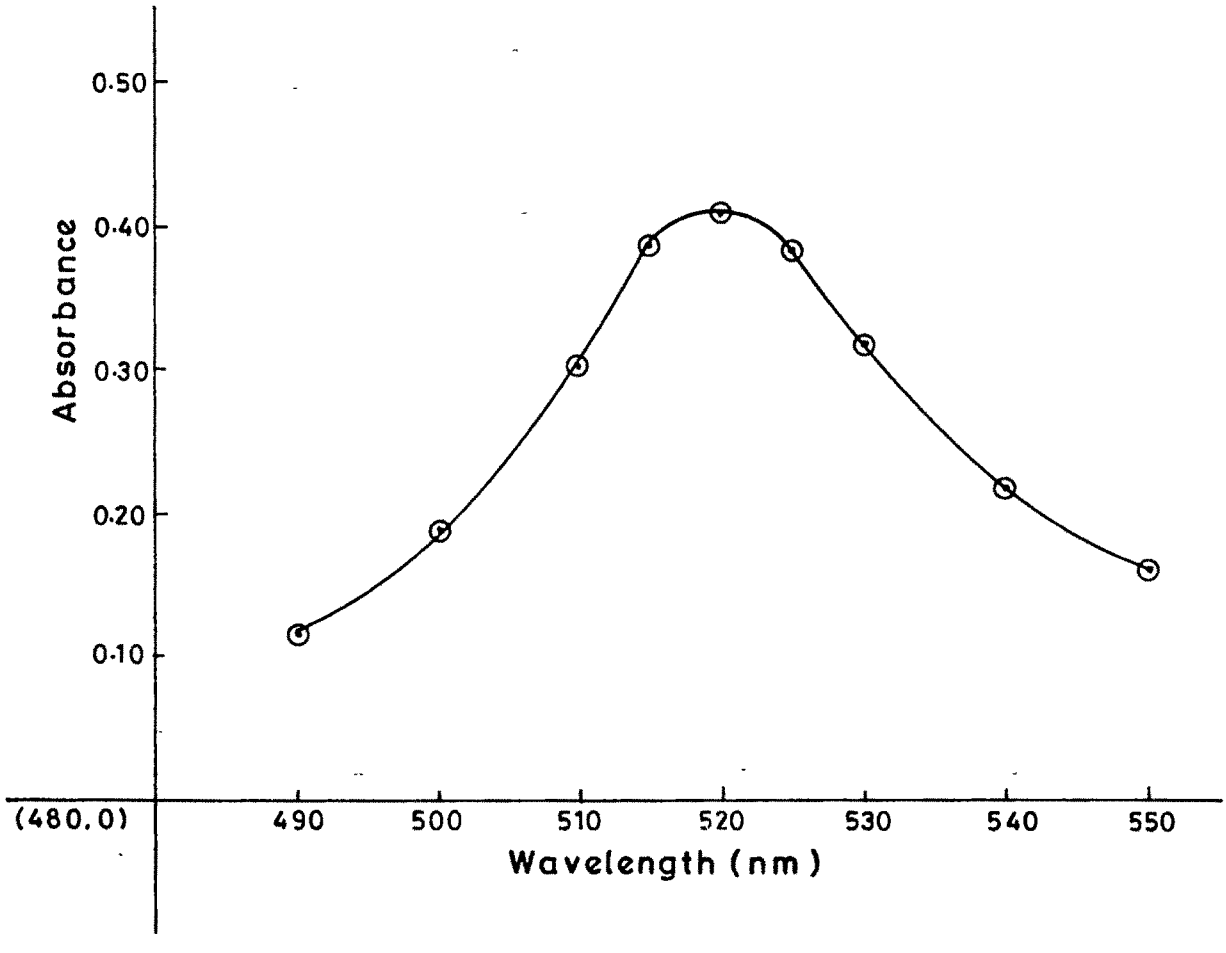


FIG. 2.23: WAVELENGTH SCAN OF MTX-HYDROXYLAMINE FERRIC CHLORIDE COMPLEX

TABLE 2.32

OPTIMISATION OF BOILING TIME

Drug concentration - 200µg/ml.
λmax - 520nm.

Time (hours)	Absorbance
0.5	0.299
1	0.411
2	0.412
3	0.411

TABLE 2.33

OPTIMISATION OF HYDROXYLAMINE HYDROCHLORIDE CONCENTRATION

Drug concentration - 200µg/ml
λmax - 520nm

Concentration of hydroxylamine HCl % w/v	Absorbance
5	0.301
10	0.359
15	0.411
20	0.411
25	0.412

TABLE 2.34

OPTIMISATION OF FERRIC CHLORIDE CONCENTRATION

Drug concentration - 200µg/ml
λmax - 520nm

Concentration of $FeCl_3$ solution % w/v	Absorbance
0.5	0.280
1.0	0.411
2.0	0.411
3.0	0.410
5.0	Interference of blank

TABLE 2.35

CALIBRATION CURVE OF MTX - HYDROXYLAMINE - FERRIC CHLORIDE COMPLEX.

λmax - 520nm.

Concentration µg/ml	Mean absorbance values (± S.D.)
100	0.202 (0.015)
200	0.411 (0.102)
300	0.592 (0.045)
400	0.753 (0.032)
500	0.939 (0.079)

TABLE 2.36

ESTIMATION OF MTX IN INJECTION, SYNTHETIC MIXTURES AND TABLETS.

Dosage form	Conc.in aliquot µg/ml	% Recovered (± S.D.) MTX - hydroxylamine-$FeCl_3$ complex λmax - 520nm
Injection	200	99.03 (0.293)
Synthetic Mixtures		
I	200	98.412 (0.486)
II	200	99.01 (0.449)
Tablets	200	98.99 (0.421)

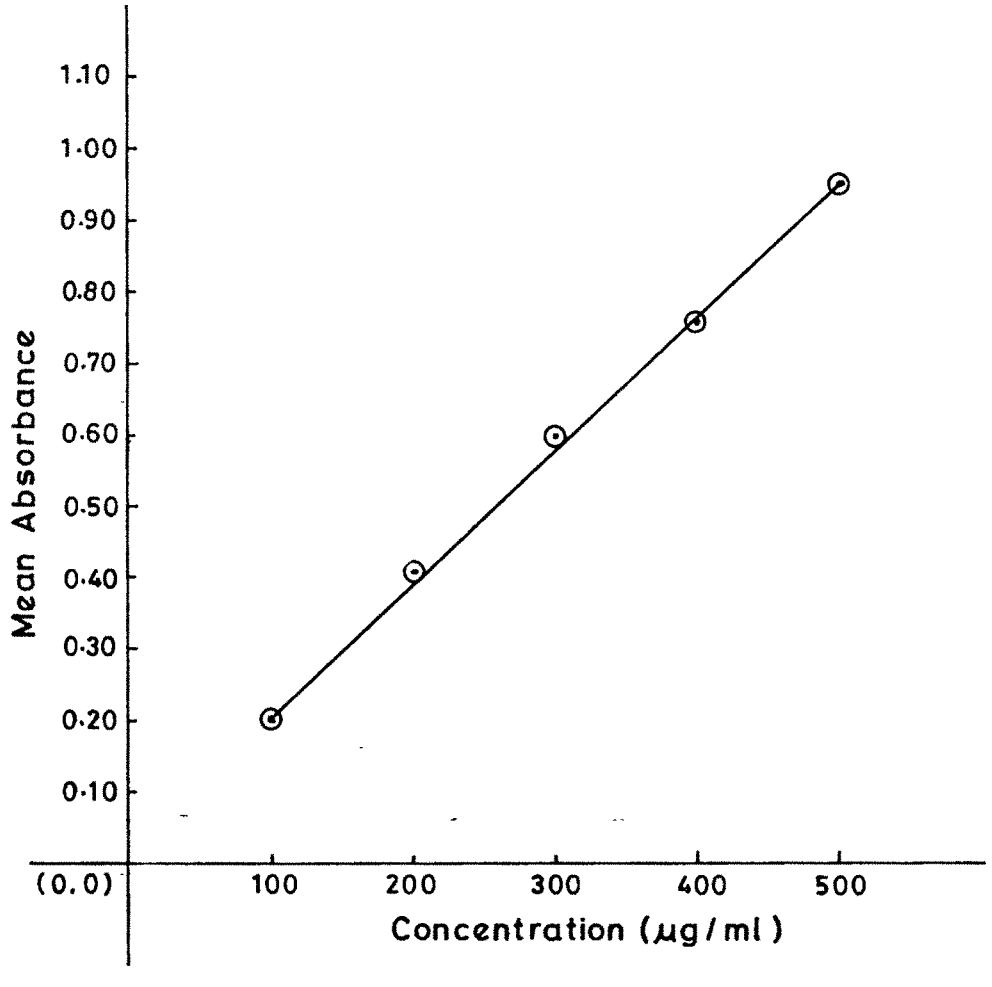


FIG. 2.24: CALIBRATION CURVE OF MTX-HYDROXYLAMINE FERRIC CHLORIDE COMPLEX

The calibration curve (Table 2.35, Fig 2.24) was rectilinear in the concentration range between 100-500µg/ml. The sensitivity was found to be 75µg/ml. The a and b values were calculated for the line of regression and were found to be a=0.048 and b=0.002. Molar absorptivity and correlation coefficient were found to be $0.933x10^{3}lmol^{-1}cm^{-1}$ and 0.999 respectively.

The percentage recoveries of MTX from injection, synthetic mixtures, and tablets (Table 2.36) lie within the range 98.41%-99.03%. From the results of analysis of MTX from synthetic mixtures it may be concluded that the commonly used excipients do not interfere with this method of estimation.

The results of estimation of MTX from formulations reveal that the method is precise and comparable with other methods.

2.4 COLORIMETRIC METHODS FOR ESTIMATION OF CYCLOPHOSPHAMIDE

2.41 Materials :

Cyclophosphamide (Cadila Laboratories, India), cobalt chloride, ammonium thiocyanate, picric acid, sodium nitrite, (E. Merck, India) hydrochloric acid (concentrated) (Qualigens, India).

2.42 Reaction of Cyclophosphamide with Cobalt thiocyanate and Ferric thiocyanate Solution

Aliphatic tertiary amines are reported to give coloured complexes with cobalt and ferric thiocyanate(9). Since one of

the nitrogens in cyclophosphamide is in a tertiary state, cyclophosphamide was reacted with cobalt thiocyanate and ferric thiocyanate in order to develop a simple colorimetric method for estimation of cyclophosphamide from bulk drug and dosage forms.

2.421 Reagents :

a) Stock solution of drug : 500 mg of cyclophosphamide was accurately weighed into a 100 ml volumetric flask, dissolved in water and volume made up with water.

b) Ammonium ferrothiocyanate solution : 2.703 g of ferric chloride hexahydrate and 3.04 g of ammonium thiocyanate were dissolved and made upto 100ml with water.

c) Cobalt thiocyanate solution : 6.8g of cobalt chloride and 4.3 g of ammonium thiocyanate were dissolved in sufficient water to produce 100 ml.

d) Buffer solutions I.P. : Buffers of pH 2-10 were prepared as per the procedure given in I.P.

e) Sodium nitrite solution (20% w/v) : A 20% w/v solution of sodium nitrite was prepared in water.

f) Hydrochloric acid solutiton (20% v/v) : A 20% v/v solution of hydrochloric acid was prepared in water.

