CHAPTER 3

RESULTS AND DISCUSSION

3.1 Determination of Diphenhydramine Hydrochloride by Spectrophotometric Flow Injection Analysis

The determination of diphenhydramine hydrochloride based on solvent extraction into chloroform of the ion-pair compound formed with bromocresol green in the buffer pH 3 solution. The excess bromocresol green in the aqueous phase is determined with FIA .

3.1.1 Absorption Spectra

The absorption spectra of the extract were investigated. Extraction was carried out by mixing 5 ml of diphenhydramine hydrochloride in different concentrations [range 3.1-15.4 p.p.m.] with 5 ml of 1.32×10^{-3}M bromocresol green solution and the DPH-BCG ion-pair compound was extraced into 10 ml of chloroform in 20 ml-vial for 1 min as following the study of Matsui and French [25].

The spectra of DPH-BCG ion-pair compound in chloroform layer and the excess bromocresol green in aqueous layer were recorded from 220 to 600 nm and from 220 to 800 nm for the aqueous layer after adjusting to be alkaline. The maximum absorption wavelength is at 415 nm of the DPH-BCG ion-pair compound in chloroform layer, at 435 nm of the excess bromocresol green in aqueous layer [pH 3] and at 615 nm of the excess bromocresol green in alkaline solution as shown in Figure 3.1, 3.2 and 3.3. The linear regressions, correlation coefficients and molar absorptivities obtained from the absorption in various conditions mentioned above are illustrated in Table 3.1 and Figure 3.4. The results show that the DPH-BCG ion-pair compound in chloroform layer gives the best absorption due to the highest molar absorptivity, 2.7×10^{4}cm^{-1}M^{-1}. [For detailed calculation, see Appendix B] In spite of the suitable condition for FIA system, the aqueous layer is considered. Thus the excess bromocresol green in aqueous layer after adjusting to be alkaline is selected condition for the determination of diphenhydramine hydrochloride since giving better molar absorptivity than the one without adjusting. The molar absorptivities of the former and the latter are 5.3×10^3 and 2.8×10^3 $cm^{-1}M^{-1}$, respectively.

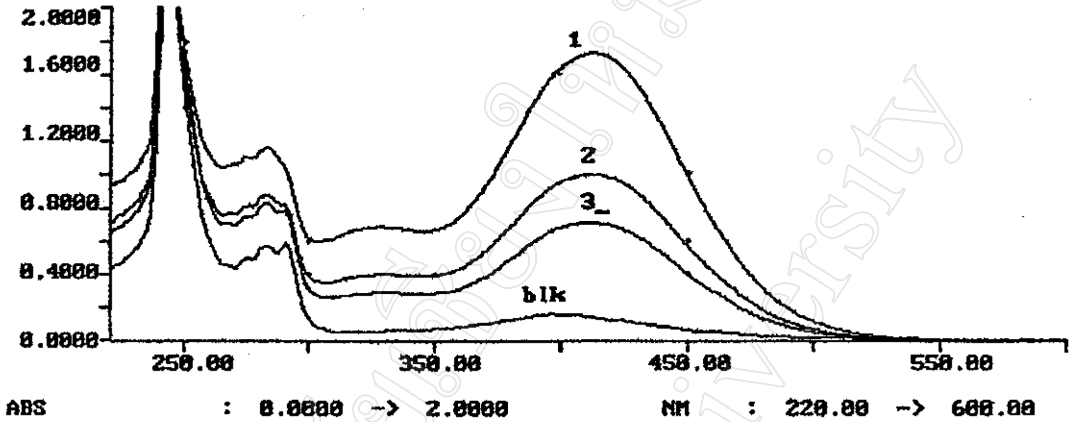

Figure 3.1 Absorption spectra of DPH-BCG ion-pair compound in chloroform layer. Diphenhydramine hydrochloride concentrations: [blank], 0; [1], 15.4 ppm; [2], 9.2 ppm; and [3], 6.2 ppm Bromocresol green concentration: 1.32×10^{-3}M.

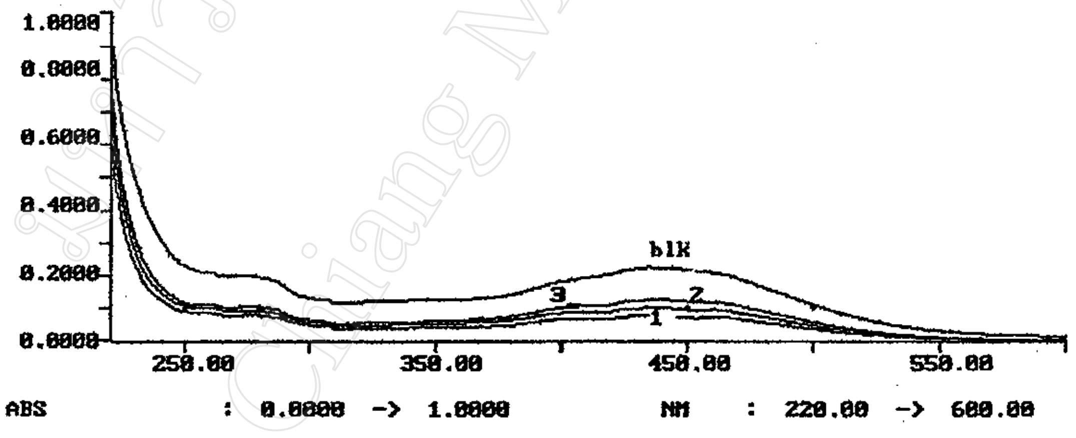

Figure 3.2 Absorption spectra of the excess bromocresol green in aqueous layer [pH 3]. Diphenhydramine hydrochloride concentrations: [blank], 0; [1], 15.4 ppm; [2], 9.2 ppm; and [3], 6.2 ppm. Bromocresol green concentration: 1.32×10^{-3}M.

