CHAPTER-9

Cephalexin

9.1 A survey of literature for Cephalexin indicated the estimation reported by the following methods in various papers, Viz., High-performance liquid chromatography , Capillary isotachophoresis. Gas chromatography-mass spectrometry, UV Spectrophotometric method , Micellar electrokinetic chromatography, Gas chromatography-mass spectrometry, HPTLC ,and High-performance liquid chromatography-electrospray ionization mass spectrometry,. The brief information on above analytical methods are follows.

Kaskhedikar SG. [235] described the analysis Simultaneous estimation of bromhexine hydrochloride and cephalexin in capsule by high performance liquid chromatography.Tsai TH [236] described the Simultaneous blood and brain sampling of cephalexin in the rat by microdialysis and microbore liquid chromatography· application to pharmacokinetics studies. Ming JL et al [237] described the High-performance liquid-chromatographic determination of cefalexin capsules.Wu ZJ et al [238] described Studies on the simultaneous measurement of several cephalosporins by reversed-phase HPLC.

Cheng FG et al [239] described Determination of cephalexin by isotachophoresis. Wang WH et al [240] described Rapid determination of cephalexin in cephalexin capsules by HPLC Coran SA et al [241] described Development of a densitometric method for the determination of cephalexin as an alternative to the standard HPLC procedure. Li YM et al [242] described Micellar electrokinetic capillary chromatography for the separation of cefalexin and its related substances. Yang TM et al [243] described Determination of cephalexin by potassium bromate dead-stop titrimetry. Shabadi CV et al [244] described Simultaneous determination of cefadroxil and cephalexin in pharmaceutical preparations by quantitative TLC. Trajkovic-Jolevska et al [245] described Derivative spectrophotometric assay of cephalexin in the presence of potassium sorbate. Gallo-Martinez et al [246] described A new derivatization procedure for the determination of cephalexin with 1,2-naphthoquinone 4-sulfonate in pharmaceutical and urine samples using solid-phase extraction cartridges and UV-visible detection. Li HK et

al [247] described Spectrophotometric determination of cephalexin. Agbaba D et al [248] described HPTLC assay of cephalexin and cefaclor in pharmaceuticals. Halkar UP et al [249] described Simultaneous determination of cephalexin and probenecid in pharmaceutical preparations by HPTLC.

Zhou MH [250] described High-performance liquid-chromatographic determination of cefalexin. Farag SA [251] described Simultaneous liquid-chromatographic analysis of the beta-lactam antibiotics cefazolin, cefadroxil, cephalexin, ampicillin, and cephradine in solution.Pecavar A et al [252] described A reversed-phase high-performance liquid chromatographic method for determination of cephalexin in human plasma.Wang Z et al [253] described Separation of drugs cephalexin, ampicillin, and their biosynthetic precursors L-Phe-L-Cys-D-Val and D-Phe-L-Cys-D-Val by capillary zone lectrophoresis. Halkar UP et al [254] described Simultaneous determination of cephalexin and probenecid in pharmaceutical preparations by reversed phase HPLC.

Erceg M et al [255] described Study of cephalexin and cefaclor adsorption at the mercury / solution interface by a.c. polarography. Shinde VM et al [256] described Simultaneous determination of cefadroxil and cephalexin from capsules by reverse phase HPLC. Campins-Falco et al [257] described Comparative study on the determination of cephalexin in its dosage forms by spectrophotometry and HPLC with UV-vis detection. Agbaba D et al [258] described Spectrophotometric determination of certain cephalosporins using ferrihydroxamate method. Argekar AP et al [259] described Simultaneous determination of cephalexin and carbocisteine from capsules by reverse-phase high-performance liquid chromatography (RP-HPLC). Hsu M et al [260] described Liquid-chromatographic determination of cephalexin preparations: inter-laboratory validation.

Yang JH et al [261] described Simultaneous determination of cephalexin and cefadroxil by using the coupling technique of synchronous fluorimetry and H-point standard additions method. Sun FS et al [262] described Direct assay of cephalexin in

human plasma by high-performance capillary zone electrophoresis. Hsu MC et al [263] described High-performance liquid-chromatographic method for potency determination of cephalexin in commercial preparations and for stability studies.Lu R et al [264] described Simultaneous determination of trimethoprim and cephalexin in compound cephalexin preparations by reversed-phase high-performance liquid chromatography. Sun N et al [265] described Quantitative determination of cephalexin capsules by polarimetry.

Hendrix C et al [266] described Quantitative analysis of cephalexin by liquid chromatography on poly(styrene - divinylbenzene). Meng L et al [267] described Determination of trimethoprim in compound cephalexin capsules by differential spectrophotometry. Dong Let al [268] described Dual-wavelength spectrophotometry for simultaneous determination of cephalexin and trimethoprim in capsule preparation. Patel IT et al [269] described spectro photometric method for determination of cephalexin in its dosage forms. Alwarthan AA et al [270] described Spectrophotometric determination of cephalexin in dosage forms with imidazole reagent Zhao Y et al [271] described Quantitative determination of cephalexin by high-performance liquid chromatography.

Wen L et al [272] described Determination of compound cephalexin capsules by tri-wavelength UV spectrophotometry. Kovach PM et al [273] described High-performance liquid-chromatographic determination of loracarbef, a potential metabolite, cefaclor and cephalexin in human plasma, serum and urine. Lee YJ et al [274] described Simultaneous determination of cefoxitin, cefuroxime, cephalexin and cephaloridine in plasma using HPLC and a column-switching technique. Murillo JA et al [275] described Determination of amoxycillin and cephalexin in mixtures by second-derivative spectrophoto metry. Izquierdo P et al [276] described Simultaneous determination of cephradine and cephalexin in serum by derivative synchronous fluorescence pectroscopy. Abdel-Gawad et al [277] described Spectrophotometric determine -ation of cefadroxil and cephalexin with ammonium vanadate. Hikida K et al [278] described Determination of cephalexin in human plasma and rabbit serum by automated column-switching HPLC.Emm TA et al [279] described High-performance liquid-chromatographic assay of cephalexin in serum and urine.

Rouan MC [280] described Micro-bore liquid-chromatographic determination of cadralazine and cephalexin in plasma with large-volume injection.Najib NM et al [281] described High-performance liquid-chromatographic analysis of cephalexin in serum and urine. Marincel J et al [282] described Comparison between HPLC and microbiological methods in assays of cephalexin in samples. Issopoulos PB [283] described Analytical investigations of beta-lactam antıbiotics in pharmaceutical preparations. Spectrophotometric determination of cephalexin, cephradine, ampicillin and amoxycillin using copper(II) acetate as a complexing agent. Morelli B et al [284] described First- and second-derivatıve spectrophotometric assay of mixtures of cefuroxime and cephalexin. Das-Gupta V et al [285] described Quantitation of cephalexin ın pharmaceutical dosage forms using high-performance liquid chromatography.