2.422 Experimental procedure :

Two sets of experiments were performed. 1 ml of drug solution was transferred into several test tubes in both the sets. 2ml of buffer solution of pH2-10 were added into each of the tubes.

To the first set of tubes, 2ml of ammonium ferro-thiocyanate solution was added followed by 2ml of chloroform.

The mixture was vortexed for 2 minutes and the chloroform layer was separated and colour formed if any was compared with that of the reagent blank.

To the second set of tubes, 2ml of cobalt thiocyanate solution was added followed by 2ml of chloroform. The mixture was vortexed for 2 minutes and the chloroform layer was separated. Colour formed if any in the chloroform layer was compared with that of reagent blank.

At pH 7 cyclophosphamide formed a red coloured complex with ferrothiocyanate solution and a blue coloured complex with cobalt thiocyanate solution. Hence attempts were made to develop colorimetric methods for estimation of cyclophosphamide from various formulations.

2.423 Development of colorimetric methods for cyclophosphamide with ferrothiocyanate and cobalt thiocyanate solutions :

A common procedure was adopted for estimation of cyclophosphamide with ferrothiocyanate and cobalt thiocyanate solutions.

a) Scanning of the spectrum :

A suitable aliquot (0.5ml) of the drug solution was transferred into a separating funnel. 2 ml of pH7 phosphate buffer and 2ml of metal thiocyanate solution were added. The chloroform extracts were collected and passed through anhydrous sodium sulphate contained in a funnel into a 5ml volumetric flask and made to volume with chloroform. The absorbance of the coloured complex

was scanned between 400-800 nm against an appropriate reagent blank. The wavelength scan is given in Figs. 2.25 and 2.26.

b) Optimisation of the volume of the buffer solution :

To a suitable aliquot (0.5 ml) of the drug solution in different separating funnels, varying volumes (0.5, 1.0...5ml) of pH7 buffer solution were added followed by 2 ml of metal thiocyanate solution. The coloured complex was extracted with 2ml of chloroform twice, made upto 5ml with the same and the absorbance was measured against a suitable reagent blank. The observations are recorded in Table 2.37.

c) Optimisation of volume of metal thiocyanate solutions :

To a suitable aliquot (0.5ml) of drug solution in different 60 ml separating funnels, 2ml of pH7 buffer solution was added followed by varying volumes (0.5, 1.0...5.0ml) of the metal thiocyanate solution. The coloured complex was extracted with chloroform twice and made upto 5ml with the same. The absorbance of the coloured solution was measured against suitable reagent blank. The observations are recorded in Table 2.38.

d) Preparation of calibration curve :

Aliquots of drug solution equivalent to 50, 100, 200... 1250 µg/ml of drug were accurately transferred into different 60 ml separating funnels, 2ml of pH7 phosphate buffer and 2ml of ferro thiocyanate/ cobaltthiocyanate solution were added. The coloured complex formed was extracted with 2ml of chloroform

twice. The chloroform extracts were collected, passed through anhydrous sodium sulphate contained in a funnel into 5ml volumetric flasks and made to volume with chloroform. The absorbance of the orange red coloured complex formed with ferrothiocyanate solution and blue coloured complex with cobaltthiocyanate solution in each flask were measured at 490 nm and 625 nm respectively. The observations are recorded in Table 2.39 and shown in Figs 2.27 and 2.28.

e) Estimation of cyclophosphamide from injection :

Cyclophosphamide is available in vials containing 100 mg, 200 mg, 500 mg, 1 and 2g of drug. The product also contains 45 mg of sodium chloride per 100 mg of drug.

A quantity of injection equivalent to 50mg of cyclophosphamide was dissolved in sufficient water to produce 100 ml. Colour was developed with a suitable aliquot (0.5ml) of the drug solution as described under preparation of calibration curve.

f) Estimation of cyclophosphamide from synthetic mixtures :

Two synthetic mixtures for cyclophosphamide tablets were prepared using commonly used excipients to see whether any of these excipients interfere with this method of estimation. The formula of synthetic mixtures is given in Table 2.40.

A quantity of the synthetic mixture equivalent to 50 mg of cyclophosphamide was extracted with four quantities each of 5ml of solvent ether and ether extract was filtered through a plug of cotton. The filtrate was

evaporated completely and the residue was dissolved in 100 ml of water. Colour was developed with a suitable aliquot (0.5ml) of drug solution as described under preparation of calibration curve.

g) Estimation of cyclophosphamide from tablets :

Twenty tablets were weighed and powdered. A quantity of powdered mixture equivalent to 25 mg of cyclophosphamide was extracted with four quantities each of 5ml of solvent ether, ether extract was filtered and the filtrate was evaporated completely. The residue was dissolved in 50 ml of water. Colour was developed with a suitable aliquot (0.5ml) of the diluted solution as described under preparation of calibration curve.

The results of the estimation of cyclophosphamide from injection, synthetic mixtures and tablets are given in Table 2.41. The percentage recoveries were compared with that of the reported method (10).

h) Reported method :

(i) Estimation from injection :

A quantity of injection equivalent to 50 mg of the drug was dissolved in sufficient water to produce 100 ml. To a suitable aliquot of drug solution (0.4ml) in a 10ml volumetric flask, 2ml of 20% w/v sodium nitrite solution and 2ml of 20% v/v hydrochloric acid solution were added. After 10 minutes the volume was made up with water and the absorbance of the yellow coloured solution was measured at 350 nm against a suitable reagent blank.

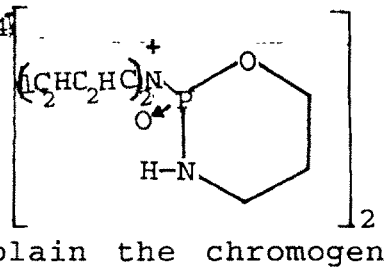
(ii) Estimation from synthetic mixtures and tablets :

A quantity of synthetic mixtures and powdered tablets equivalent to 25 mg of the drug was extracted separately with four quantities each of 5ml of solvent ether; ether extract was filtered and the filtrate was evaporated completely. The residue was dissolved in 50 ml of water and colour was developed as described under estimation from injection by reported method.

The results of the estimation of cylcophosphamide from injection, synthetic mixtures and tablets by the reported method are recorded in Table 2.41.

(i) Results and discussion :

The orange-red coloured complex formed with cyclophosphamide and ferrothiocyanate and blue coloured complex formed between cyclophosphamide and cobaltthiocyanate in chloroform show a λ max at 490nm and 625nm respectively (Figs 2.25 and 2.26). It is quite probable that cobalt may be existing in organic phase in the form of anion complex with cyclophosphamide by ion association of the type $[CO(CNS)_6]^{4}$



A similar mechanism may explain the chromogenic reaction between cyclophosphamide and ferrothiocyanate.

The ion pair complexes get extracted into chloroform phase at pH7. From Tables 2.37 and 2.38 it may be observed that 2ml of each of pH7 phosphate buffer and