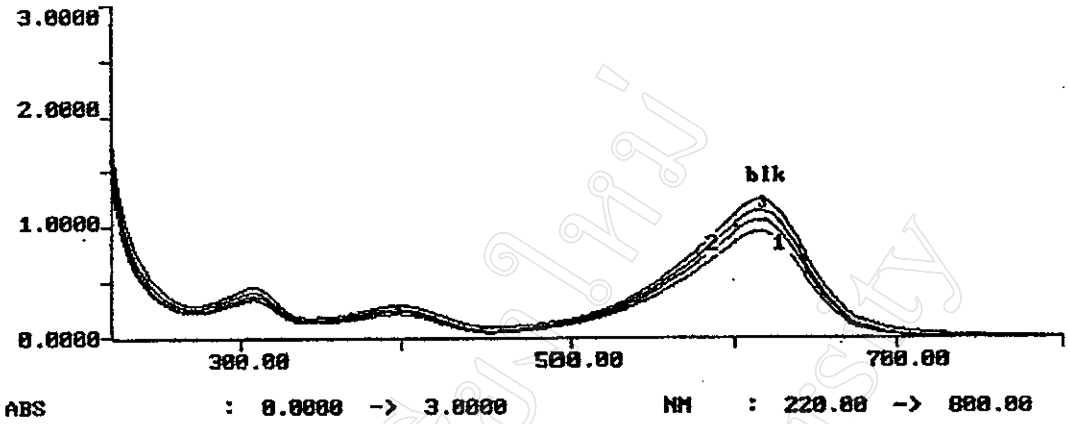

Figure 3.3 Absorption spectra of the excess bromocresol green in aqueous layer after adjusting to be alkaline. Diphenhydramine hydrochloride concentrations: [blank],0; [1], 15.4 ppm; [2], 9.2 ppm; and [3], 3.1 ppm. Bromocresol green concentration: 1.32x10^{-3}M.

Table 3.1 Absorption of the series of diphenhydramine hydrochloride [0-15.4 ppm] obtained from various conditions.

DPHH [ppm]	Absorbance		
	Conditions		
	$CHCl_3$ at 415 nm	aq. at 435 nm	Alkalined aq. at 615 nm
0	0.1602	0.2270	1.2387
3.1	-	-	1.1403
6.2	0.6660	0.1247	-
9.2	0.9234	0.1007	1.0526
15.4	1.5812	0.0778	0.9453
Y = ax + b	Y = 0.092x + 0.125	Y = -0.009x + 0.207	Y = -0.018x + 1.220
R^2	0.994	0.878	0.980
Molar absorptivity [cm^{-1} M^{-1}]	2.7x10^4	2.8x10^3	5.3x10^3

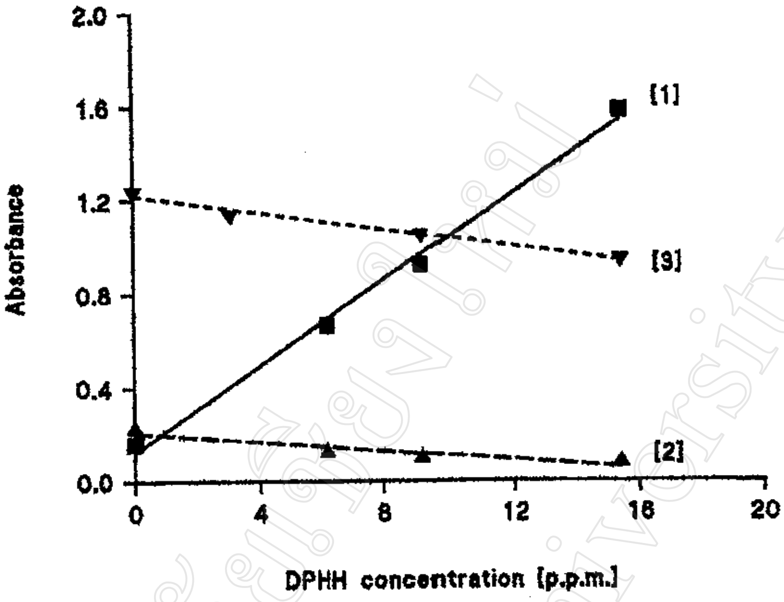

Figure 3.4 Study of various conditions on absorption; [1] $CHCl_3$ layer at 415 nm, [2] aqueous layer at 435 nm, [3] basicified aqueous layer at 615 nm.

3.1.2 Study of extraction time

The absorbances of the excess bromocresol green in aqueous layer after extraction and adjusting the solution to be alkaline were measured at 615 nm.

The extraction was carried out by mixing 5 ml of different dipenhydramine hydrochloride concentrations [4.4-22.1 ppm], 5 ml of 1.04×10^{-4}M bromocresol green solution and 5 ml of chloroform in 20 ml-vial for various extraction times. The results are shown in Table 3.2 and Figure 3.5. From the results, the absorption obtained in various extraction time have slight difference. In order to complete the extraction and shorten the analysis time, 1 min. is considered for optimum extraction time.