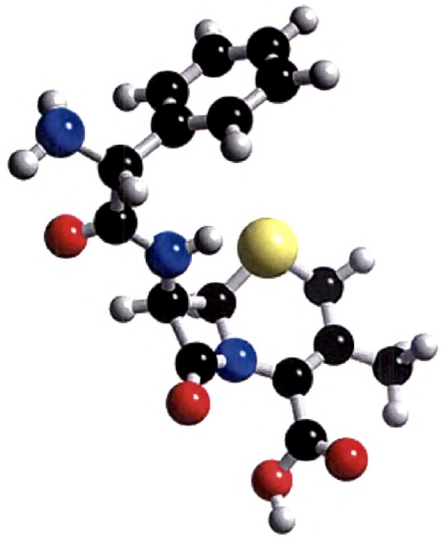
Issopoulos PB [286] described Analytical investigations of beta-lactam antibiotics ın pharmaceutıcal preparations. Spectrophotometric determination of cephalexin, cephradıne, ampicillin and amoxycillin using paramolybdate anion. McAteer JA et al [287] described Liquid-chromatographic determınation of five orally active cephalosporins, cefixime, cefaclor, cefadroxil, cephalexin and cephradine, ın human serum. Kovacic-Bosnjak et al [288] described Reversed-phase HPLC separation of. DELTA.2- and .DELTA.3-ısomers of 7-ADCA [7-aminodeacetoxycephalosporanic acid] and cephalexin monohydrate. Shingbal,-DM et al [289] descrıbed Spectrophoto -metric analysis of cephalexin and ıts dosage forms. Emmannuel J et al [290] described simple and accurate spectrocolorimetric method for the quantitatıve estimation of cephalexin and ıts dosage forms.Kennedy JH et al [291] described Investıgation of perchlorate, phosphate and ion-pairing eluent modifiers for the separation of cephalosporin epimers. Hernandez-Mendez J et al [292 described Differential pulse polarographic determination of cephalexin based on the catalytic pre-wave of nickel(II).Miyazaki K et al [293] described Determination of ampicillin, amoxycillın, cephalexin and cephradine in plasma by high-performance liquid chromatography usıng fluorimetric detection. Mori I et al [294]descrıbed Spectrophotometric determination of cephalexın and ampicillin using o-hydroxyquınolphthalein and palladium(II). Lecaillon JB et al [295] described Determinatıon of cefsulodin, cefotiam, cephalexin, cefotaxime, deacetylcefotaxime, defuroxime and cefroxadin in plasma and urine by high-performance liquid

chromatography. Nunez-Vergara et al [296] described Polarography of an acidic degradation product from cephalexin.Tsutsumi K et al [297] described Determination of serum cephalexin by high-performance liquid chromatography.

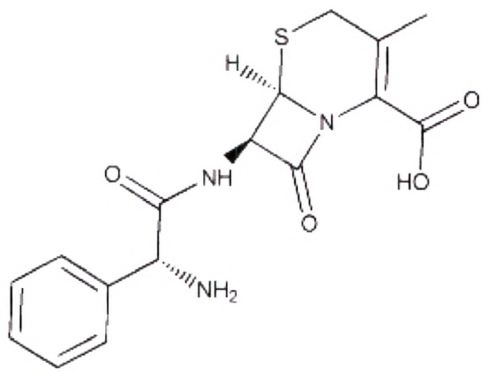
Takagishi Y et al [298] described High-performance liquid chromato graphic assay of cephalexin in biological fluid. Nahata MC et al [299] described High-performance liquid-chromatographic determination of cephalexin in human plasma, urine and saliva. Fabregas JL et al [300] described Simultaneous determination of cephalexin and lysine in their salt using high-performance liquid chromatography of derivatives. Mangia A et al [301] described Quantitative determination of cephalexin by proton magnetic resonance spectroscopy. Matousova O et al [302] described Fluorimetric determination of cefalexin. Quercia V et al [303] described Application of high-pressure liquid chromatography to cephalosporin analysis Matousova O et al [304] described Spectrophotometric determination of cephalexin. Fogg AG et al [305] described Differential pulse-polarographic determination of cephalexin after hydrolysis in neutral phosphate buffer Also described [306] Differential pulse polarographic study of the degradation of cephalexin.

Even though there are several methods reported to estimate sertraline from plasma samples as well as matrices but no where Cephalexin separations and estimations from its related compound and process impurities are reported. Hence we have under taken this problem for current work Subsequently we have proposed to develop a simple and cost effective stability indicating assay method on HPLC, which will suit even small scale manufacturers keeping ICH guide lines and various regulatory requirements mind. Developed HPLC methods are validated thoroughly to check the suitability and corrective ness as per ICH guidelines.