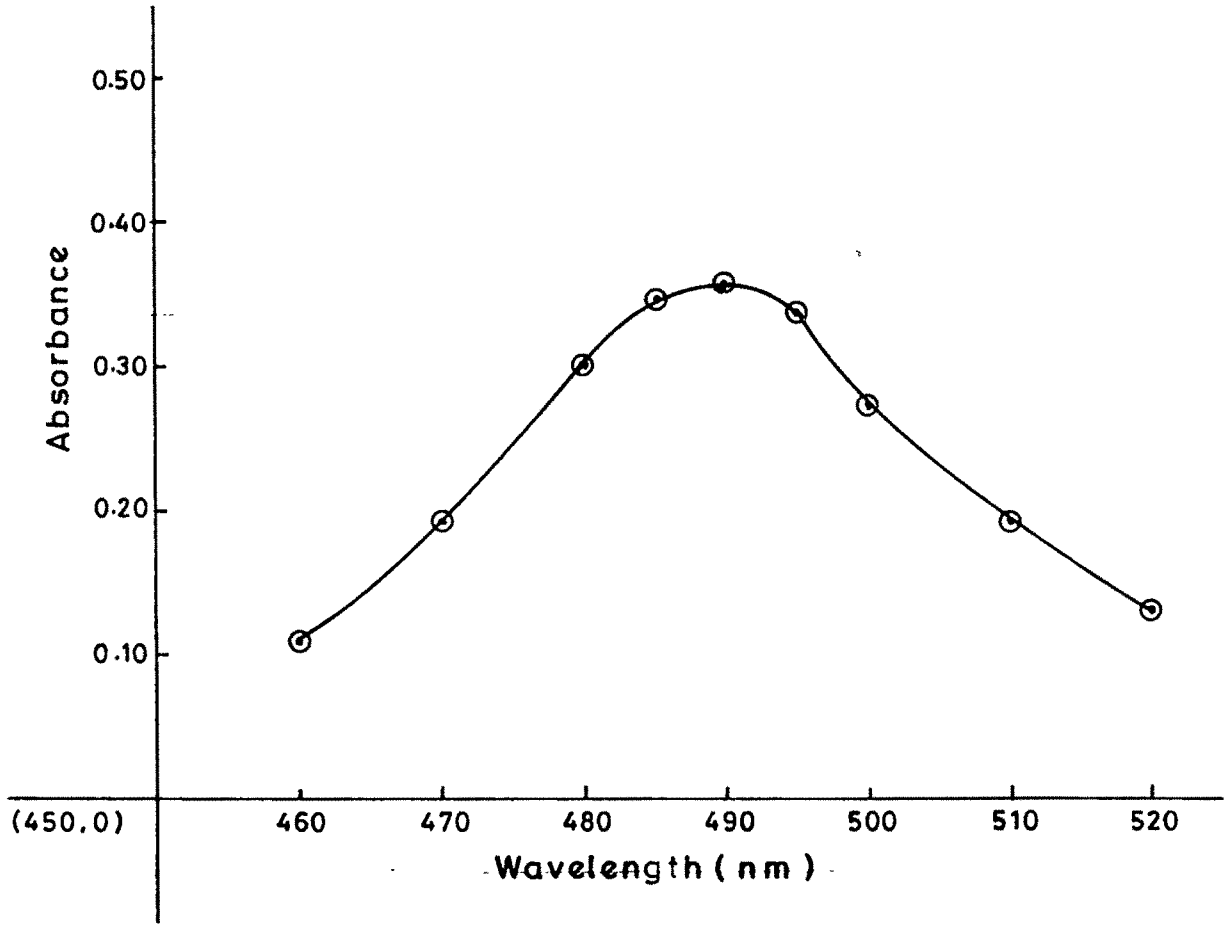


FIG. 2.25: WAVELENGTH SCAN OF CYCLOPHOSPHAMIDE - FERROTHIOCYANATE COMPLEX

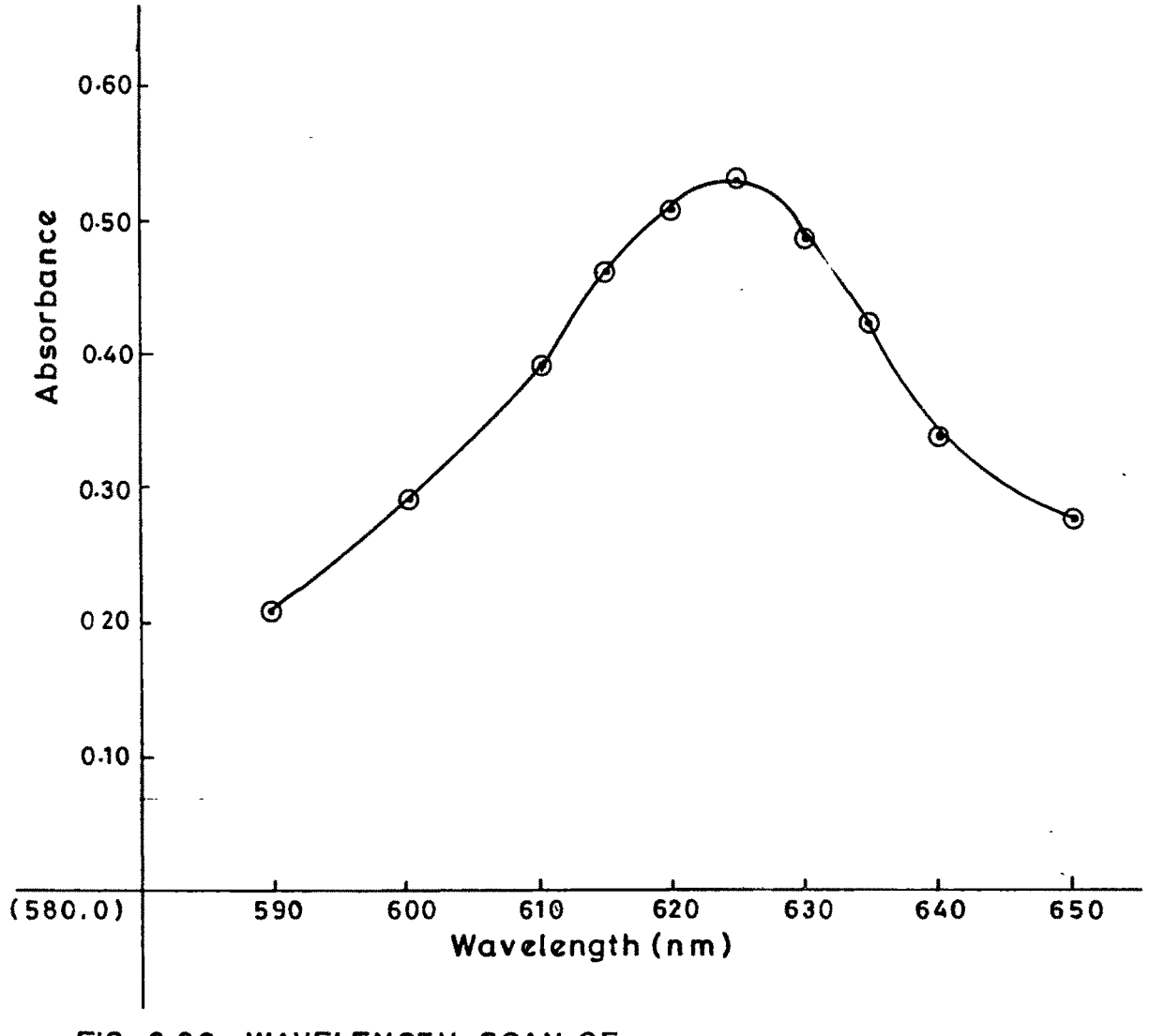


FIG. 2.26: WAVELENGTH SCAN OF CYCLOPHOSPHAMIDE - COBALT THIOCYANATE COMPLEX

TABLE 2.37

OPTIMISATION OF VOLUME OF pH7 BUFFER

Drug Concentration = 50 µg/ml

Volume of buffer ml	Absorbance	
	Ferrothiocyanate method	Cobalt thiocyanate method
0.5	0.202	0.195
1.0	0.275	0.262
2.0	0.308	0.301
3.0	0.307	0.301
5.0	0.308	0.301

TABLE 2.38

OPTIMISATION OF VOLUME OF FERROTHIOCYANATE/COBALT THIOCYANATE SOLUTION.

Drug concentration = 50 µg/ml

Volume of thiocyanate solution ml	Absorbance	
	Ferrothiocyanate method	Cobalt thiocyanate method
0.5	0.211	0.209
1.0	0.290	0.285
2.0	0.308	0.301
3.0	0.308	0.301
5.0	0.306	0.301

ferrothiocyanate/cobaltthiocyanate solutions were optimum for colour development. The colour produced was stable for more than 4 hours in both the cases.

The calibration curves were found to be rectilinear in the concentration range of 10-250 µg/ml in case of cyclophosphamide-ferrothiocyanate complex and 20-200 µg/ml in case of cyclophosphamide-cobalt thiocyanate complex (Table 2.39, Figs. 2.27 and 2.28). The sensitivity in the earlier case was 5µg/ml and in latter case 20µg/ml.

The a and b values for line of regression for ferrothiocyanate and cobaltthiocyanate method were calculated and found to be a=0.069, 0.81 and b=0.0075, 0.0074 respectively. The molar absorptivity and correlation coefficient 'r' for cyclophosphamide ferrothiocyanate method were found to be $1.72 \times 10^{3} lmol^{-1} cm^{-1}$, r=0.997 respectively while that for cyclophosphamide-cobaltthiocyanate were $1.68 \times 10^{3} lmol^{-1} cm^{-1}$ and r=0.998 respectively.

From Table 2.41 it may be observed that the percentage recoveries of cyclophosphamide from injection, synthetic mixtures and tablets lie between 99.077%-99.627% comparable with that of the reported method (99.35%-99.92%).

The result of estimation of cyclophosphamide from synthetic mixtures reveal that the commonly used excipients do not interfere with the method of analysis of cyclophosphamide by cobalt and ferrothiocyanate

TABLE 2.39

CALIBRATION CURVES OF CYCLOPHOSPHAMIDE - FERROTHIOCYANATE / COBALTTHIOCYANATE COMPLEX

Concentration µg/ml	Mean Absorbance (±.S.D.)	
	Ferrothiocyanate method	Cobalt thiocyanate method
10	0.043 (0.007)	-
20	0.097 (0.013)	0.082 (0.003)
40	0.231 (0.009)	0.215 (0.009)
50	0.308 (0.005)	0.301 (0.009)
80	0.539 (0.002)	0.533 (0.009)
100	0.693 (0.007)	0.682 (0.011)
120	0.871 (0.015)	0.811 (0.008)
160	1.155 (0.013)	1.112 (0.013)
200	1.463 (0.015)	1.423 (0.011)
250	1.848 (0.007)	-