Table 3.2 Absorption of the series of diphenhydramine hydrochloride [4.4-22.1 ppm] obtained in various extraction times.

DPHH [ppm]	Absorbance		
	Extraction time		
	0.5 min	1 min	2 min
4.4	0.3688	0.3864	0.3815
8.8	0.2958	0.3156	0.3129
13.2	0.2268	0.2555	0.2389
17.7	0.1643	0.1998	0.1765
22.1	0.1147	0.1328	0.1170
$Y = ax + b$	$Y = 0.014x + 0.425$	$Y = -0.014x + 0.444$	$Y = -0.015x + 0.444$
R^2	0.996	0.998	0.998

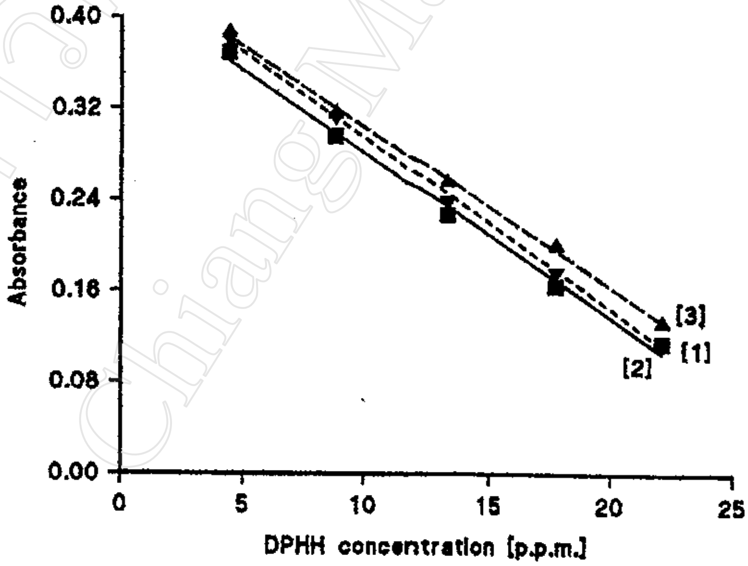

Figure 3.5 Study of extraction time; [1] 0.5 min, [2] 1 min and [3] 2 min

3.1.3 Manifold

A simple flow diagram of the system is shown in Figure 3.6.

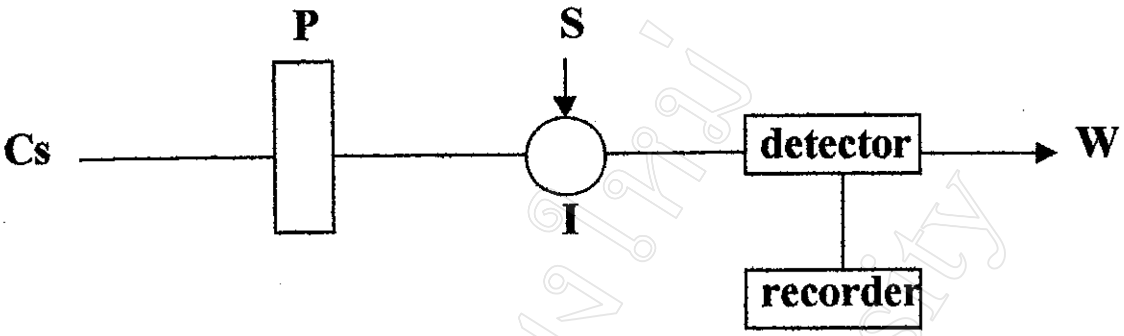

Figure 3.6 Flow diagram of the system; CS:carrier solution, P:peristaltic pump, I:six-port injection valve, S:sample, W:waste

3.1.4 Optimization of Flow Injection Determination of Diphenhydramine Hydrochloride

Preliminary conditions were used as following:

Flow rate of carrier solution	5.0 ml/min
Sample volume (teflon tube)	100 µl
Mixing coil length (teflon tube, i.d.= 0.8 mm)	50 cm
Flow through cell volume	80 µl (1 cm path length)
Measurement wavelength	610 nm
Sensitivity of recorder	1 V
Chart speed of recorder	5 mm/min

3.1.4.1 Effect of carrier solution

Using the manifold as shown in Figure 3.6 of which the different concentrations of diphenhydramine hydrochloride (4.4-22.1 ppm) were extracted as described in 3.1.2 using 1.05x10^{-4} M of bromocresol green solution and aqueous layer was injected. Various concentrations of borax and sodium hydroxide solution were varied. The results are shown in Table 3.3 and Figure 3.7. It was found that the more concentration of carrier solution, the higher peak height and slope obtained. Not only giving the appropriate peak height and slope but also having the reproducible responses, 0.01M borax was chosen.

Table 3.3 Effect of carrier solution on peak height; mean of triplicate injections.