9.2 Analytical Method For Determination Of Related Substances In Cephalexin By HPLC

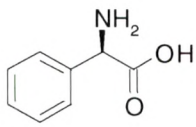


Cephalexin 3D structure STR-24

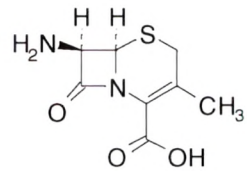


Chemical Formula $C_{16}H_{17}N_3O_4S$

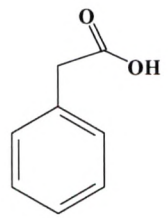
Cephalexin STR-25

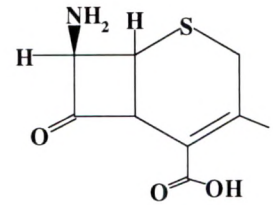


D-Phenyl glycine STR-26



7-ADCA STR-27

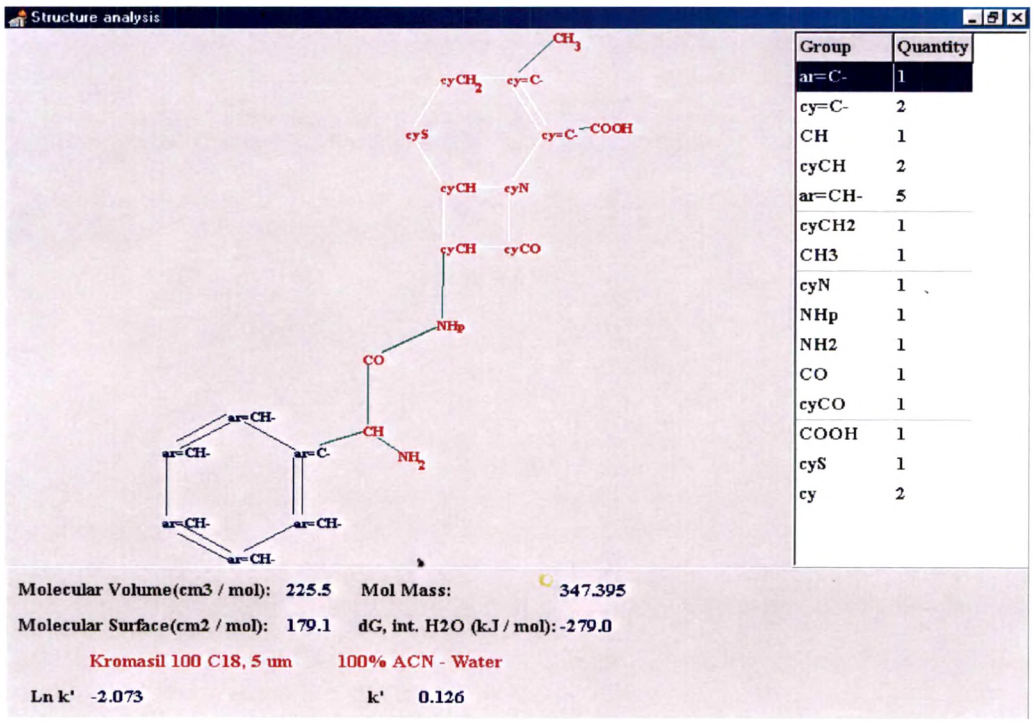




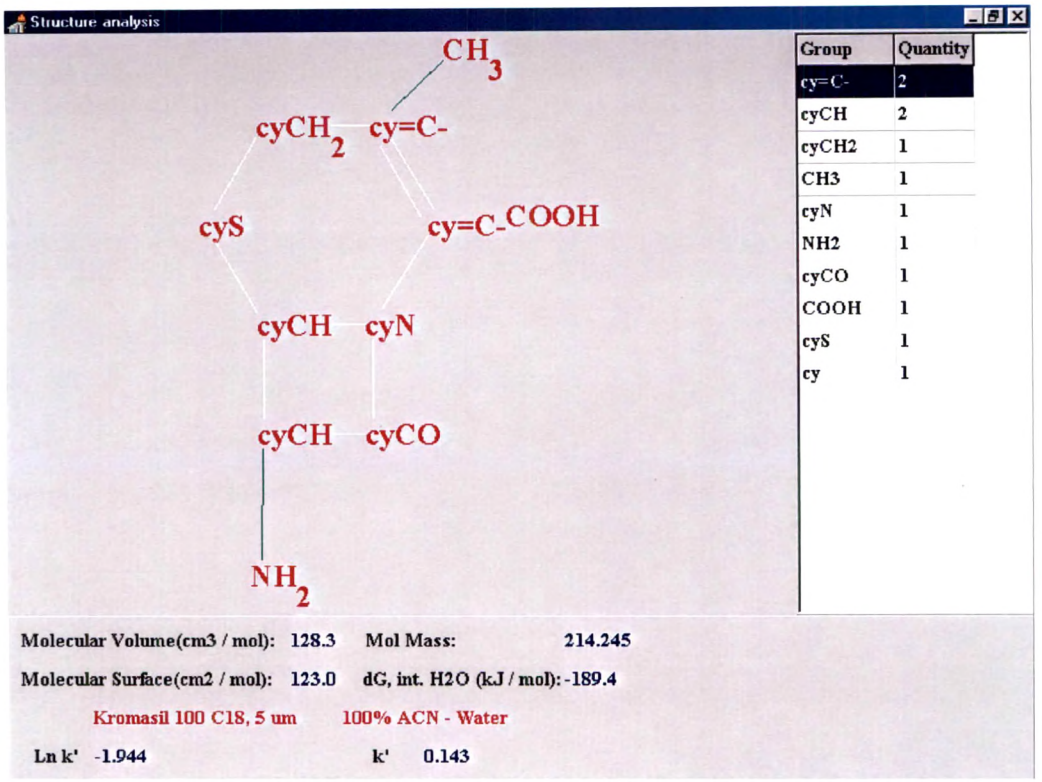
Phenyl acetic acid STR-28

Δ^2-7-ADCA STR-29

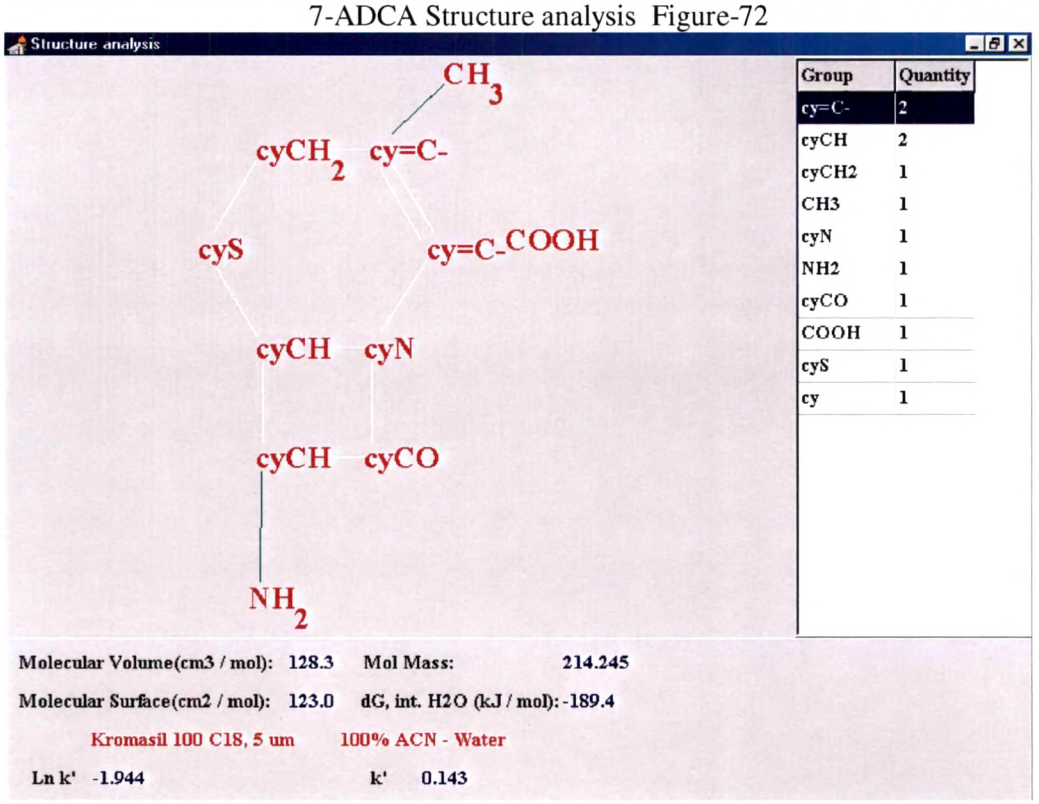
All structures are loaded into chromsword HPLC method development software to deduce structure analysis for method development. Figure-71 to 75 shows cephalexin and its related compounds structure analysis charts.