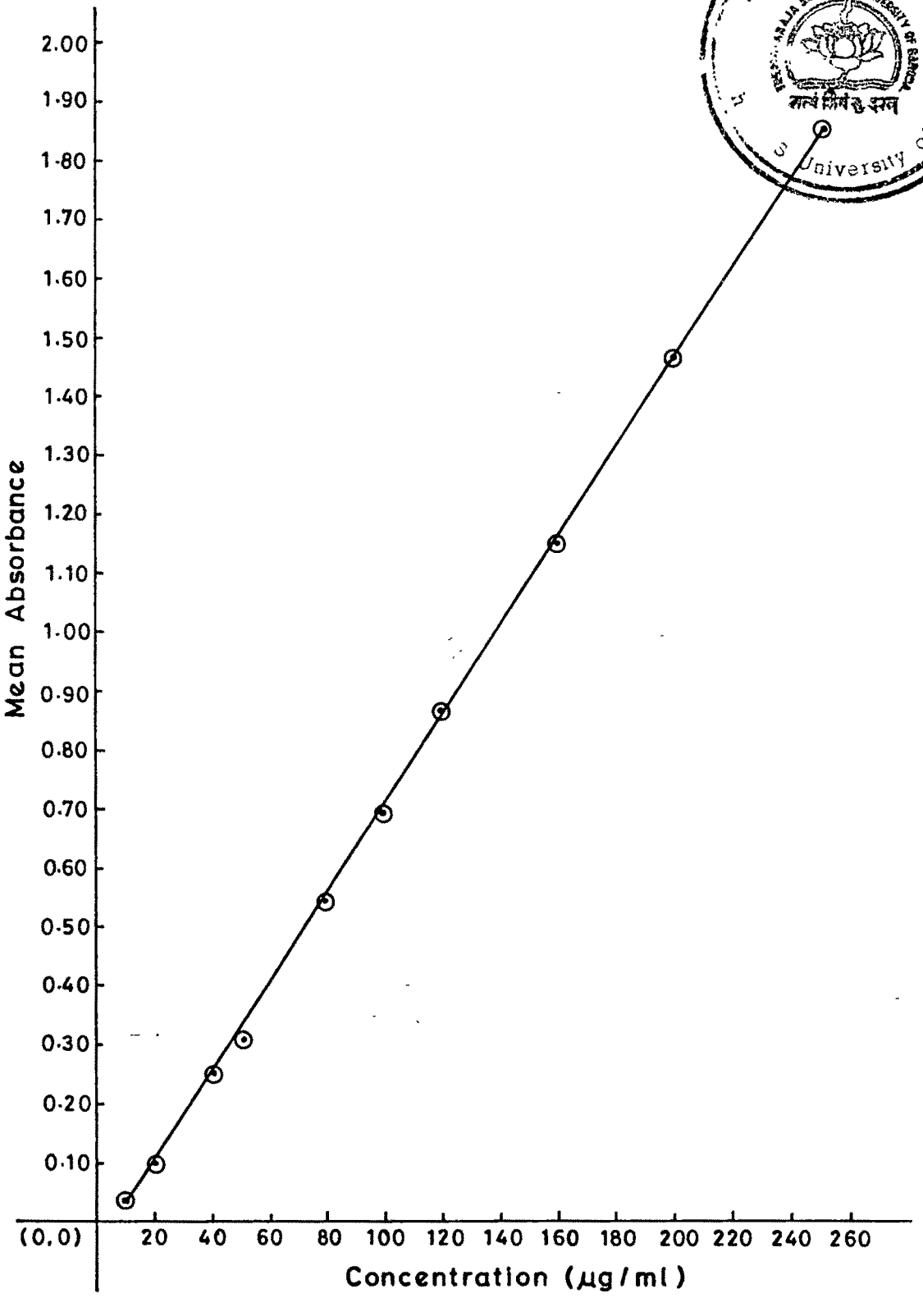


FIG. 2.27: CALIBRATION CURVE OF CYCLOPHOSPHAMIDE - FERROTHIOCYANATE COMPLEX

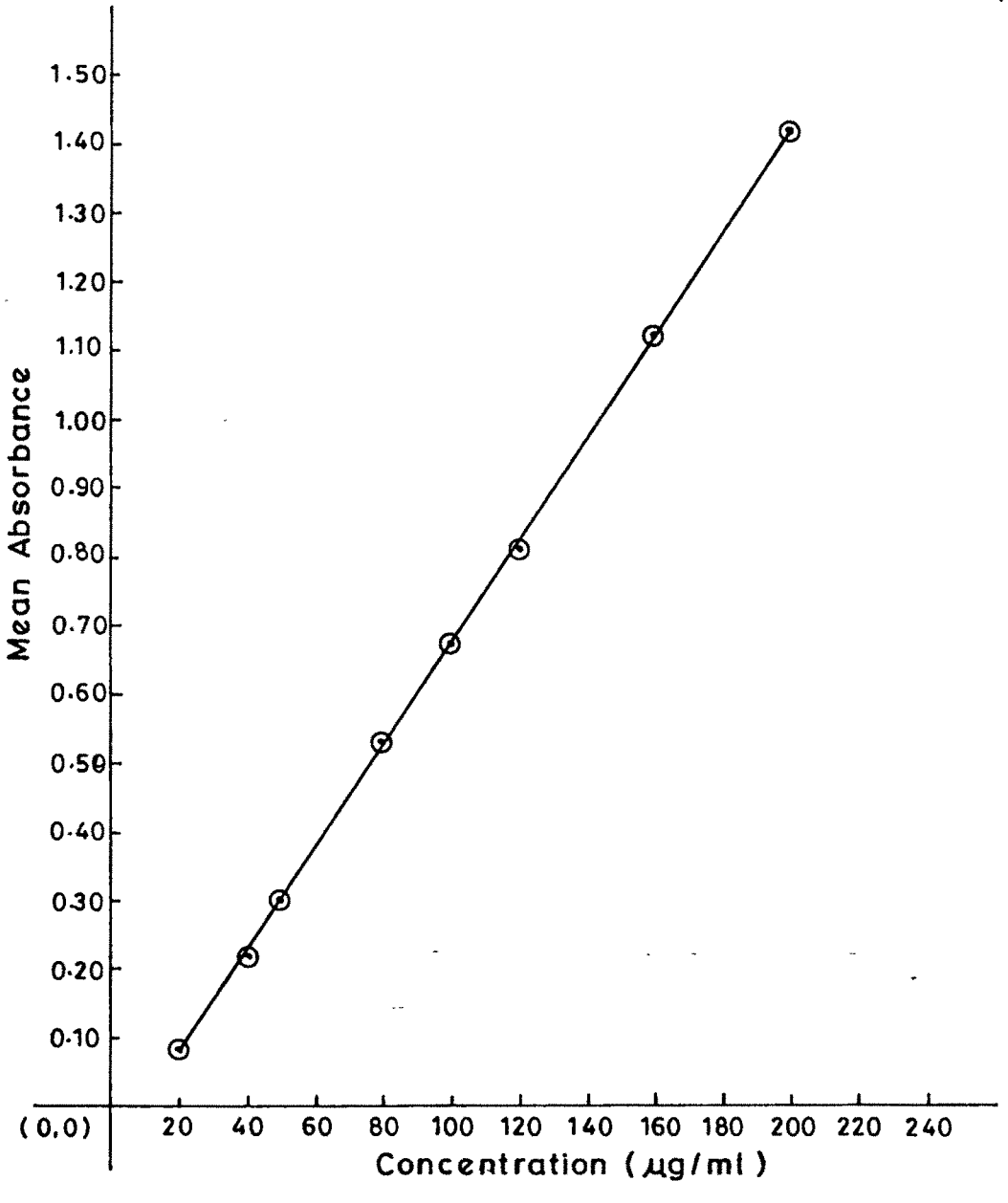


FIG. 2.28 : CALIBRATION CURVE OF CYCLOPHOSPHAMIDE - COBALT THIOCYANATE COMPLEX

TABLE 2.40

FORMULAE FOR SYNTHETIC MIXTURES OF CYCLOPHOSPHAMIDE

Ingredients	Quantity/100 tablets (mg)	
	Formula I (mg)	Formula II (mg)
Cyclophosphamide	2500	2500
Starch	3000	-
Lactose	3250	-
Polyvinyl pyrrolidone	1000	-
Dicalcium phosphate	-	3000
Avicel	-	3250
Ethyl cellulose	-	1000
Aerosil	50	50
Talc	100	100
Magnesium stearate	100	100

TABLE 2.41

ESTIMATION OF CYCLOPHOSPHAMIDE IN INJECTION, SYNTHETIC MIXTURES AND TABLETS.

Dosage form	Reported Method		Proposed analytical methods % Recovered (± S.D.)		
	Conc.in aliquot µg/ml	%Recovered ± .S.D.	Conc.in aliquot µg/ml	Ferrothiocyanate method	Cobalt thiocyanate method
Injection	20	99.92 (0.211)	50	99.575 (0.331)	99.63 (0.295)
Synthetic mixture					
I	20	99.52 (0.195)	50	99.10 (0.215)	99.45 (0.299)
II	20	99.35 (0.293)	50	99.29 (0.327)	99.32 (0.217)
Tablets	20	99.57 (0.217)	50	99.38 (0.279)	99.08 (0.375)

solutions. It may therefore be concluded that both these methods are simple, reproducible and comparable with that of the standard method.

2.43 Reaction of Cyclophosphamide with Picric Acid :

The fact that many organic compounds containing tertiary nitrogen form coloured picrates was exploited to develop a simple colorimetric procedure for estimation of cyclophosphamide.

2.431 Reagents :

a) Stock solution of drug : 50 ml of cyclophosphamide was accurately weighed into a 100ml volumetric flask, dissolved and diluted to volume with chloroform.

b) Picric acid solution : 0.5% w/v solution of picric acid was prepared in chloroform.

2.432 Experimental procedure :

To 2ml of drug solution, 2ml of picric acid solution was added, the solution was thoroughly mixed and noted for formation of any colour.

Yellow colour was formed between cyclophosphamide and picric acid. An attempt was made to quantify this colour reaction.

2.433 Development of colorimetric method for estimation of cyclophosphamide with picric acid :

a) Scanning of the spectrum :

To a suitable aliquot (0.5ml) of drug solutin in a 5ml volumetric flask, 2ml of picric acid solution was added and made to volume with chloroform. The absorbance

of the yellow colour formed was scanned between 350-600 nm against the reagent blank. The wavelength scan is shown in Fig. 2.29.

b) Optimisation of concentration of picric acid solution :

To suitable aliquot (0.5ml) of drug solution in separate 5ml volumetric flasks, 2ml of varying concentrations (0.25, 0.5, 5.0%w/v) of picric acid solution were added, the volume was made up with chloroform and the absorbance of the yellow colour was measured at 410 nm against the reagent blank. The observations are recorded in Table 2.42.

c) Preparation of calibration curve :

Aliquots of drug solution equivalent to 200µg, 400µg, 500µg....2000µg/ml were transferred into separate 5ml volumetric flasks, 2ml of picric acid solution was added and the volume was made up with chloroform. The absorbance of yellow coloured solution in each flask was measured at 410nm against the reagent blank. The observations are recorded in Table 2.43.

d) Estimation of cyclophosphamide in injection :

A quantity of the drug equivalent to 50mg was dissolved in sufficient chloroform to produce 100ml, and then filtered. With a suitable aliquot (0.5ml) of the filtrate, colour was developed as described under preparation of calibration curve.

f) Estimation of cyclophosphamide from synthetic mixtures :

Two synthetic mixtures were prepared, the formula of which is given in Table 2.40. A quantity of the

synthetic mixture equivalent to 25 mg of cyclophophamide was extracted with four quantities each of 5ml of solvent ether, the ether extract was filtered and then evaporated to dryness. The residue was dissolved in 50 ml of chloroform. Colour was developed with a suitable aliquot (0.5ml) of this solution as described under preparation of calibration curve.

(g) Estimation of cyclophosphamide from tablets :

20 tablets were weighed and powdered. A quantity of the powdered mixture equivalent to 25mg of cyclophosphamide was extracted with four quantities each of 5ml of solvent ether. The ether extract was filtered and the filtrate was evaporated to dryness, The residue was dissolved in 50ml of chloroform. Colour was developed with a suitable aliquot of (0.5ml) this solution as described under preparation of calibration curve.

The results of estimation of cyclophosphamide from injection, synthetic mixtures and tablets are recorded in Table 2.44. The percentage recoveries were compared with that of the reported method (section 2.423h).

h) Results and discussion :

When cyclophosphamide was reacted with picric acid, a yellow colour was produced. The absorption spectrum of the yellow coloured solution showed a λ max at 410nm (Fig. 2.29). From Table 2.42 it may be observed that 2ml of 0.5% w/v solution of picric acid was optimum for colour development. The calibration curve was linear in the concentration range of 20-200 µg/ml. The colour formed was stable for several hours.

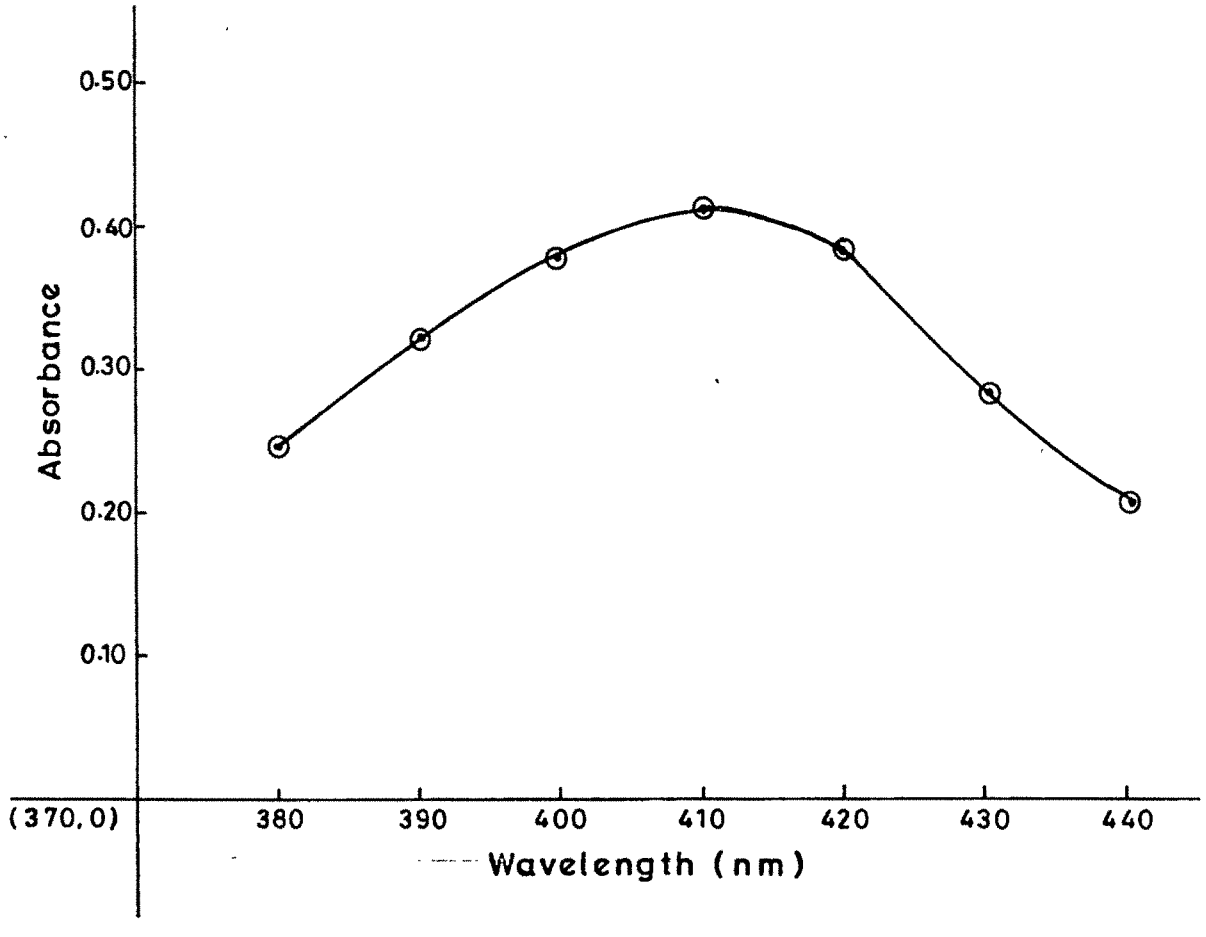


FIG. 2.29 : WAVELENGTH SCAN OF CYCLOPHOSPHAMIDE - PICRIC ACID SOLUTION

TABLE 2.42

OPTIMISATION OF CONCENTRATION OF PICRIC ACID SOLUTION

Drug concentration - 50 µg/ml.
λmax - 410nm

Concentration of picric acid solution %w/v	Absorbance
0.25	0.175
0.5	0.252
1.0	0.252
2.0	0.250
5.0	0.250