DPHH (ppm)	Peak height (mV)						
	0.002M Borax	0.005M Borax	0.01M Borax	0.1M Borax	0.005M NaOH	0.01M NaOH	0.1M NaOH
4.4	250.0	321.5	350.0	365.0	266.5	306.5	361.5
8.8	212.5	265.0	303.0	307.5	210.0	251.5	306.5
13.2	195.0	235.0	256.5	265.0	190.0	215.0	265.0
17.7	130.0	168.0	186.5	205.0	137.5	172.5	205.0
22.1	100.0	138.0	161.5	165.0	110.0	136.5	166.5
Y=ax+b	Y=-8.64x +291.88	Y=-10.48x +364.21	Y=-11.14x +399.04	Y=-11.34x +411.70	Y=-8.70x +298.03	Y=-9.46x +341.61	Y=-11.10x +407.81
R^2	0.973	0.987	0.986	0.996	0.981	0.994	0.993

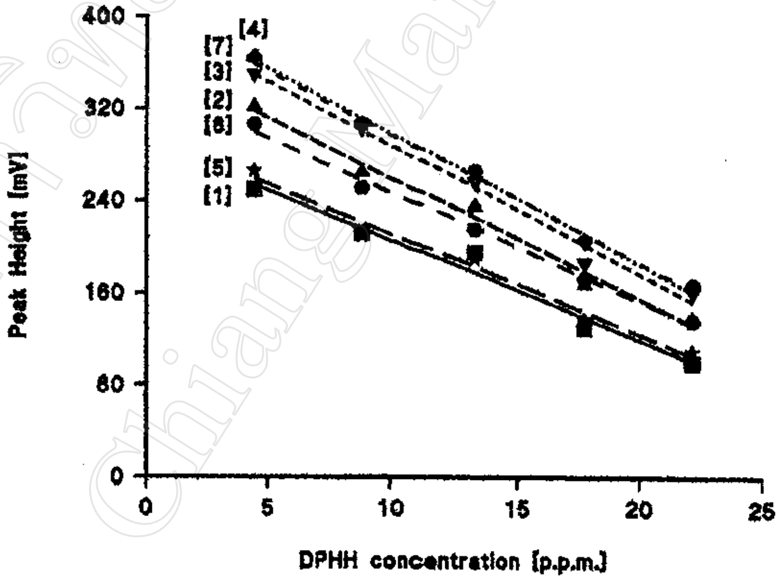

Figure 3.7 Effect of carrier solution on peak height; [1]0.002M borax, [2] 0.001M borax, [3]0.01M borax, [4]0.1M borax, [5]0.005M NaOH, [6]0.01M NaOH and [7]0.1M NaOH

3.1.4.2 Effect of pH of reagent

The effect of pH of reagent was studied by preparing the bromocresol green solution with different acid phthalate buffer as described in 2.2 using the same conditions in 3.1.4.1, a blank and a series of standard diphenhydramine hydrochloride solutions were injected after extraction with various pH of bromocresol green solution. The results are shown in Table3.4 and Figure 3.8. It was found that the higher pH of reagent, the higher peak height obtained. According to the study of Maghssoudi and et al.[20] and the results obtained indicate that the acid phthalate buffer pH 3 is considered for preparing the bromocresol green solution as giving the acceptable slope and correlation coefficient.

Table 3.4 Effect of pH of reagent on peak height; mean of triplicate injections.

DPHH	Peak height (mV)			
(ppm)	pH 5.0	pH 4.0	pH 3.0	pH 2.2
4.4	625.0	548.0	361.5	136.0
8.8	600.0	506.0	306.5	98.0
13.2	546.5	438.0	265.0	81.5
17.7	503.0	393.0	205.0	73.0
22.1	448.0	303.0	166.5	61.5
Y=a(x)+b	Y=-10.18x+679.31	Y=-13.61x+617.80	Y=-11.10x+407.81	Y=-3.92x+141.96
R^2	0.988	0.983	0.996	0.901

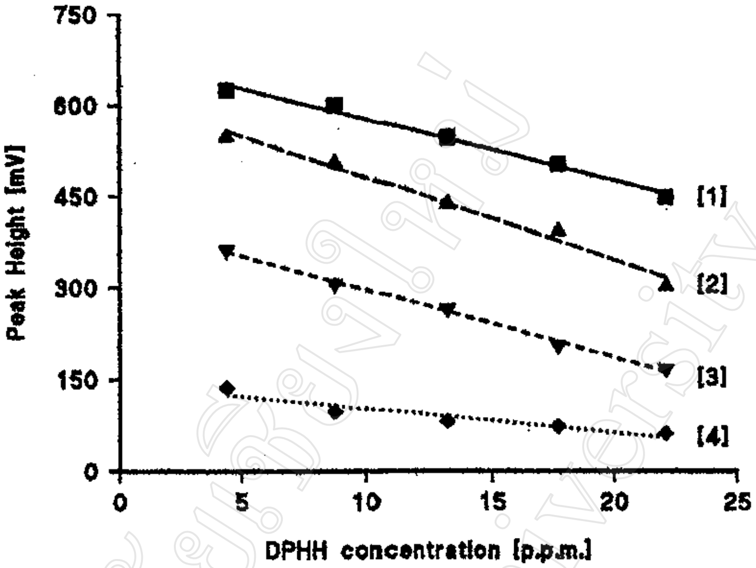

Figure 3.8 Effect of pH of reagent on peak height; pH of [1] 5.0, [2] 4.0, [3] 3.0 and [4] 2.2

3.1.4.3 Study of wavelength of measurement

Using the condition described in 3.1.4.2, a blank and a series of diphenhydramine hydrochloride standard solution were injected in the system after extraction and the various measurement wavelengths were investigated. The results are illustrated in Table 3.5 and Figure 3.9. It was found that the wavelength at 610 nm should be used because of the appropriate slope and correlation coefficient.