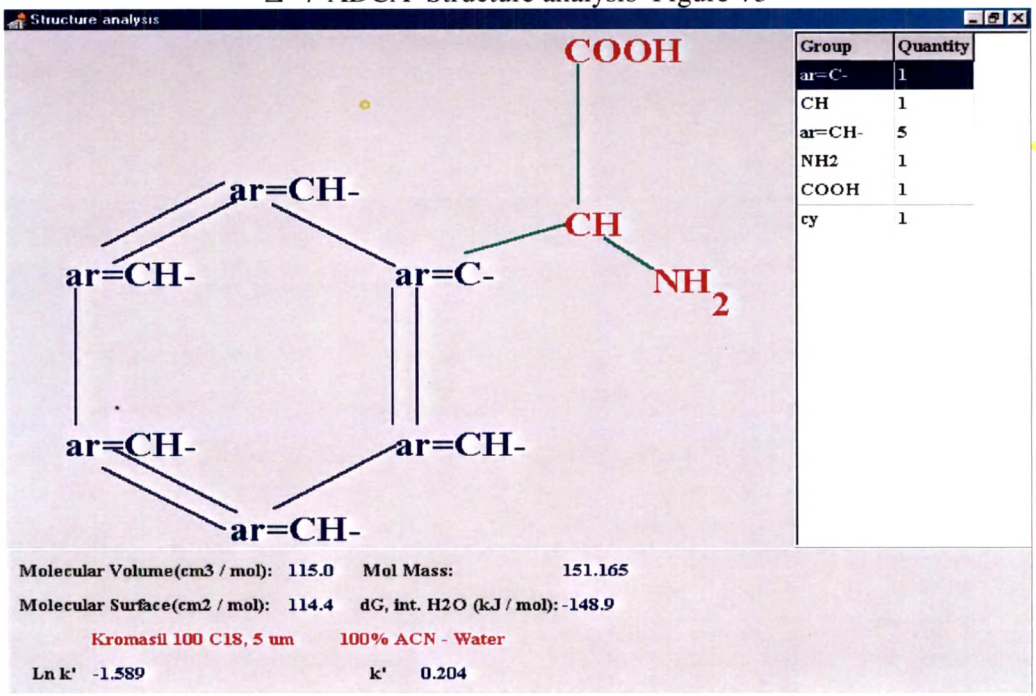
Cephalexin Structure analysis Figure-71



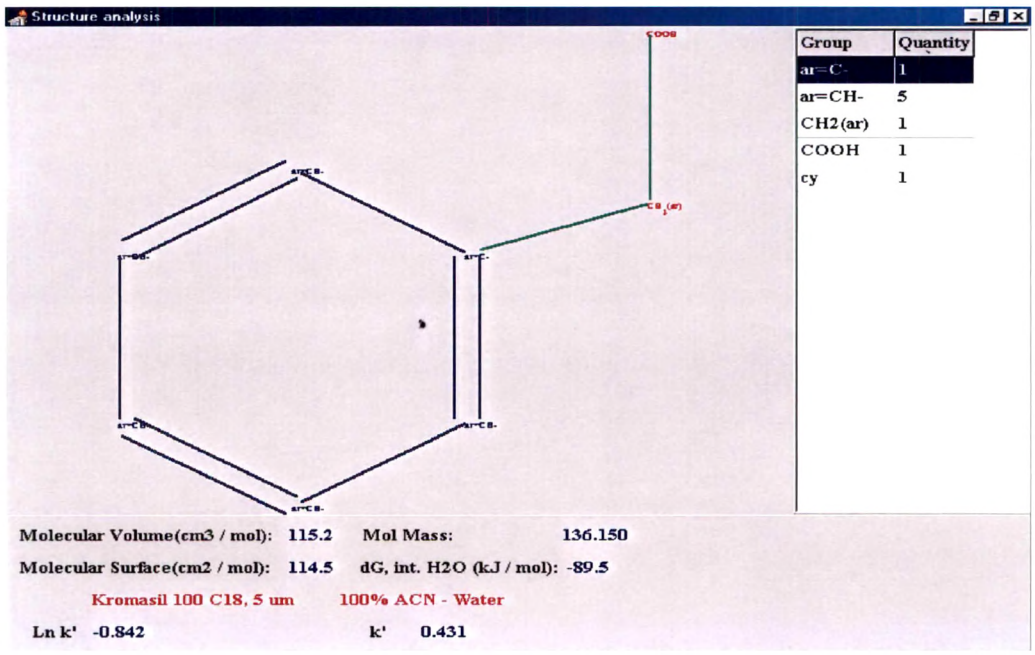
7-ADCA Structure analysis Figure-72



Δ^2-7-ADCA Structure analysis Figure-73

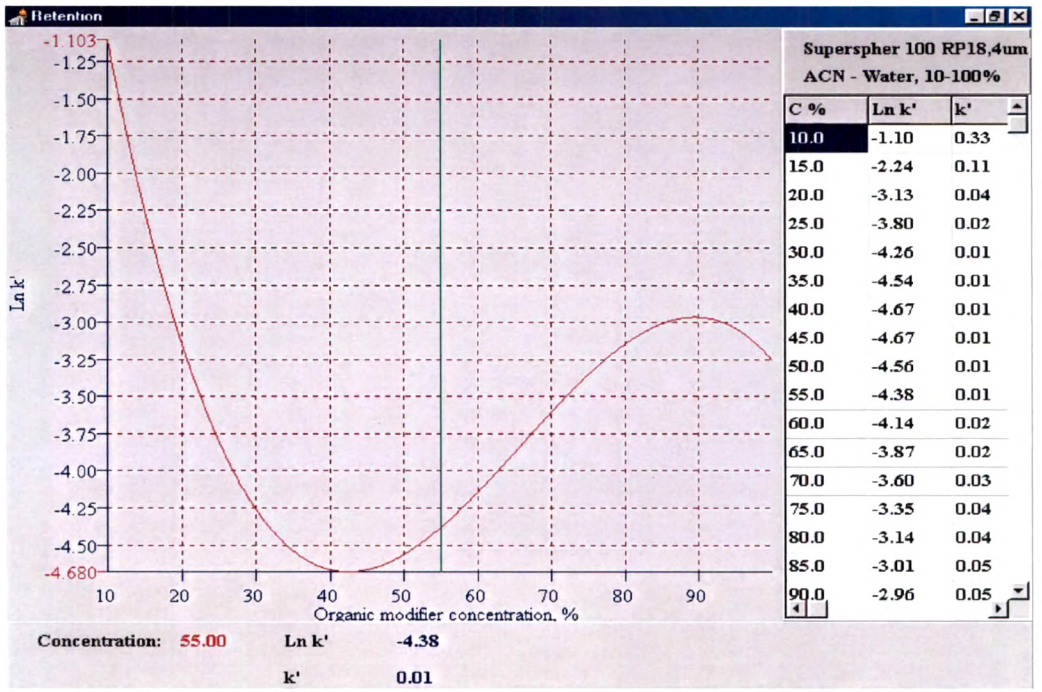


Phenyl glycine Structure analysis Figure-74

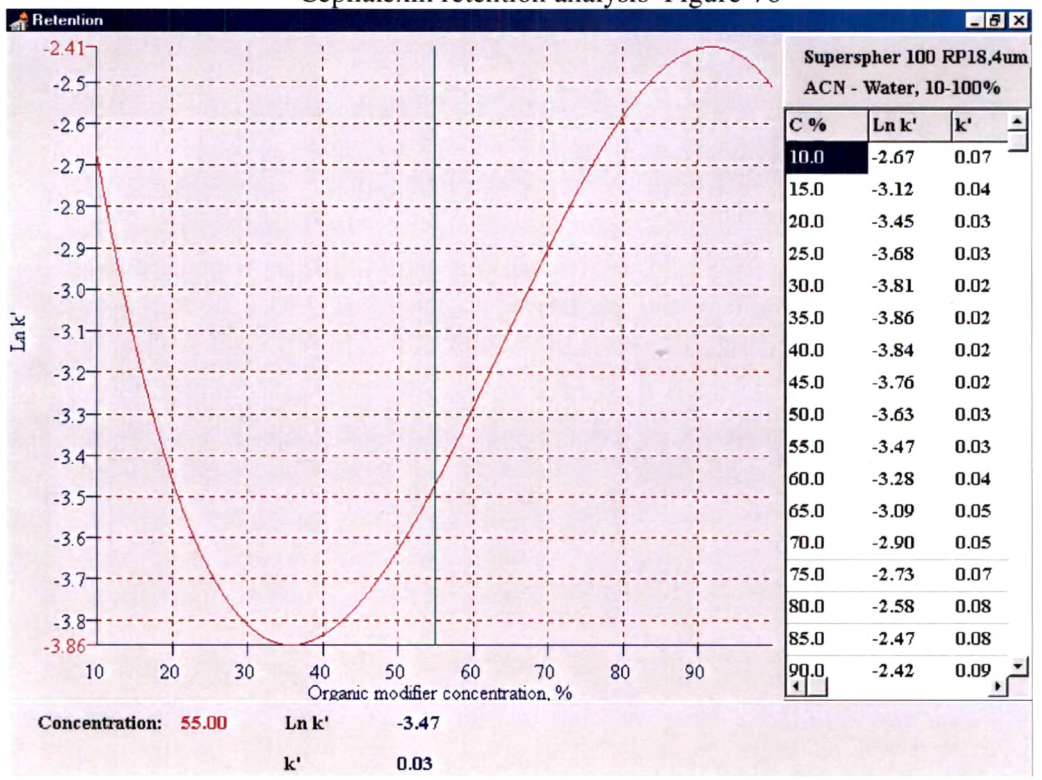


Pheyl Acetic acid Structure analysis Figure-75

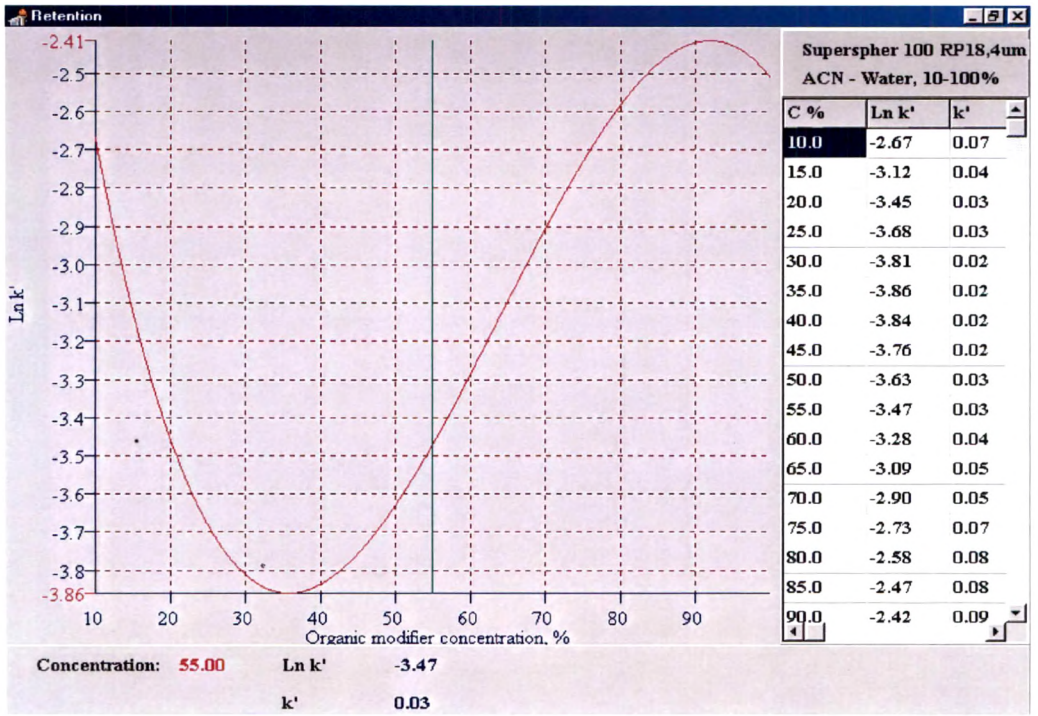
Figure-76to 80 shows cephalexin and its related compounds retention analysis charts.