TABLE 2.43

CALIBRATION CURVE OF CYCLOPHOSPHAMIDE - PICRIC ACID SOLUTION

Concentration µg/ml	Absorbance (± S.D.)
20	0.082 (0.005)
40	0.198 (0.011)
50	0.252 (0.007)
60	0.301 (0.032)
80	0.403 (0.029)
100	0.492 (0.013)
160	0.791 (0.005)
200	1.015 (0.005)

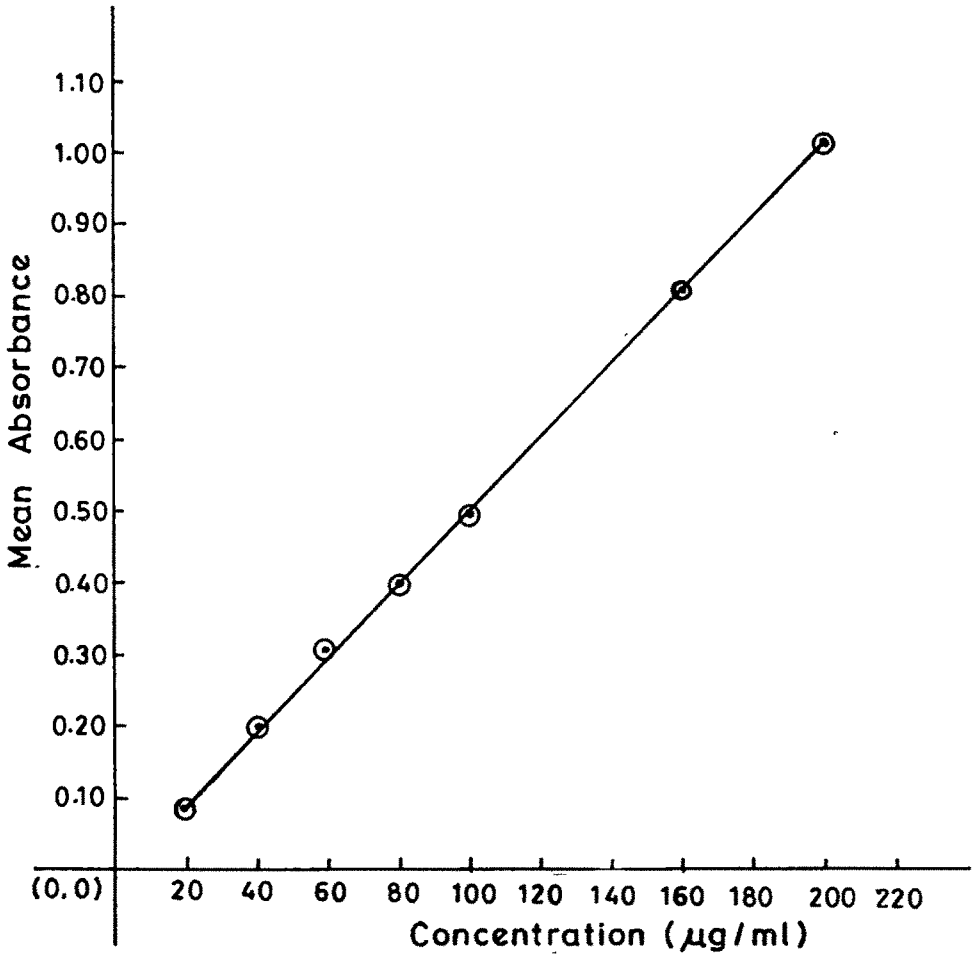


FIG. 2.30: CALIBRATION CURVE OF CYCLOPHOSPHAMIDE-PICRIC ACID SOLUTION

TABLE 2.44

ESTIMATION OF CYCLOPHOSPHAMIDE IN INJECTION, SYNTHETIC MIXTURES AND TABLETS.

Dosage form	Reported Method		Picric acid method	
	Conc.in aliquot µg/ml	%Recovered (± S.D.)	Conc.in aliquot µg/ml	%Recovered (± S.D.)
Injection	20	99.92 (0.211)	50	99.60 (0.259)
Synthetic mixture				
I	20	99.52 (0.195)	50	98.97 (0.527)
II	20	99.35 (0.293)	50	99.21 (0.375)
Tablets	20	99.57 (0.217)	50	99.16 (0.425)

The a and b values for line of regression were found to be -0.0034 and 0.005 respectively. Molar absorptivity value and correlation co efficient 'r' were found to be $1.41x10^3$ $lmol^{-1}$ cm^{-1} and r=0.999 respectively.

The percentage recoveries of cyclophosphamide from injection, synthetic mixtures and tablets were found to lie between 99.16%-99.97% (Table 2.44). These values lie with the pharmacoepial limits (92.5%-107.5%) and were comparable with the reported method (Table-2.41). From the results of estimation of the drug from synthetic mixtures it may be concluded that the commonly used excipients do not interfere with this method of estimation.

On the basis of estimation of cyclophosphamide from various formulations it may be concluded that the method is simple, accurate and precise and does not involve any complicated chemical reaction and therefore can be used for routine analysis of cyclophosphamide.

2.5 FLUORIMETRIC METHOD OF ANALYSIS FOR 5FU

The fluorescence of 5FU has been reported in methanol(11). However, this property of 5FU has not been studied in aqueous medium. Hence an attempt was made to study the fluorescence property of 5FU in aqueous solutions at different pH values in order to develop a simple fluorimetric method for estimation of the drug in various dosage forms.

EXPERIMENTAL

2.51 Equipment and Operating Conditions :

Spectrophotofluorimeter	- Shimadzu RF540
Scan speed	- Fast
Sensitivity	- High
Excitation and emission slit(NM)	- 10.

2.52 Reagents :

(a) Stock solution of 5FU : 100mg of 5FU was accurately weighed into a 100ml volumetric flask, dissolved and the volume was made up with water.

(b) pH solutions (1.2-12) :

About 200ml of water was taken in separate 250ml beakers the pH was adjusted to 1.2,2,4 and 5 with 0.2N hydrochloric acid solution and to 8,9,10 and 12 with 0.2N sodium hydroxide solution.

2.53 Scanning of Fluorescence of 5FU Solution at Different pH Values :

1ml of drug solution was transferred into different 10ml volumetric flasks and the volume was made up with various pH solutions.

1. At each pH value excitation wavelength was fixed at the absorption maxima (266nm) in UV region and emission was varied to determine the optimum wavelength.
2. Optimum emission wavelength found if any in step 1 was fixed and excitation wavelength was varied to determine the effect of the latter on fluorescence intensity.

3. Optimum excitation found in step 2 was fixed and emission wavelength was varied to determine the effect of latter on the fluorescence intensity. All spectra were recorded against suitable reagent blanks.

It was observed that 5FU did not exhibit fluorescence in acidic pH. However fluorescence was observed in pH 9 and above. Maximum fluorescence was exhibited at pH 9, therefore pH 9 was selected for development of a simple fluorimetric method for estimation of 5FU.

2.54 Development of Fluorimetric Method for Estimation of 5FU in Dosage forms :

2.541 Preparation of calibration curve :

Aliquots of drug solution equivalent to 0.5,1,2,....80µg/ml were transferred accurately into separate 10ml volumetric flasks, diluted and made upto volume with pH9 solution. The fluorescence spectra was recorded with excitation wavelength at 300nm and emission wavelength at 400nm against the blank solution. The data of peak height obtained for each concentration is recorded in Table 2.45 and calibration curve is shown in Fig. 2.32.

2.542 Estimation of 5FU from injection :

An aliquot of the injection equivalent to 100mg of 5FU was accurately transferred into a 100ml volumetric flask and made to volume with pH 9 solution. An aliquot of the solution was suitably diluted with pH 9 solution to give final concentration of 5µg/ml. The fluorescence spectra was recorded as described in the preparation of calibration curve.

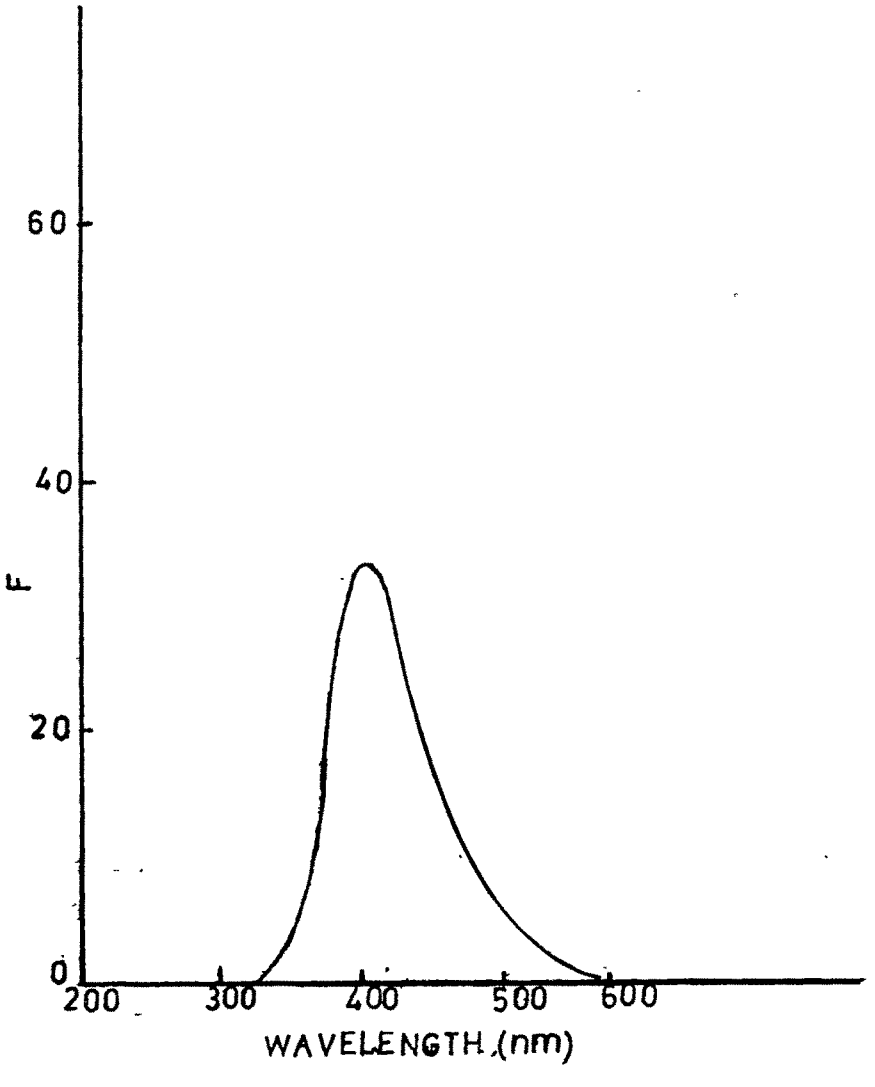


FIG-2·31 WAVELENGTH SCAN OF 5FU BY FLUORIMETRIC METHOD

TABLE 2.45

CALIBRATION CURVE OF 5FU BY FLUORIMETRIC METHOD AT pH 9.0

Conc. in µg/ml	Peak height in cms (± S.D.)
0.05	0.43
0.2	0.51
0.4	0.65
0.5	0.71
1.0	1.15
2.0	1.78
4.0	3.41
5.0	4.01
8.0	6.35