Table 3.5 Study of measurement wavelength; mean of triplicate injections.

DPHH (ppm)	Peak height (mV)			
	590 nm	600 nm	610 nm	620 nm
4.4	284.0	330.0	361.5	409.0
8.8	241.5	271.5	306.5	338.0
13.2	202.5	235.5	265.0	275.0
17.7	183.0	181.5	205.0	249.0
22.1	130.0	138.0	166.5	172.5
Y=ax+b	Y=-8.27x+317.69	Y=-10.70x+372.97	Y=-11.10x+407.81	Y=-12.68+456.58
R^2	0.984	0.996	0.996	0.981

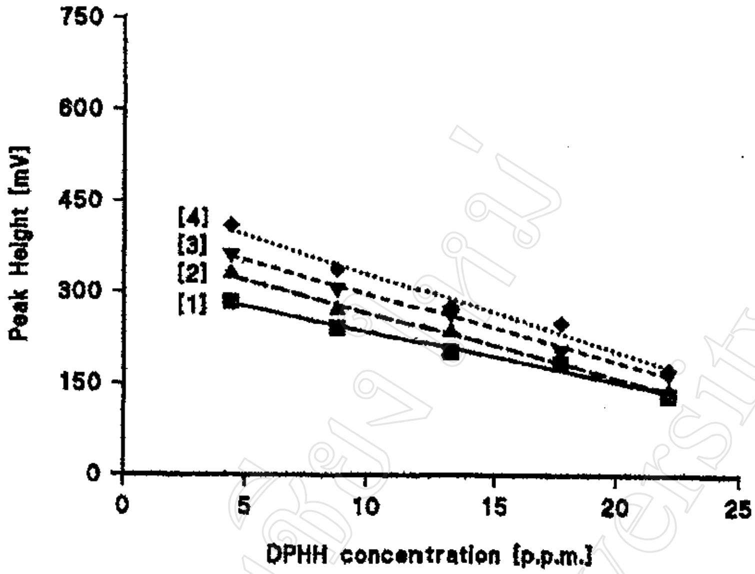

Figure 3.9 Study of measurement wavelength; [1]590 nm, [2]600 nm, [3] 610 nm and [4]620 nm.

3.1.4.4 Effect of mixing coil length

The effect of mixing coil length was investigated using the condition described in 3.1.4.3, a blank and a series of standard solution were injected in the system after extraction. The results are shown in Table 3.6 and Figure 3.10. The results indicate that the system without mixing coil give higher peak height, slope and sample throughput than the one with mixing coil, due to the influence of the travelled distance between the injection and the detection point which the longer distance, the higher dispersion obtained and so do the start-up and residence times.

Table 3.6 Effect of mixing coil length on peak height; mean of triplicate injections.

DPHH (ppm)	Peak height (mV)		
	0 cm	50 cm	100 cm
4.4	470.0	380.0	301.0
8.8	441.5	326.0	253.0
13.2	342.5	273.0	215.0
17.7	286.5	225.0	173.0
22.1	211.0	158.0	125.0
Y=ax+b	Y=-15.19x+551.44	Y=-12.38x+435.27	Y=-9.75x+342.50
R^2	0.981	0.997	0.998

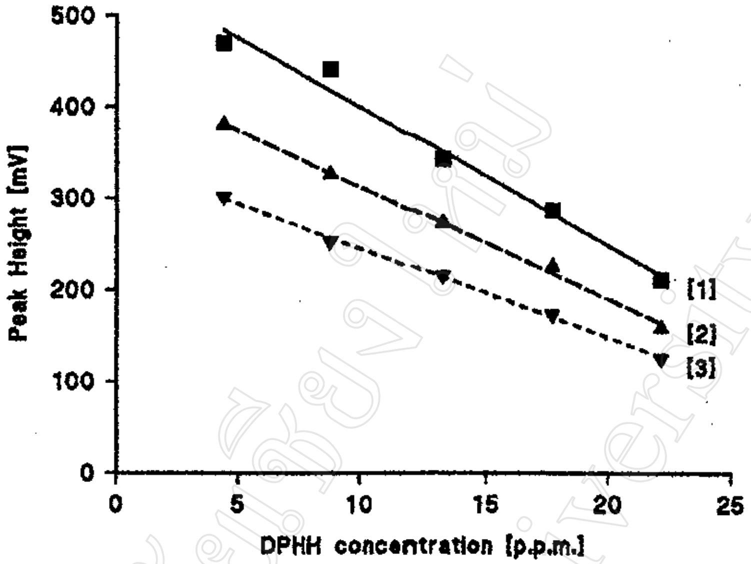

Figure 3.10 Effect of mixing coil length on peak height; [1]0 cm, [2]50 cm and [3]100 cm

3.1.4.5 Effect of sample volume

Using the condition described in 3.1.4.4 except the sensitivity of recorder, 0.5V was used, a blank and a series of standard diphenhydramine hydrochloride were injected after extraction with various sample volume. The results are shown in Table 3.7 and Figure 3.11. It was found that the much more sample volume, the higher peak height obtained. In this study the sample volume of 100 μl is considered as giving the acceptable peak height and correlation coefficient.

Table 3.7 Effect of sample volume on peak height; mean of triplicate injections.