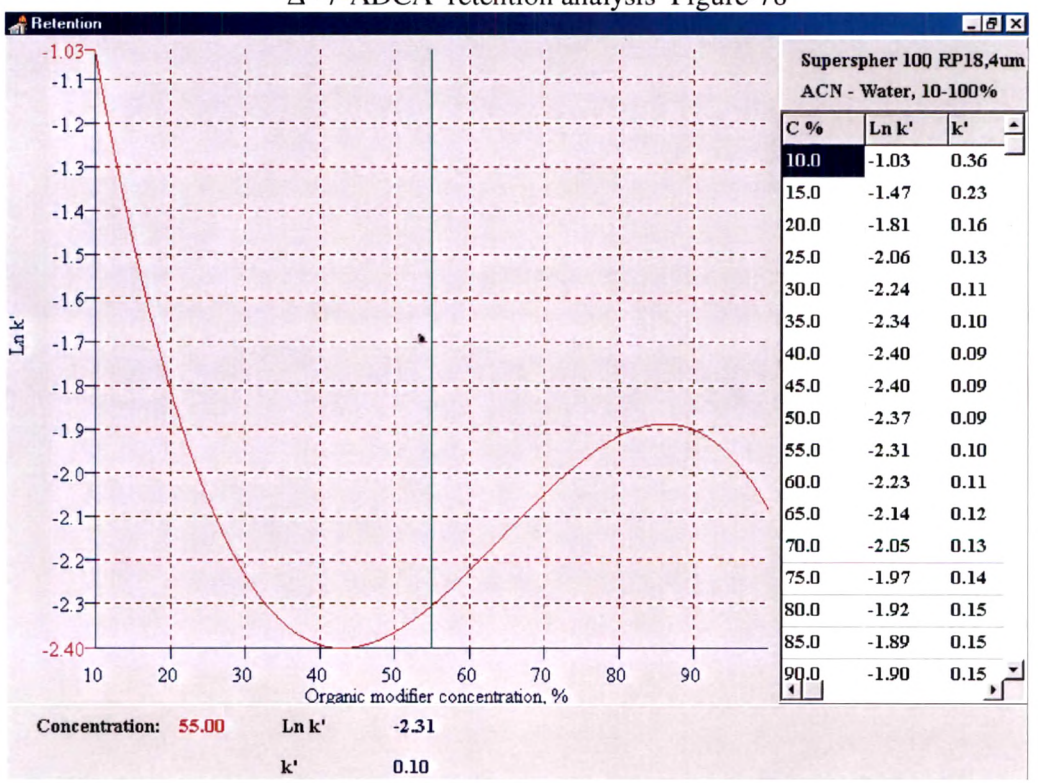
Cephalexin retention analysis Figure-76



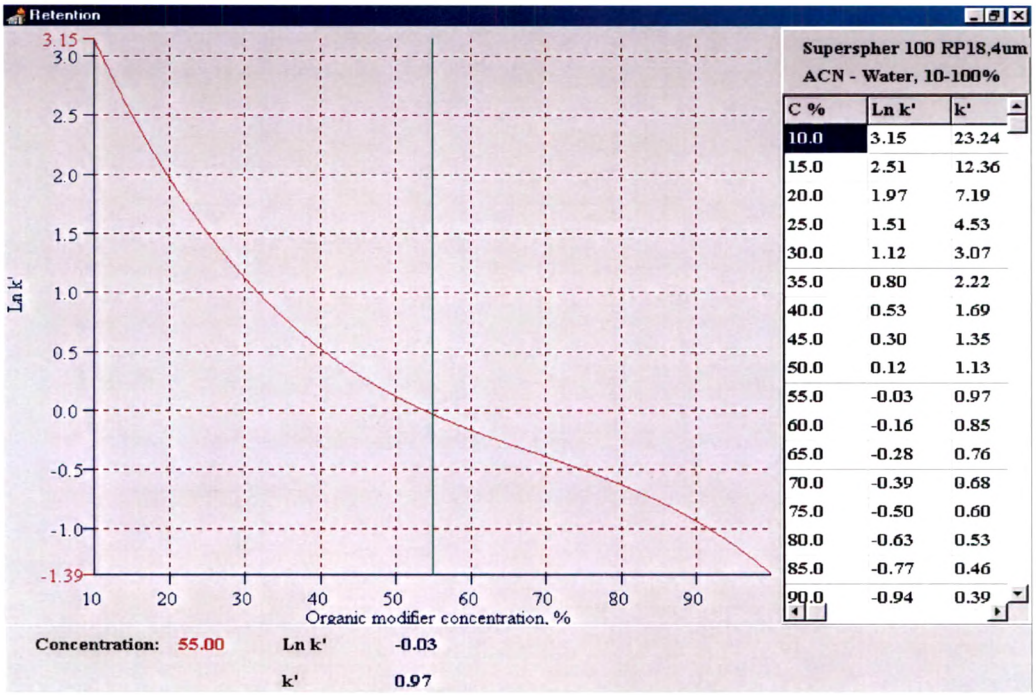
7-ADCA retention analysis Figure-77



Δ^2-7-ADCA retention analysis Figure-78



Phenyl glycine retention analysis Figure-79



PAA retention analysis Figure-80

Simulated chromatogram Figure-81

Figure-81 shows cephalexin and its related compounds simulated chromatogram

Analysis: Software had predicted only three peaks which are shown in Figure-81. Table mentioned in figure -81 indicating all five components but three components are at one place ie at 2.08 min .These analysis were done based on at molecular volumes and molecular surfaces as given in structure analysis charts.

At this point software failed to predict the correct simulated chromatogram due to closeness between the structures Hence software failed to predict the correct condition. Experiment was performed to verify the software prediction. Mixed phosphate buffer solution with pH to 6.0.0 ± 0.1 is selected as buffer and methanol as organic modifier. Wavelength 220 nm is selected to detect all impurities. Found gradient analysis is a better choice to resolve closely eluting peaks .Final conditions are given below.

Analytical Method :

Reagents and chemicals :

Potassium dihydrogen orthophosphate	: AR grade
Di-potassium hydrogen orthophosphate	: AR grade
Methaol	: HPLC grade
Orthophosphoric acid	: AR grade
Water	: Milli-Q grade

Mobile phase A :Transfer 1.05g dipotassium hydrogen orthophosphate and 5.98g potassium dihydrogen orthophosphate into a 1000ml volumetric flask. Dilute upto the mark with water. Adjust the pH of the solution to 6.0 with orthophosphoric acid. Filter and degas prior to use.

Mobile phase B :HPLC grade methanol .Filter and degas prior to use.

Diluent : Transfer 18.0g potassium dihydrogen orthophosphate into a1000ml volumetric flask dissolve and dilute to volume with Milli Q water.

System suitability :Transfer about 5 mg accurately weighed, each of Δ^2-7-ADCA and Phenyl acetic acid (PAA) into a 25 ml volumetric flask. Dissolve in about 2 ml of diluent

(sonicate if necessary). Dilute to volume with diluent (200 µg/ml each of Δ^2-7-ADCA and PAA).

Sample preparation :Transfer about 50 mg accurately weighed cephalexin sample into a 50 ml volumetric flask. Dissolve in and dilute upto mark with diluent (1000 µg/ml).

Standard solution preparation :Prepare a solution containing about 10 µg/ml of each impurity and cephalexin individually (1.0% of sample concentration).

Chromatographic conditions :Use a suitable high pressure liquid chromatography (HPLC) system equipped with a UV detector set to 220 nm and a column of 250 mm x 4.6mm containing 5µ C18, BDS packing material (suggested column : Hypersil C-18, BDS, Themoquest U.K).