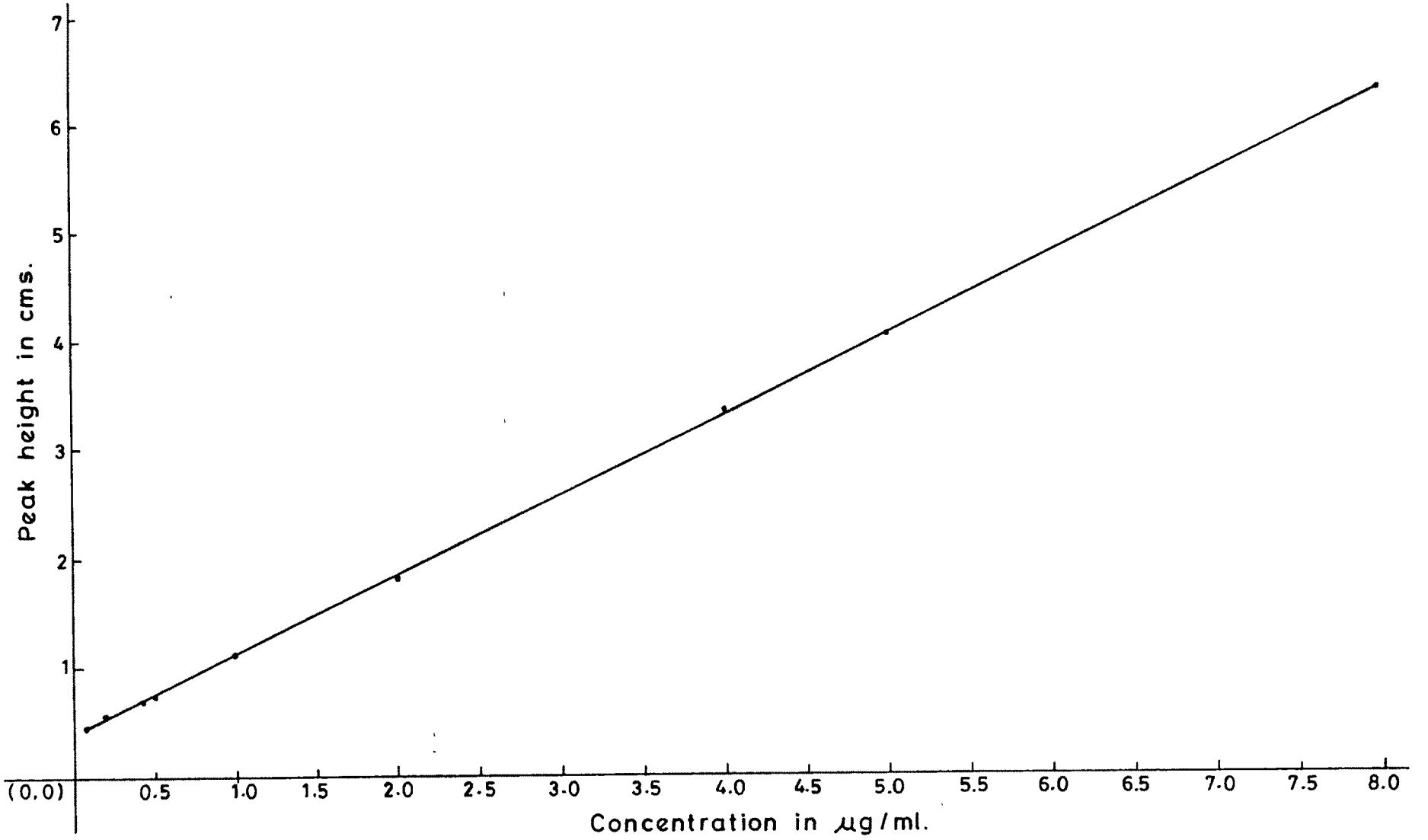


FIG. 2.32: CALIBRATION CURVE OF 5 FU BY FLUORIMETRIC METHOD AT pH 9.0

TABLE 2.46

ESTIMATION OF 5FU FROM INJECTION AND CREAM BY FLUORIMETRIC METHOD

Dosage form	Conc. µg/ml	% Recovered (± S.D.)
Injection	5.0	99.82 (0.795)
Cream	5.0	99.11 (0.821)

2.543 Estimation of 5FU from cream :

A quantity of the cream equivalent to 500mg of 5FU was accurately weighed and the drug was extracted from the cream base as per the U.S.P. method described earlier. A suitable aliquot of the aqueous extract containing 50 µg/ml of 5FU was transferred into a 10ml volumetric flask and diluted to volume with pH 9 solution. The fluorescence spectra was recorded as described in the preparation of calibration curve.

The percentage recoveries of 5FU from injection and cream along with the standard deviation are recorded in Table 2.46.

2.55 Results and Discussion :

5FU exhibited fluorescence at pH 9 solution. The excitation wavelength and emission wavelengths were found to be 300nm and 400nm respectively (Fig. 2.31). The calibration curve (Fig. 2.32) was linear in the concentration range of 0.05ug-8µg/ml. The a and b values for the line of regression were found to be a = 0.0042 and b = 0.0017. The correlation coefficient was found to be r = 0.995.

From Table 2.46 it can be observed that the percentage recoveries of 5FU from injection and cream were within the range of 99.11-99.82%. These values were found to be comparable with those obtained by I.P. and U.S.P. methods respectively.

Hence it may be concluded that the proposed fluorimetric method is simple, reproducible and sensitive.

2.6 HPLC METHOD FOR ANALYSIS OF 5FU

A number of HPLC methods have been reported for the estimation of 5FU from (chapter-1, Table 1.1) dosage forms, plasma and other body fluids. The reported methods use systems like water, water-acetonitrile, water-methanol (pH adjusted with buffers) and various buffers. However some of these procedures are lengthy. Recently, a stability indicating method was reported for 5FU which involves the use of tetrabutyl ammonium hydroxide as mobile phase (12). Since columns and pumps are damaged by prolonged use of buffers and other corrosive reagents; it was proposed to develop a simple solvent system for estimation of 5FU from bulk, dosage forms, bodyfluids and for accelerated stability studies.

2.61 Experimental :

2.611 Materials :

Methanol, HPLC grade (SRL, India.), water (double distilled and filtered through 0.45µm filter), thymine A.R grade (Qualigens, India).

2.612 Equipment :

Liquid chromatograph	-	Waters HPLC.
Column	-	Bondapak C_{18}.
Detector	-	Waters 440 fixed wavelength (254nm.)
Integrator	-	Waters 746 Data Module.

2.613 Reagents :

a) Preparation of mobile phase : A freshly degassed solution of water and methanol(80:20) was prepared.

b) Internal standard solution : 50mg of thymine was accurately weighed into a 50ml volumetric flask dissolved and made up to volume with the mobile phase.

2.614 Procedure :

Operating conditions : The liquid chromatograph was equipped with a fixed wavelength UV detector at 254nm. The flow rate was 1ml per minute (pressure 1000psi). Chart speed was 0.5 mm/min. The attenuation was fixed at 4,128,512 and 1024 in the concetration range of 0.05-1.0,1.0-20,20-50,50-200 µg/ml respectively.

Aliquots of the drug solution equivalent to 0.5µg, 1µg...2000µg/ml were accurately transferred into separate clean 10ml volumetric flasks. An aliquot of the internal standard solution equivalent to 100µg/ml was added into each flask and the volume was made up with mobile phase. A 20µl aliquot of each of these solutions were injected into the injection port and the chromatograms were recorded. Each solution was injected thrice and their chromatograms were recorded. The ratio of peak areas of the drug to that of the internal standard for each sample was calculated and the data is recorded in Table 2.48 and shown in Fig. 2.33.

2.615 Estimation of 5FU from injection :

An aliquot of the injection equivalent to 100mg of 5FU was transferred into 100ml volumetric flask, diluted and made up to volume with mobile phase.

An aliquot of this solution equivalent to 100µg/ml of 5FU was accurately transferred into a 10ml volumetric flask, spiked with 100µg/ml of internal standard solution and made

upto volume with the mobile phase. A 20µl aliquot of this solution was injected into the injection port and the chromatogram was recorded. The ratio of peak area of the sample to that of the internal standard was calculated and the concentration of the drug was determined from the calibration curve.

2.616 Estimation from cream :

A 5% w/w cream of 5FU was prepared in PEG base.

A quantity of the cream equivalent to 500mg of drug was accurately weighed and the drug was extracted from the cream base by the U.S.P method as described under estimation of 5FU by copper acetate method (chapter 2.2, section 2.223e). The ethyl acetate-isopropanol (7:3) mixture containing drug was evaporated on a water bath and the residue was dissolved in the mobile phase. Other operations were carried out as described under estimation of 5FU from injection starting from words "An aliquot of this solution equivalent to 100µg/ml of 5FU...".

The percentage recoveries of 5FU from injection and cream are recorded in Table 2.63.

2.617 Stability studies of 5FU :

Accelerated stability studies for 5FU was carried out at pH10 and at different temperatures viz. 60°C, 45°C and 25°C.

About 100mg of 5FU was accurately weighed out into a 50ml volumetric flask. The drug was dissolved in and diluted to volume with pH10 borate buffer. The solution was filtered and the initial concentration was determined by the procedure

given under estimation of 5FU from injection. 5ml of the filtered solution was filled in clean, amber coloured glass ampoules, the ampoules were sealed and stored at 60°C, 45°C, and 25°C. Samples were withdrawn at 1,3,7,15,30,60 and 90 days interval and the concentration of the drug was determined in triplicate. The chromatographic peaks at each sampling time are shown in Fig. 2.34. The data of percentage drug remaining at each sampling point is recorded in Table 2.50. The first order rate constant 'K' was calculated for each temperature from plot of log% drug remaining vs time (Fig. 2.35). The t1/2 and t90 were calculated from K values. The K values, t1/2 and t90 values are recorded in Table 2.51. The data obtained by HPLC method was statistically compared by student's 't' test with that of UV method reported in chapter 2.2 (section 2.223e).

2.62 Results and Discussion :

a) Optimisation of solvent system :

The retention times for the various ratios of water : methanol system which were tried as the mobile phase are given in Table 2.47.