DPHH (ppm)	Peak height (mV)			
	60 μl	100 μl	150 μl	200 μl
4.4	131.2	164.2	221.2	217.0
8.8	90.0	148.8	173.2	194.2
13.2	70.0	110.0	151.2	153.8
17.7	52.5	85.0	96.2	112.5
22.1	38.8	68.8	82.5	95.8
Y=ax+b	Y=-5.02x+142.90	Y=-5.75x+191.45	Y=-8.00x+250.81	Y=-7.32x+251.55
R^2	0.947	0.979	0.972	0.983

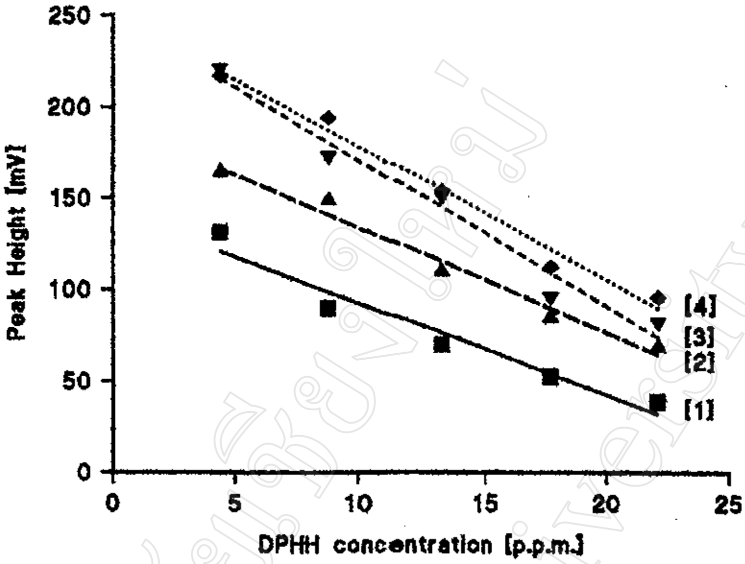

Figure 3.11 Effect of sample volume on peak height; [1]60 µl, [2]100 µl, [3]150 µl and [4]200 µl

3.1.4.6 Summary of condition used.

The recommended FIA manifold is depicted in Figure 3.6 and the optimum condition are summarized in Table 3.8

Table 3.8 Condition used for the determination of diphenhydramine hydrochloride.

Carrier solution	0.01 M Borax
Flow rate of carrier solution	5.0 ml/min
Sample volume	100 µl
Mixing coil length	0 cm
Flow through cell volume	80 µl
Measurement wavelength	610 nm
Sensitivity of recorder	1 V
Chart speed of recorder	5 mm/min

3.1.4.7 Calibration curve and detection limit.

The optimum FIA system described in 3.1.4.6 was used. The calibration curve and detection limit of the condition used were investigated. The results are shown in Table 3.9-3.10, and Figure 3.12-3.15. The results are obtained in the linear range of 5.2-21.0 ppm and 75.1-187.8 ppm of diphenhydramine hydrochloride with the detection limit of 1.0 ppm and 15.3 ppm. of diphenhydramine hydrochloride, respectively. (described in Appendix C) The sample throughput in this study was 100 injections/h. It was found that the limiting factor of the calibration range was bromocresol green, the anionic dye, concentration used.

Table 3.9 Calibration curve; mean of triplicate injections (5.2-21.0 ppm of diphenhydramine hydrochloride, 1.05x10^{-4} M of bromocresol green solution)