The system is also equipped to deliver the two phases in a programmed manner as shown in the Table below :

Total flow rate : 1.0 ml / min

Time (in min.)	*Mobile Phase A (% v/v)*	*Mobile Phase B (% v/v)*
0	100	0
10	100	0
20	85	15
35	40	60
36	100	0
42	100	0

Procedure :

Inject 20 µl system suitability solution into the chromatograph set to above conditions and record the chromatogram upto 42 min. Test is valid only when the resolution between Δ^2-7-ADCA and phenyl acetic acid is not less than 4.

Inject 20 µl of standard solution preparation in duplicate into the chromatograph set to above conditions and record the chromatograms upto 42 min. Calculate the average area of individual component and RSD. Test is valid only when the RSD is not more than 5.0 %.

Inject 20 µl test preparation in duplicate into the chromatograph set to above conditions and record the chromatograms upto 42 min. Calculate the amount of related substances using the formula given in calculations:

Retention times of the impurities & cephalexin are:

Sr.No	*Name of the component*	*RT(min)*
2	Phenyl glycine	5
3	7-ADCA	9
4	Δ^2-7-ADCA	20
5	Phenyl acetic acid	22
6	Cephalexin	32
7	7-PADCA	36

Calculations :

i) Calculate the percentage of Impurity, individually using the formula

$$\frac{C_{im}}{C_S} \times \frac{r_s}{r_{im}} \times 100$$

Where

C_{im} = Concentration of impurity individually in standard solution (µg/ml)

C_S = Concentration of sample solution (µg/ml)

r_s = Detector response for impurity individually in sample preparation

r_{im} = Detector response for impurity individually in standard solution

ii) Calculate the percentage of any other impurity, individually using the formula :

$$\frac{C_P}{C_S} \times \frac{r_s}{r_P} \times 100$$

Where :

C_P = Concentration of cephalexin in standard solution (µg/ml)

C_S = Concentration of sample solution (µg/ml)

r_s = Detector response for impurity individually in sample preparation

r_P = Detector response for cephalexin in standard solution

Limit of impurities :

Any impurity, individually not more than 1.0 %.

Total impurity not more than 5.0 %

9.3 Validation Protocol

Purpose :The purpose of this document is to establish the precision, accuracy, linearity of detector response and ruggedness of the analytical method through number of scientific studies and discussions of the data.

Scope :This method upon validation can be used in the analysis of related substances in cephalexin.

Analytical method: As described in section 9.2

9.3.1 The following experiments are designed to study

a System suitability

b.Instrument precision

c Identification of individual component

d.Solution stability

e.Method precision

f Linearity and range

g Accuracy

h.Minimum detection limit

i.Minimum quantitation limit

j.Ruggedness of the method

9.3.2 Stock Solutions :

Solution 1 : Transfer about 25 mg accurately weighed each of Phenyl glycine (PG), 7-ADCA, Δ^2-7-ADCA, Phenyl acetic acid, and 7-PADCA, into a 100 ml volumetric flask. Add about 2 ml 1M hydrochoric acid ,2ml diluent and 2ml methanol (sonicate if necessary) and dilute to volume with diluent [250 µg/ml each of Phenyl glycine (PG), 7-ADCA, Δ^2-7-ADCA, phenyl acetic acid and 7-PADCA].

Solution 2 : Transfer about 25mg, accurately weighed cephalexin into a 100ml volumetric flask. Dissolve in and dilute to volume with water.

Standard solution :Pipette out 2.0 ml of solution 1 and solution 2 into a 50 ml volumetric flask. Dilute to volume with water. (10 µg/ml of each impurities, individually and 10 µg/ml of cephalexin).

System suitability :

System Suitability solution :Prepare a system suitability solution as mentioned under the procedure (section 9.2.e).

Set up the system as mentioned under the chromatographic conditions.

Inject 20 µl of the system suitability solution in duplicate and record the chromatograms upto 42 min Calculate the resolution between Δ^2-7-ADCA and Phenyl acetic acid peak.

Acceptance limit :

The mean resolution factor R between Δ^2-7-ADCA and Phenyl acetic acid peak. is not less than 4.0

9.3.4 Identification : Inject 20 µl each of the following solutions A to F (about 100 µg/ml), individually and record the chromatograms upto 42 min Note the retention time of each component for identification.

Solution A : Transfer 5mg of Phenyl glycine into a 25 ml volumetric flask, dissolve and dilute to volume with water.

Solution B : Transfer 5mg of 7-ADCA into a 25 ml volumetric flask, dissolve in 1N HCl and dilute to volume with water.

Solution C : Transfer 5mg of Δ^2-7-ADCA into a 25 ml volumetric flask dissolve in 1N HCl and dilute to volume with water.

Solution D : Transfer 5mg of Phenyl acetic acid into a 25 ml volumetric flask, dissolve and dilute to volume with water.

Solution E · Transfer 5mg of cephalexin into a 25 ml volumetric flask, dissolve and dilute to volume with water.

Solution F . Transfer 5mg of 7-PADCA into a 25 ml volumetric flask, dissolve in 1 ml methanol and dilute to volume with water.

9.3.5 Instrument precision :

System precision solution :

Transfer about 50 mg accurately weighed cephalexin WRS into a 50 ml volumetric flask Pipette out 2.0 ml of Stock Solution 1 into it Dissolve in and dilute upto mark with diluent. (1000 µg/ml of cephalexin and 10 µg/ml each of impurities individually).

i) Set up the system as mentioned under the chromatographic conditions.

ii) Inject 20 µl of the system precision solution six times and record the chromatograms upto 42 min

iii) Calculate the relative standard deviation for the detector response for each component individually.

Acceptance limit :

RSD (%) of detector response for each component is not more than 5.0 %

Solution stability :

Stability solution preparation :

Transfer 5ml each of stock solution 1 and stock solution 2 into a 10ml volumetric flask, mix and dilute to volume with diluent.

- Inject 20 µl of the stability solution in duplicate periodically and record the chromatograms upto 42 min.

- Calculate the relative standard deviation of the detector response for each component individually over the entire period.

Acceptance limit :
RSD (%) of detector response for each component is not more than 5 0 %

9.3.6 Method precision :

i) Prepare a sample solution as directed under the procedure (about 1000 µg/ml)

ii) Set the chromatographic conditions as mentioned under the method, inject 20 µl of the standard solution in duplicate and record the chromatograms upto 42 min.

iii) Inject 20 µl of standard solution in duplicate and record the chromatograms upto 42 min Use this for calculations.

iv) Inject 20 µl of the sample solution in duplicate and record the chromatograms upto 42 min.

v) Calculate the amount of the impurities present in the sample

vi) Prepare six sets of this sample as directed under the method Spike impurities upto the target levels in each of the sample preparation

vii) Inject 20 µl of each sample preparation in duplicate into the chromatograph set to the condition mentioned under the method and record the chromatograms upto 42 min

viii) Calculate the amount of each impurity from the six sets subtract the amount of any impurity already present. Calculate the RSD of each impurity percentage (corrected values) from the six sets.

Acceptance limit :
RSD (%) of the calculated impurities in the six sets $\leq$ 5.0 %.

Linearity and range :

i) Use solution 1 and 2 (mentioned under section 9.3.2) for preparing the following linearity solutions

ii) Linearity solutions.

Table No.9.3.6.1. Dilution and concentration for Linearity study

Level	*% of target*	*Vol. of. Solution 1*	*Vol. of. Solution 2*	*Final Dilution*	*Final concentration of each impurity, individually and cephalexin*
		ml	*ml*	*ml*	*µg/ml*
L1	50	1.0	1.0	50	5.0
L2	75	1.5	1.5	50	7.5
L3	100	2.0	2.0	50	10.0
L4	125	2.5	2.5	50	12.5
L5	150	3.0	3.0	50	15.0

iii) Inject 20 µl each of the linearity solution, in triplicate, into the chromatographic system set to the conditions mentioned under the method and record the chromatograms upto 42 min.

iv) Calculate the mean and RSD (%) of the detector responses for each linearity level individually of each component.

v) Plot a graph of the concentration versus mean area count and perform mathematical regression analysis for each component individually.