From the data given, it can be observed that 80:20 of water : methanol system gave a satisfactory retention time (3.38) for 5FU and a good resolution between the drug and internal standard.

b) Linearity :

From Table 2.48 and Fig. 2.33 it may be observed that the calibration curve is linear in the concentration

TABLE 2.47

RETENTION TIMES OF 5FU AND THYMINE IN VARIOUS MOBILE PHASES

Mobile phase	Retention time	
	5FU	Thymine
Water	6.20	7.74
Water : MeOH		
80 : 20	3.38	4.49
60 : 40	3.24	4.18
50 : 50	3.01	3.88
40 : 60	2.93	3.78
20 : 80	2.82	3.63
MeOH	2.75	3.55

TABLE 2.48

CALIBRATION CURVE OF 5FU BY HPLC METHOD

Concentration of internal standard (thymine) =10µg/ml
Mean area of the peak of thymine - 1016049

Conc. in aliquot µg/ml	Mean peak area of 5FU(±S.D.)	Ratio of the peak of 5FU to that of thymine
0.05	4369.00 (0.0052)	0.0043
0.1	9246.00 (0.0073)	0.0091
0.2	18593.00 (0.0095)	0.0183
0.4	34740.00 (0.0021)	0.0342
0.5	42466.00 (0.0067)	0.0418
0.8	66246.00 (0.0081)	0.0652
1.0	92275.00 (0.017)	0.0908
2.0	175320.00 (0.135)	0.173
4.0	339082.00 (0.112)	0.334
5.0	434557.00 (0.097)	0.428
10.0	874005.00 (0.213)	0.860
20.0	1738920.00 (0.019)	1.711
40.0	3763721.00 (0.075)	3.704
50.0	4925651.00 (0.235)	4.848
100.0	9262852.00 (0.035)	9.117
200.0	18414475.00 (0.097)	18.124

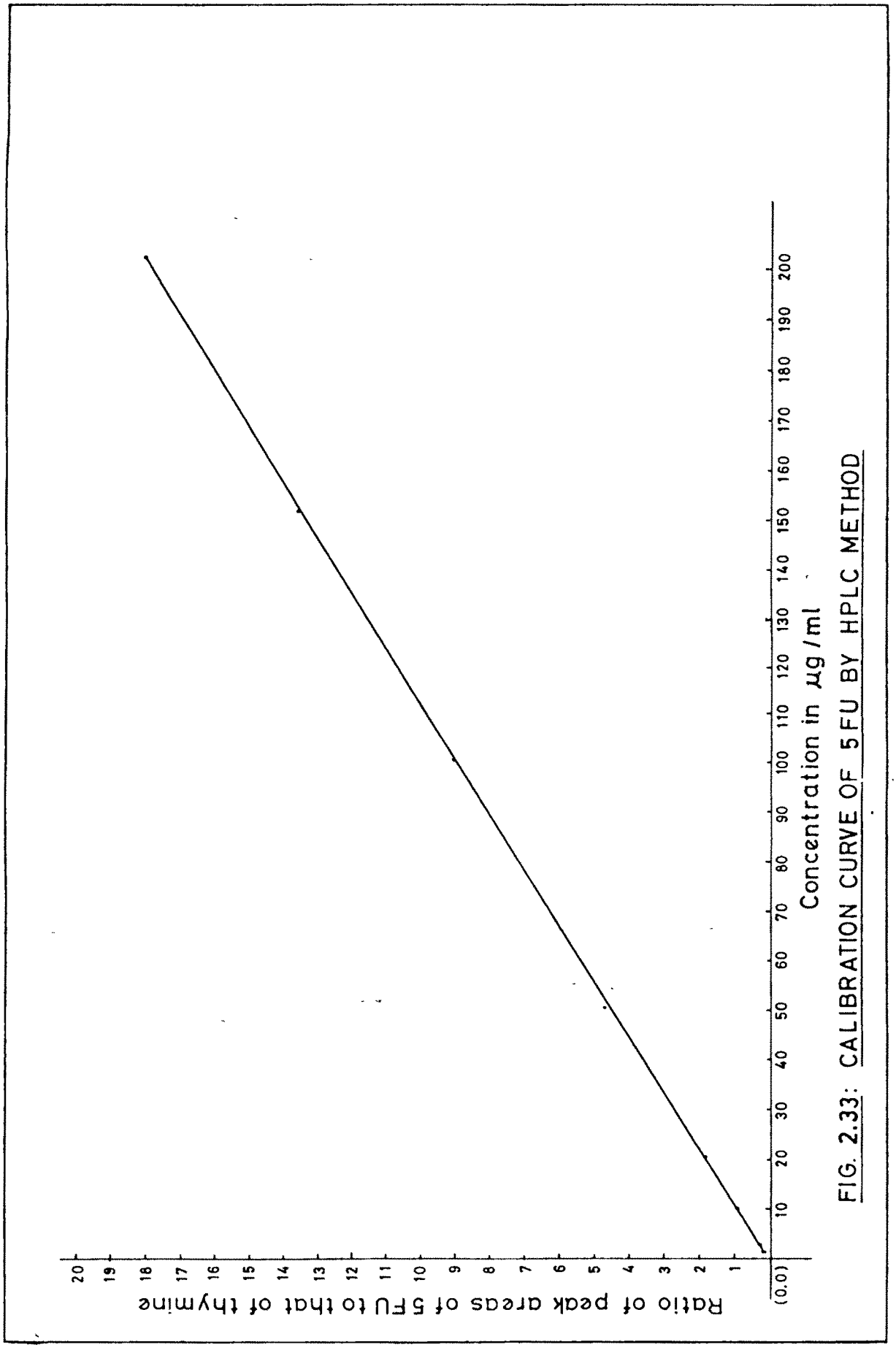


FIG. 2.33: CALIBRATION CURVE OF 5FU BY HPLC METHOD

TABLE 2.49

ESTIMATION OF 5FU FROM INJECTION AND CREAM BY HPLC METHOD

Dosage form	Concentration in aliquot µg/ml of 5FU	% Recovered (± S.D.)	
		UV spectrophoto metric method λmax = 266nm	HPLC method
Injection (50mg/ml)	10.0	100.38 (0.543)	99.69 (0.625)
Cream (5 % w/w)	10.0	99.31 (0.796)	99.00 (0.479)

FIG. 2.34 STABILITY CHROMATOGRAMS OF 5FU BY HPLC METHOD (AT pH 10.0)

TABLE 2.50

STABILITY DATA FOR 5FU AT pH 10.0 BY HPLC METHOD.

Time in days	% drug remaining undergraded (± S.D)		
	Room temp.	45°C	60°C
0	100.00 (-)	100.00 (-)	100.00 (-)
1	99.95 (0.195)	99.65 (1.065)	98.12 (0.935)
3	99.92 (1.017)	99.25 (1.002)	95.92 (1.082)
7	99.85 (0.997)	98.01 (1.732)	91.59 (0.993)
15	99.69 (1.472)	95.59 (2.013)	83.55 (0.775)
30	99.32 (0.875)	93.01 (1.117)	70.54 (1.345)
60	98.65 (2.011)	86.42 (0.232)	49.15 (0.965)
90	98.07 (0.979)	80.79 (0.777)	34.49 (1.132)

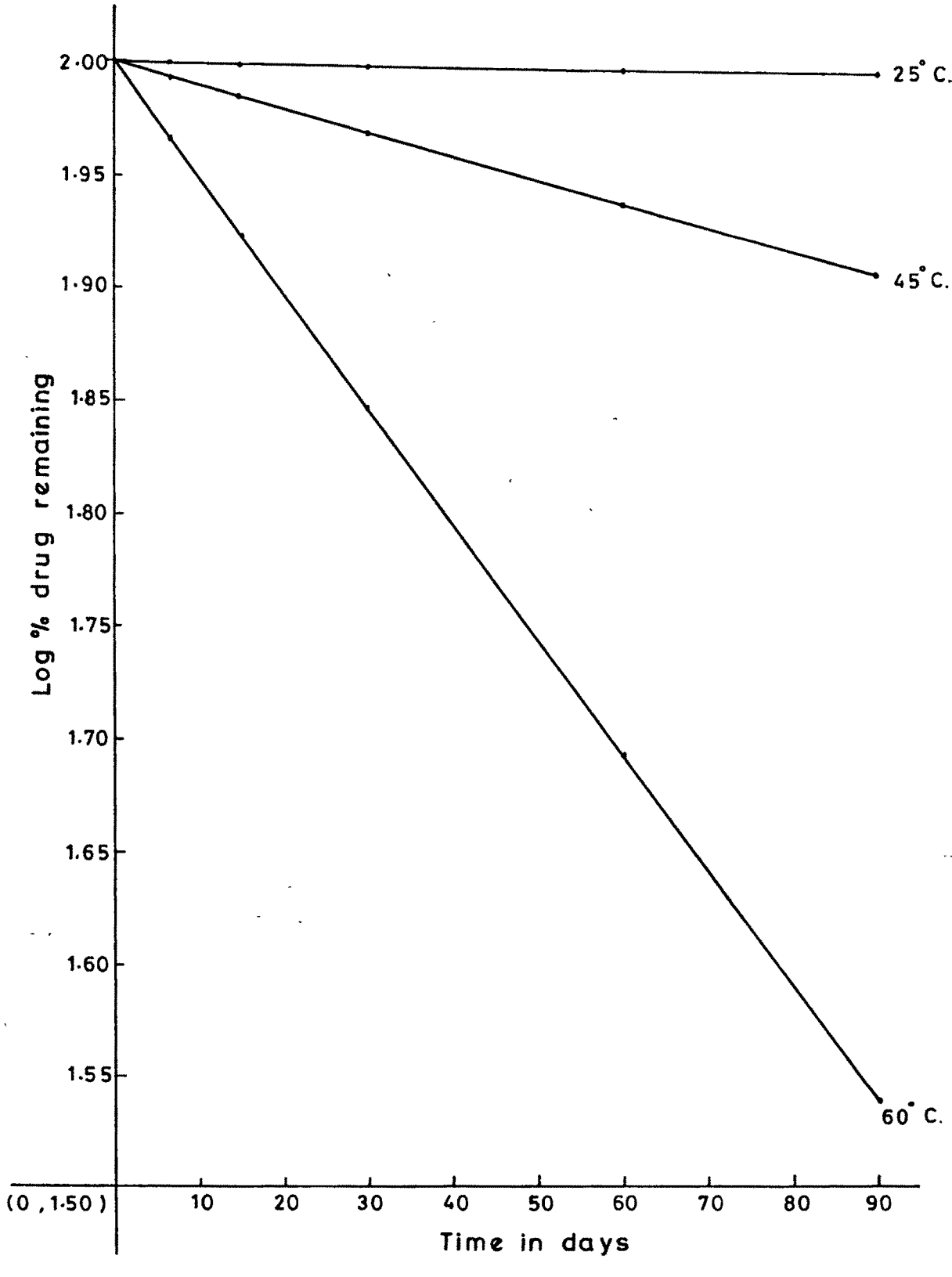


FIG. 2.35: LOG % DRUG REMAINING VS TIME PLOT OF 5 FU AT pH 10 BY HPLC METHOD

TABLE 2.51

STABILITY PARAMETERS (K, t 1/2, t90) OBTAINED FOR 5FU AT VARIOUS TEMPERATURES BY HPLC METHOD

Temperature ^{o}C	K s^{-1}	t 1/2 days	t_{90} days
60	1.31×10^{-7}	61.23	9.276
45	2.53×10^{-8}	317.03	48.030
25	1.87×10^{-9}	4289.22	649.880

range of 0.05µg - 200µg/ml. The a and b values for the line of regression y=a+bx were calculated and found to be a = 0.004 and b=0.091. The correlation coefficient was found to be r=0.999.

c) Estimation of 5FU from injection and cream :

From Table 2.49 it can be observed that the percentage recoveries of 5FU from injection and cream were found to be in the range of 99.01% and 99.697%. These values were statistically comparable with those obtained by the standard UV spectrophotometric method.

d) Data of stability studies :

From Table 2.50 and Fig. 2.35 it may be inferred that no statistically significant difference exists in the degradation patterns obtained by the HPLC and standard spectrophotometric method. The stability rate constant (K), t 1/2 and t90 values (Table 2.51) obtained by the HPLC method and the standard spectrophotometric method are statistically similar ($P < 0.05$).