DPHH (ppm)	Peak height (mV)
5.2	26.6
10.5	22.5
15.7	16.6
21.0	12.5

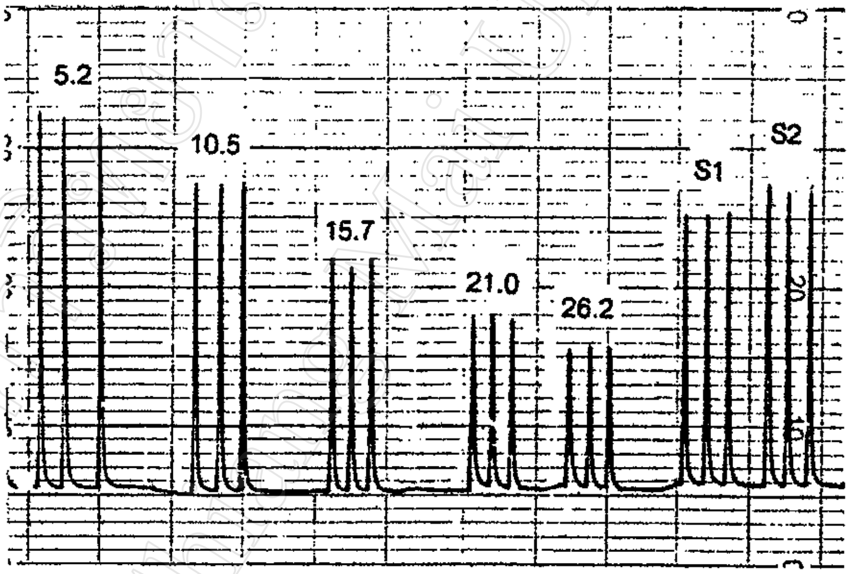

Figure 3.12 FIA signals for determination of diphenhydramine hydrochloride

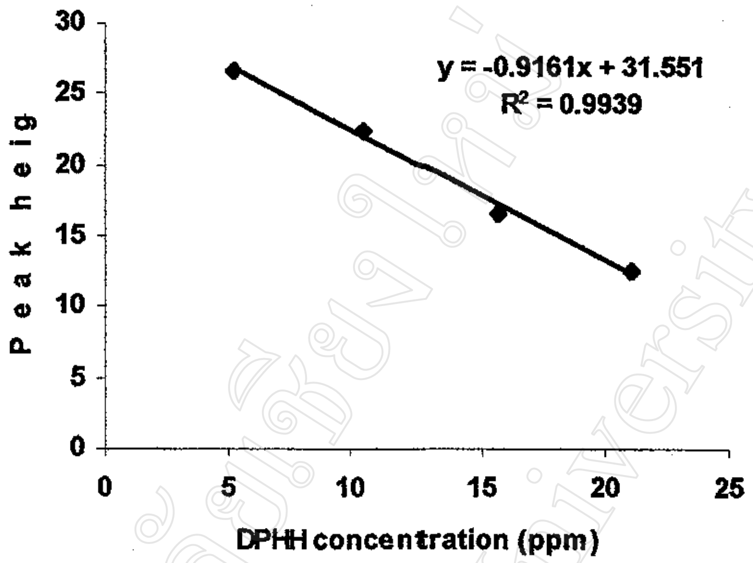

Figure 3.13 Calibration curve for 5.2-21.0 ppm of diphenhydramine hydrochloride (n=3), 1.05×10^{-4} M of bromocresol green solution.

Table 3.10 Calibration curve; mean of triplicate injections (75.1-187.8 ppm of diphenhydramine hydrochloride, 5.42×10^{-4} M of bromocresol green solution)

DPHH (p.p.m.)	Peak height (mV)
75.1	768.0
112.7	557.0
150.3	285.0
187.8	120.0

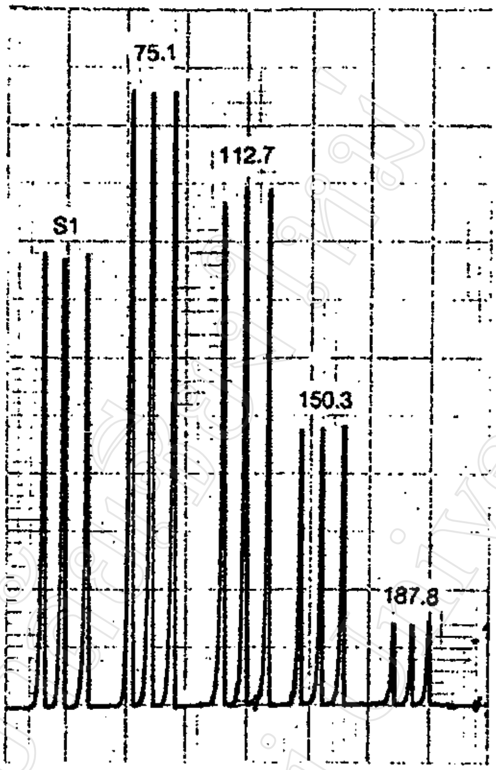

Figure 3.14 FIA signals for determination of diphenhydramine hydrochloride

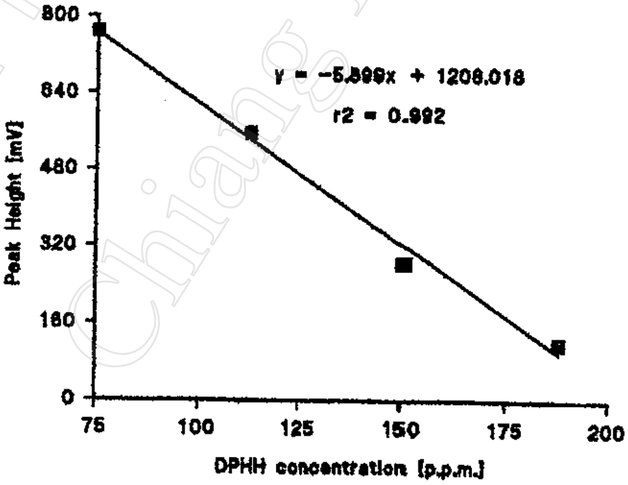

Figure 3.15 Calibration curve for 75.1-187.8 ppm of diphenhydramine hydrochloride (n=3), 5.42×10^{-4} M of bromocresol green solution.

3.1.4.8 Precision of FIA system

The optimum FIA system described in 3.1.4.6 was used. The precision of the conditions used was determined by 11 replicate injections of 106.7 ppm of diphenhydramine hydrochloride, using the calibration curve range 75.1-187.8 ppm and 5.42×10^{-4} M of bromocresol green solution. The results are shown in Table 3.11. The relative standard deviation (RSD) was found to be 1.6%.

Table 3.11 Precision study of 106.7 ppm of diphenhydramine hydrochloride; n=11

Peak height (mV)	Mean (mV)	SD	%RSD
685.0, 676.0, 685.0, 685.0 685.0, 685.0, 676.0, 666.0 685.0, 666.0, 704.0	681.6	10.6	1.6

3.1.4.9 Reproducibility and efficiency of the extractions

Using the optimum FIA system described in 3.1.4.6 and the same calibration curve in 3.1.4.8, the reproducibility and efficiency of the extractions was investigated by triplicate injections of each determination (n=7) of 106.7 ppm of diphenhydramine hydrochloride. The results are shown in Table 3.12. The relative standard deviation was found to be 1.5% and the efficiency of extraction was 92.6%.

Table 3.12 Reproducibility and efficiency of the extractions of 106.7 ppm of diphenhydramine hydrochloride; n=7.