Acceptance limits :

RSD (%) of area counts for individual components at each level ≤ 5 0 %

Plot of concentration versus detector response for each component is linear. The regression correlation coefficient (r) ≥ 0.99

9.3.7 Accuracy :

Use the solution 1 (250 µg/ml each of impurity as mentioned under section 9.3.2) for preparing the following solutions.

i) Prepare five sets for five level (70, 85, 100, 115 and 130% of target concentration) recovery study by transferring about 50mg, accurately weighed, cephalexin standard into five 50 ml volumetric flasks separately. Pipette out appropriate volumes of Solution 1 as shown in the Table 9.3.7.1 and dilute to volume with water. (Solution 1 = 250 µg/ml of each impurity)

Table 9.3.7.1. Dilution and concentration for Accuracy study

Level	*% of target*	*Vol. of. Solution 1*	*Final Dilution*	*Final concentration of each impurity, individually*
		ml	*ml*	*µg/ml*
R1	70	1.4	50	7.0
R2	85	1.7	50	8.5
R3	100	2.0	50	10.0
R4	115	2.3	50	11.5
R5	130	2.6	50	13.0

ii) Inject 20 µl of standard solution, prepared as mentioned in section 9.3.3, in triplicate and record the chromatograms upto 42 min. Calculate the mean area counts of the components in the standard solution.

iii) Inject 20 µl each of the recovery solution R1, R2, R3, R4 and R5 into the chromatograph in triplicate and record the chromatograms upto 42 min.

v) Calculate the mean and RSD (%) of the detector responses for each component from each recovery set.

vi) Calculate the amount of each spiked impurity in each set of the recovery sample and calculate the percentage recovery.

Acceptance limit :

Percentage recovery is not less than 95.0 % and not more than 105.0%. RSD (%) of detector response for each component is ≤ 5.0 %.

9.3.8 Minimum Quantitation level and Minimum Detection level

Prepare a stock solution for this study by pipetting out each of 1.0 ml of Solution 1& Solution 2 (10 µg/ml each of impurities + Cephalexin) into a 50ml volumetric flask. Dilute to volume with diluent [5 µg/ml each component].**(MDL stock solution)**

Prepare subsequent diluted solutions as shown in the table below and inject them in triplicate and record the chromatograms upto 42 min.

Calculate the RSD (%) of the triplicate injections for each level.

Table 9.3.8.1. Dilution and concentration for Limit of detection and limit of quantitation study

Level	*Vol. Of soln MDL stock solution (ml)*	*Final dilution (ml)*	*Final concentration of each impurity, individually (µg/ml)*
D1	-	-	5.000
D2	5.0	10	2.500
D3	2.5	10	1.250
D4	1.2	10	0.600
D5	0.6	10	0.300
D6	0.3	10	0.150
D7	0.1	10	0.050
D8	0.1	25	0.020
D9	0.1	50	0.010

Acceptance limit :

The MQL of each component is the lowest concentration at which the RSD (%) of the triplicate injections ≤ 5.0 %.

The MDL of each component is that concentration at which the detector shows a positive response.

9.3.9 Ruggedness of the method :

A sample previously analysed for related substances is reanlaysed by another analyst independently by this method and the results are compared.

9.4 Experimental Data

Reagents and chemicals :

1.Potassium dihydrogen orthophosphate : AR grade (S.d. Fine Chem.)

2.Di-potassium hydrogen orthophosphate : AR grade (S.d. Fine Chem.)

3 Methanol : HPLC grade (Merck)

4. Water : Milli-Q grade

5. Orthophosphoric acid : AR grade (S.d. Fine Chem.)

Working standards and sample :

Cephalexin WRS : # ws 04 (Supplied by Gujarat Lyka Labs.)

Phenyl glycine : (Supplied by Gujarat Lyka Labs.)

7-ADCA	: # ws 08 (Supplied by SPARC Org. Synth)
Δ^2-7-ADCA	: (Supplied by Gujarat Lyka Labs.)
Phenyl acetic acid	: (Supplied by Gujarat Lyka Labs.)
7-PADCA	: (Supplied by Gujarat Lyka Labs.)
Cephalexin	Sample. # 221/2000 (Supplied by Gujarat Lyka Labs.)

9.4.1 Chromatographic system :

Column	.	4 6 mm x 25 cm, 5 µm, Hypersil C-18, BDS (Thermoquest, U.K)
Detector	:	UV-220 nm
Injection volume	:	20 µl

The system is also equipped to deliver the two phases in a programmed manner as shown in the Table 9.4.1.1 .

Total flow rate : 1.0 ml / min.

Table 9.4.1.1 : Gradient programme

Time (in min.)	*Mobile Phase A (per cent v/v)*	*Mobile Phase B (per cent v/v)*
0	100	0
10	100	0
20	85	15
35	40	60
36	100	0
42	100	0

- Two Shimadzu LC-10AT$_{vp}$ solvent delivery pumps with SIL-10AD$_{vp}$Autoinjector
- Shimadzu SPD-10A$_{vp}$ UV detector with CLASS vp software.

9.4.2 Mobile phase :

Mobile phase A : Transfer 5.25g di-pottasium hydrogen orthophosphate and 29 9g. potassium dihydrogen orthophosphate transferred into a 5.0 lit. beaker. Dissolved it into a1000ml milli Q water by sonication, then 4000ml of milli Q water was added and

mixed. The pH of the solution was adjusted to 6.01 by orthophosphoric acid (85%). Filtered and degassed.

Mobile phase B:2000ml of HPLC grade methanol. Filtered and degassed and used for the analysis.

9.4.3 Stock Solutions :

Diluent :36.0g. potassium dihydrogen orthophosphate was transferred into a 2000ml milli Q water Dissolved it and filtered.

Solution 1 : 25.06mg of α-phenyl glycine, 25.14mg of 7-ADCA, 25.01mg of Δ^2-7-ADCA, 25.26mg of phenyl acetic acid and 25.36 mg 7-PADCA was transferred into a 100ml volumetric flask, dissolved it in 2 ml 1M hydrochoric acid ,2ml diluent and 2ml methanol (sonicated) and dilute to volume with diluent (250.06µg/ml of α-phenyl glycine,251.4 µg/ml of 7-ADCA, 250 1 µg/ml Δ^2-7-ADCA, 252.6µg/ml of phenyl acetic acid and 253.6µg/ml of 7-PADCA.

Solution 2 : 25.02 mg of cephalexin WRS was transferred into a 100ml volumetric flask, dissolved it and diluted it upto the mark with diluent. (250.2 µg/ml of cephalexin)

Standard solution: 2.0 ml of solution 1 and solution 2 was pipetted out into a 50 ml volumetric flask. Diluted to volume with diluent. (10 µg/ml of each impurity, individually and 10 µg/ml of cephalexin).

9.4.4 System suitability :

System Suitability solution :5.24mg of Δ^2-7-ADCA and 5.14mg of Phenyl acetic acid transferred into a 25 ml volumetric flask. Dissolved and diluted to volume with diluent.

- Set up the system as mentioned under the chromatographic conditions.
- Injected 20 µl of the system suitability solution in duplicate and recorded the chromatograms upto 42 min.

Calculated the resolution between Δ^2-7-ADCA and phenyl acetic acid peak.

Acceptance limit :

The mean resolution factor R between Δ^2-7-ADCA and phenyl acetic acid peak is not less than 4.0 .

9.4.5 Identification :

Stock solutions for identification :

Solution A : Transfer 5.02mg of α-Phenyl glycine into a 25 ml volumetric flask, dissolve and dilute to volume with diluent.

Solution B : Transfer 5.11mg of 7-ADCA into a 25 ml volumetric flask, dissolve in 1ml 1N hydrochloric acid and dilute to volume with diluent.

Solution C : Transfer 5.06mg of Δ^2-7-ADCA into a 25 ml volumetric flask, dissolve in 1N HCl and dilute to volume with diluent.