The activation energy constant (Ea) was determined from the slope of the Arrhenius plot. The Ea value obtained by HPLC method was compared with that of UV method and they were found to be comparable.

On the basis of analysis of the stability data and the data for estimation of drug from formulations, it may be concluded that the method is stability indicating, reproducible and comparable with the pharmacoepial methods.

2.7 ESTIMATION OF 5FU, MTX AND CYCLOPHOSPHAMIDE IN BODY FLUIDS BY PROPOSED ANALYTICAL METHODS

2.71 Estimation of 5FU in Human Plasma and Rat Blood by the Proposed Analytical Methods :

5FU was estimated in human plasma and rat blood by the following analytical methods to determine their applicability in estimating the drug in blood and other body fluids.

i) 5FU-metal complexation (copper acetate method)

ii) 5FU-diazotised primary amines (sulfanilic acid)

iii) 5FU-Hg-diphenyl carbazone method.

iv) Fluorimetric method.

v) HPLC method.

2.711 Experimental :

a) From human plasma :

Human plasma was obtained from Blood Bank (Sri Sayaji Government Hospital, Baroda). 25ml of the plasma was spiked with 25 mg of 5FU and the mixture was thoroughly shaken. Proteins were precipitated with 3% v/v trichloroacetic acid - 10%v/v sulphuric acid mixture (100:6) (13) and the mixture was centrifuged for 5 minutes. The clear supernatant was analysed for 5FU content by the procedures given under methods of analysis of 5FU from body fluids. Sample estimation were carried out in triplicate.

b) From rat blood :

Wistar rats of either sex having an average weight of 250g were fasted overnight. They were anaesthetised with

pentobarbitone sodium (40mg/kg, i.p) and the femoral vein was exposed by careful dissection. 5FU, 50mg/kg, body weight (14) was injected into the femoral vein and 1ml of blood was withdrawn at 5,15, 30 and 60 minutes from the tail vein. The proteins were precipitated from the samples by trichloroacetic acid-sulphuric acid mixture (100:6), centrifuged for 5 minutes and the clear supernatant was analysed for 5FU content by the procedure given under methods of analysis of 5FU from blood. For each method three rats were used and the sample analysis carried out in triplicate.

2.712 Method of estimation of 5FU in blood by the proposed analytical methods :

a) Copper acetate method :

100µl aliquot of human plasma and 500µl of rat plasma were transferred into separate 5 ml volumetric flasks using a micropipette and made up to volume with normal saline. In each case the drug was extracted from the entire 5ml with ethylacetate-isopropanol mixture as described earlier in section 2.223e. The organic extract containing the drug was evaporated, the residue was dissolved in 2ml of $CHCl_3$: MeOH (3:2) mixture and colour was developed with this solution as described under preparation of calibration curve by copperacetate method (Section 2.223.d). The absorbance of the coloured solutions were measured against appropriate plasma blank treated similarly.

b) Diazotised sulfanilic acid method :

500µl aliquots of human plasma and rat plasma after removal of proteins were transferred into separate 10ml volumetric flasks and colour was developed by the procedure described under preparation of calibration curve with, diazotised primary amines (section 2.233e). The colour developed was measured against the plasma blank treated similarly.

c) Diphenyl carbazone method :

500µl aliquots of proteins precipitated human plasma and rat plasma were transferred into different 60ml separating funnels, pH of the solution was adjusted to 8 with 0.01N sodium hydroxide solution and colour was developed as described under preparation of calibration curve by the diphenyl carbazone method (Section 2.243.d). The absorbance of the coloured solutions were measured against the plasma blank treated similarly.

d) Fluorimetric method :

100µl aliquot of protein removed human plasma and 500µl aliquot of rat plasma were transferred into separate 10ml volumetric flasks, pH of the solution was adjusted to 9 with 0.01N sodium hydroxide solution and made upto volume with the same. Fluorescence of the solution was measured against the plasma blank as described under preparation of calibration curve by fluorimetric method. (Section 2.541)

e) HPLC method :

100µl aliquot of human plasma and 500µl of rat plasma after removal of proteins were transferred into separate

10ml volumetric flasks, an aliquot of internal standard solution (thymine) was added, volume was made up with mobile phase i.e., water : methanol (80:20) and the chromatogram was recorded as described under preparation of calibration by HPLC method (Section 2.614.b)

f) UV method :

100µl aliquot of human plasma and rat plasma after removal of proteins were transferred into separate 10ml volumetric flasks, the volume was made up with pH 7.4 phosphate buffer and the absorbance was measured at 266nm against plasma blank.

2.713 Results and discussion :

a) From human plasma:

The mean percentage recovery of 5FU obtained from human plasma by different methods are recorded in Table 2.52. The percentage recoveries were obtained between 98.91%-99.72% for all the methods. These values were comparable with that of the UV method . From the data it may also be concluded that the plasma components do not interfere with any of the proposed analytical methods.

b) From rat plasma :

The concentration of 5FU obtained from rat plasma at various time intervals by different methods are given in Table 2.53.

From the data obtained it may be observed that blood levels of 5FU in rats at the given dose can be detected by HPLC, fluorimetric and copper acetate methods upto 60 minutes, by UV method upto 30 minutes. Blood levels in

TABLE 2.52

MEAN PERCENTAGE OF 5FU RECOVERED FROM HUMAN PLASMA BY VARIOUS METHODS.

Method	Conc. in aliquot (µg/ml)	Mean % recovered (± S.D.)
Copper acetate	10.0	99.23 (0.323)
diazotised sulfanilic acid	50.0	98.91 (0.254)
diphenyl carbazone	50.0	99.29 (0.312)
fluorimetric	10.0	99.95 (0.272)
HPLC method	10.0	99.72 (0.277)
UV method	10.0	99.91 (0.213)

TABLE 2.53

CONCENTRATION OF 5FU IN RAT PLASMA OBTAINED BY VARIOUS METHODS AT VARIOUS TIME INTERVALS.

Time in minutes	Concentration of 5FU in µg/ml (± S.D)			
	Method			
	Copper acetate	fluorimetric	HPLC	UV method
5	52.95 (0.251)	55.30 (0.352)	55.05 (0.275)	53.80 (0.319)
15	34.20 (0.301)	35.05 (0.311)	35.91 (0.209)	32.51 (0.214)
30	23.05, (0.305)	23.97 (0.319)	24.10 (0.229)	22.09 (0.271)
60	11.72 (0.312)	11.78 (0.219)	12.85 (0.207)	-

Note : Blood level of 5FU in rats could not be detected by diazotised sulfanilic acid method and diphenyl carbazone method.

rat could not be measured by diphenyl carbazone method and diazotised sulfanilic acid method at any time interval.

From this study it is observed that the pharmacokinetics of 5FU in laboratory animals like rats can be studied by using the proposed HPLC, fluorimetric and copper acetate methods.

2.72 Estimation of MTX in Human Plasma by the Proposed Analytical Methods :

MTX was estimated from human plasma by the following analytical methods :

i. Folin-ciocalteau method.

ii. Nessler's reagent method.

iii. Nitric acid method.

2.721 Experimental :

25ml of plasma was spiked with 25mg of purified MTX and the mixture was thoroughly shaken. Proteins were precipitated with 3% v/v trichloro acetic acid - 10% v/v sulphuric acid mixture (100:6) and the mixture was centrifuged for 5 minutes. The clear supernatant was analysed for drug content by the procedure given under the method of analysis of MTX from human plasma (given below). A plasma blank sample was prepared similarly. The estimations were carried out in triplicate.

2.722 Method of estimation of MTX in human plasma by proposed analytical methods :

a) Folin-ciocalteau method and Nessler's method :

100µl aliquot of the supernatant solution was transferred into separate 10ml volumetric flasks, pH of

the solution in each case was adjusted to 7 with 0.01N sodium hydroxide solution and colour was developed as described under preparation of calibration curve by Folin-ciocalteau method and Nessler's method respectively. The absorbance was measured against plasma blank treated similarly.

b) Nitric acid method :

A chromogenic reaction was carried out by taking an aliquot (100µl) as described under the method for preparation of calibration curve by nitric acid method (section 2.353.e). The absorbance was measured against plasma blank treated similarly.

c) UV method :

100µl aliquot of the sample was transferred into 10ml volumetric flask, made upto volume with pH 7.4 phosphate buffer and the absorbance was measured at 305 nm against plasma blank.

2.723 Results and discussion :

The mean percentage recovery of MTX obtained from human plasma by different methods are recorded in Table 2.54. The percentage recoveries were obtained between 99.01% - 99.92% for all methods. These values were comparable with that of the UV method. From the above data it may be concluded that the plasma components do not interfere with any of the proposed analytical methods.

TABLE 2.54

MEAN PERCENTAGE RECOVERY OF MTX FROM HUMAN PLASMA

Method	Conc. in aliquot µg/ml	% Recovered (± S.D.)
Folin ciocalteau method	10	99.90 (0.195)
Nessler's method	10	99.01 (0.232)
Nitric acid	10	99.81 (0.352)
UV method	10	99.92 (0.211)

2.73 Estimation of Cyclophosphamide in Human Plasma by the Proposed Analytical Methods :

Cylophosphamide was estimated from human plasma by the following analytical methods :

i. Ammonium ferrothiocyanate method

ii. Cobaltthiocyanate method.

iii. Nitroso method.

2.731 Experimental :

25ml of the plasma was spiked with 25mg of cyclophosphamide and the mixture was shaken well. Proteins were precipitated with trichloroacetic acid - sulphuric acid mixture and the mixture was centrifuged for 5 minutes. The clear supernatant was analysed for drug content by the procedure given under method of analysis of cyclophosphamide from plasma. The sample analysis was carried out in triplicate. Plasma blank was also treated similarly.

2.732 Method of analysis of cyclophosphamide from human plasma :

i. Ammonium ferrothiocyanate and cobaltthiocyanate method :

A common procedure was adopted for both the methods. 500µl aliquot of the clear supernatant solution was transferred into different 60ml separating funnels, pH of the solution was adjusted to 7 with 0.01 N sodium hydroxide solution and colour was developed as described under preparation of calibration curve by ammonium ferrothiocyanate and cobaltthiocyanate method (section 2.423, d). Absorbance in each case was measured against plasma blank.

TABLE 2.55

MEAN PERCENTAGE RECOVERY OF CYCLOPHOSPHAMIDE FROM HUMAN PLASMA BY DIFFERENT METHODS

Method	Conc. in aliquot µg/ml	% recovered (± S.D.)
Ammonium ferro thiocyanate method	50	99.95 (0.145)
Cobalt thiocyanate method	50	98.95 (0.211)
Nitroso method	50	99.15 (0.138)

ii. Reported Nitroso Method :

500µl of the plasma after removal of proteins was transferred into a 10 ml volumetric flask and colour was developed as described in chapter 2.4 (Section 2.423 h). The absorbance of the coloured solution was read against a reagent blank treated similarly.

2.733 Results and discussion :

The mean percentage of drug recovered by different method is recorded in Table 2.55. The percentage recoveries obtained by the proposed analytical methods were comparable with the reported method.

From the above data it may be concluded that the plasma components do not interfere with any of the proposed analytical methods.

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