Amount found (ppm)	Mean (ppm)	SD	%RSD	%extraction[a]
97.5, 97.9, 99.0, 96.9 100.0, 99.7, 100.9	98.8	1.5	1.5	92.6

[a] %extration = $\frac{\text{Amount found}}{\text{Amount added}} \times 100$

3.1.4.10 Interference studies

The tolerance of the method to foreign compounds that may be found in typical pharmaceutical samples containing diphenhydramine hydrochloride was studied by using condition and solutions similar to those used for the reproducibility studies (containing 106.7 ppm of diphenhydramine hydrochloride) and adding various concentrations of the interfering compounds. The results obtained for various interferences are shown in Table 3.13. The results indicate that the compounds commonly present with diphenhydramine hydrochloride in pharmaceutical preparations containing single tertiary alkylamine drug do not interfere in the determination, except the compounds effecting the pH of solution such as sodium citrate (pH 7.5). The effect of pH can be resolved by adjusting the solution with acid phthalate buffer pH 3.0 before extraction.

Table 3.13 Effect the interfering compounds on the determination of 106.7 ppm of diphenhydramine hydrochloride; mean of 11 replicate injections.

Substances	Amount ratio (by weight)	%Relative error
Sucrose [170]	370000	0.8
Lactose [4]	179	3.5
Ammonium chloride [10]	42	0.009
Citric acid [3.2]	13	2.5
Sodium citrate[a] [4]	8	3.6
Camphor [0.1]	38	1.7
Dextromethophan.HBr [0.75]	0.3	35.4
	0.6	67.1
Bromhexine.HCl [1]	0.05	11.8
	0.27	34.2
Glyceryl guaiacolate [12.5]	21	3.5

[a] adjusted with acid phthalate buffer pH 3.0

[ ] amount ratio by weight may be found in pharmaceutical preparations

3.1.4.11 Determination of diphenhydramine hydrochloride in pharmaceutical preparations

The optimized FIA system was applied to the determination of diphenhydramine hydrochloride in pharmaceutical preparations (The details of samples were shown in Appendix D) The accurately weighed quantity of samples was dissolved with deionized water, shaked for 15 min. and adjusted with water to obtain a solution having a concentration in the range of calibration curve used. 5 ml of sample solution was transferred to 20-ml vial, added with 5 ml of bromocresol green solution and chloroform, extracted by shaking for 1 min and then the aqueous layer was aliquoted to centrifuge for complete separation and clarification before injecting into the system. The results obtained were compared to the ones determined with HPLC following the method mentioned in the United States Pharmacopoeia [35] and the other ones by JAOAC method [19] as shown in Table 3.14. The differences among the means and accuracy obtained from the proposed FIA method, HPLC method and JAOAC method were evaluated by t-test (For detailed calculation, see Appendix E) It was indicated that the results from all recommended methods are not significantly different at confidence interval of 95%. From the study, it was found that the high pH of samples(pH >7.0) decreased the efficiency of extraction. Using the acid phthalate buffer pH 3.0 diluted the samples before extraction gave the better results as shown in Table 3.15.

Table 3.14 Determination of diphenhydramine hydrochloride in various pharmaceutical preparations

sample	Labelled Amount	FIA^a		FIA^b		HPLC^c		JAOAC^d	
		Amount found	%L.a	Amount found	%L.a	Amount found	%L.a	Amount found	%L.a
Benadryl®	25.0mg/cap	25.4±1.0	101.6	23.5±1.3*	94.5	25.0±0.5	100.0	23.9±0.4	95.7
Benadryl®	12.5mg/5ml	12.8±0.1	102.4	11.0±0.5*	88.0	12.6±0.1	100.8	10.0±0.4	80.0
Cotussin®	12.5mg/5ml	11.5±0.6	92.0	10.5±0.2	84.0	11.6±0.6	93.2	9.3±0.3	74.1
Coldanyl®	12.5mg/5ml	-	-	11.4±0.4	91.2	-	-	-	-
Broncho-prex®	10.0mg/5ml	-	-	15.5±0.4	155.0	-	-	-	-
Broncho-prex®	4.0mg/5ml	-	-	6.3±0.1	157.2	-	-	-	-
caladryl®	1%w/w	0.698±0.1	69.8	0.667±0.02*	66.7	0.969±0.01	96.9	0.962±0.02	96.2
Caldamine®	1%w/v	-	-	0.844±0.05	84.4	-	-	-	-
Cadramine-V®	1%w/v	-	-	0.537±0.01	53.7	-	-	-	-

[a] using calibration curve range 5.2-26.2 ppm; mean of 3 determinations.

[b] Using calibration curve range 75.1-187.8 ppm; mean of 3 determinations.

[c] USP 1995; mean of 3 determinations.

[d] JAOAC; mean of 2 determinations.

* mean of 11 determinations.

\- not determined

Table 3.15 Effect of pH of samples on determination of diphenhydramine hydrochloride by the proposed FIA method; mean of 3 determinations.

Sample	pH	Diluted with water		Diluted with buffer pH 3.0	
		Amount found	%L.a	Amount found	%L.a
caladryl®	7.55	0.667±0.02*	66.7	0.829±0.02*	82.9
caldramine®	7.24	0.844±0.05	84.4	0.843±0.05	84.3
Cadramine-V®	8.55	0.537±0.01	53.7	0.604±0.03	60.4

* mean of 11 determinations.