Solution D : Transfer 5.05mg of Phenyl acetic acid into a 25 ml volumetric flask, dissolve and dilute to volume with diluent.

Solution E : Transfer 5.15mg of cephalexin into a 25 ml volumetric flask, dissolve and dilute to volume with diluent.

Solution F : Transfer 5.09mg of 7-PADCA into a 25 ml volumetric flask, dissolve in 1ml methanol and dilute to volume with diluent.

Injected 20 µl each of the impurity stock solutions A to F (about 200 µg/ml), individually in duplicate and recorded the chromatograms upto 42 min. Noted the retention time of components for identification.

9.4.6 Instrument precision :

System precision solution :Transferred 49.96mg, of cephalexin WRS into a 50 ml volumetric flask. Pipetted out 2.0 ml each of stock solution 1 into it. Dissolved in and diluted upto mark with diluent. (999 2 µg/ml of cephalexin and about 2 µg/ml of each of impurity).

Table 9.4.6.1 Dilutions & concentrations for system precision

Vol. of stock solution 1 (ml)	*Wt. Of Cephalexin (mg)*	*Final dilution (ml)*	*Final Concentrations*		
			α-Phenyl glycine µg/ml	*7-ADCA µg/ml*	*Δ^2-7-ADCA µg/ml*
2.0	49.96	50	10.024	10 056	10.004

Contd.

Vol. of stock solution 1 (ml)	*Wt. of Cephalexin (mg)*	*Final dilution (ml)*	*Final Concentrations*		
			Phenyl acetic acid µg/ml	*7-PADCA µg/ml*	*Cephalexin µg/ml*
2 0	49.96	50	10.104	10.144	999 2

i) Set up the system as mentioned under the chromatographic conditions.

ii) Injected 20 µl of the system precision solution for six times and recorded the chromatograms upto 42 min.

iii) Calculated the relative standard deviation for the detector response for each component.

Acceptance limit:

%RSD of detector response for each component is not more than 5.0

9.4.7 Solution stability :

Stability study solution preparation:

Transferred 5ml each of solution 1 and solution 2 into a 10ml volumetric flask, mixed and diluted upto the mark with diluent

i) Injected 20 µl of the stability study solution in duplicate, periodically and recorded the chromatograms upto 42 min.

Calculated the relative standard deviation for the detector response for each component over the period.

Acceptance limit:

RSD (%) of detector response for each component is not more than 5.0 %

9.4.8 Method precision :

i) Prepared a sample solution as directed under the procedure (about 1000 µg/ml)

ii) Set the chromatographic conditions as mentioned under the method, injected 20 µl of the system suitability in duplicate and recorded the chromatograms upto 42 min.

iii) Injected 20 µl of standard solution in duplicate and recorded the chromatograms upto 42 min. Used this for calculations.

iv) Injected 20 µl of the sample solution in duplicate and recorded the chromatograms upto 42 min.

v) Calculated the amount of the impurities present in the sample.

vi) Prepared six sets of this sample as directed under the method. Spiked impurities upto the target levels in each of the sample preparation.

vii) Injected 20 µl of each sample preparation in duplicate into the chromatograph set to the condition mentioned under the method and recorded the chromatograms upto 42 min .

viii) Calculated the amount of each impurity from the six sets. Calculated the RSD (%) of each impurity percentage from the six sets.

Acceptance limit:

RSD of the calculated impurities in the six sets should be ≤ 5.0%.

9.4.9 Linearity and range :

i) Used **Solution 1 & Solution 2** (mentioned under section 9.4.3) for preparing the following linearity solutions.

ii) Linearity solutions :

Table 9.4.9.1 Dilutions & concentrations for Linearity study

Level	*% of target*	*Vol. of. solution 1*	*Vol. of. solution 2*	*Final dilution*	*Final Concentrations*		
		ml	*ml*	*ml*	*α-Phenyl Glycine µg/ml*	*7-ADCA µg/ml*	*Δ^2-7-ADCA µg/ml*
L1	50	1.0	1.0	50.0	5.01	5.03	5.00
L2	75	1.5	1 5	50.0	7 52	7.54	7.50
L3	100	2.0	2 0	50.0	10.02	10.06	10.00
L4	125	2.5	2 5	50.0	12.53	12.57	12.51
L5	150	3.0	3.0	50.0	15.04	15.08	15 01

Level	*% of target*	*Vol. of. solution 1*	*Vol. of. solution 2*	*Final dilution*	*Final Concentrations*		
		ml	*ml*	*ml*	*α-Phenyl Glycine µg/ml*	*7-ADCA µg/ml*	*Δ^2-7-ADCA µg/ml*
L1	50	1.0	1.0	50.0	5.01	5.03	5.00
L2	75	1 5	1.5	50.0	7.52	7.54	7.50
L3	100	2.0	2.0	50.0	10.02	10.06	10.00
L4	125	2.5	2.5	50.0	12.53	12.57	12.51
L5	150	3.0	3 0	50.0	15.04	15.08	15.01

Contd....

Level	*% of target*	*Vol. of. solution 1*	*Vol. of. solution 2*	*Final Dilution*	*Final Concentrations*		
		ml	*ml*	*ml*	*Phenyl acetic acid µg/ml*	*7-PADCA µg/ml*	*Cephalexin µg/ml*
L1	50	1.0	1.0	50.0	5.05	5.07	5.00
L2	75	1.5	1.5	50.0	7.58	7.61	7.51
L3	100	2.0	2.0	50.0	10.10	10.14	10.01
L4	125	2.5	2.5	50.0	12.63	12.68	12.51
L5	150	3.0	3.0	50.0	15.16	15.22	15.01

Injected 20 µl each of the linearity solution, in triplicate, into the chromatographic system set to the conditions mentioned under the method and record the chromatograms upto 42 min.

Calculated the mean and RSD (%) of the detector responses for each linearity level, individually, for each component.

v) Plotted a graph of the concentration versus mean area count and performed mathematical regression for each component individually.

Acceptance limits :

RSD (%) of area counts at for each level for individual components ≤ 5.0 %

Plot of concentration versus detector response for each component is linear. The regression correlation coefficient (r) ≥ 0.99

9.4.10 Accuracy :

Used the **Solution 1** (about 10 µg/ml each of impurity as mentioned under section 9.4.3) for preparing the following solutions.

i) Prepared five sets for five level (70, 85, 100, 115 and 130% of target concentration) recovery study by transferring about 50 mg, accurately weighed, cephalexin working standard into five 50ml volumetric flasks separately. Pipetted out appropriate volumes of Solution 1 as shown in the Table 9.4.10.1 and diluted to volume with mobile phase.

Solution 1

Component	*α-Phenyl glycine*	*7-ADCA*	*Δ^2-7-ADCA*	*Phenyl acetic acid*	*7-PADCA*
Conc. (µg/ml)	250.6	251.4	250.1	252.6	253.6

Table 9.4.10.1 Dilutions & concentrations for Accuracy study

level	*% of target*	*Vol. of. Solution 1*	*Wt of Cephalexin*	*Final Dilution*	*Final Concentrations(µg/ml)*		
		ml	*mg*	*ml*	*α-Phenyl glycine*	*7-ADCA*	*Δ^2-7-ADCA*
R1	70	1.4	50.09	50	7.017	7.039	7.003
R2	85	1.7	50.41	50	8.520	8.548	8.503
R3	100	2.0	50.11	50	10.024	10.056	10.004
R4	115	2.3	50 24	50	11.528	11.564	11.505
R5	130	2.6	50.07	50	13.031	13.073	13.005

Contd....

Sr. No.	*Level % of target*	*Vol. of. Soln. 1*	*Wt of Cephalexin*	*Final Dilution*	*Final Concentrations (µg/ml)*		
		ml	*mg*	*ml*	*Phenyl acetic acid µg/ml*	*7--PADCA µg/ml*	*Cephalexin µg/ml*
R1	70	1.4	50.09	50	7.073	7.101	1001.8
R2	85	1.7	50.41	50	8.588	8.622	1008.2
R3	100	2.0	50.11	50	10.104	10.144	1002.2

R4	115	2.3	50.24	50	11.620	11.666	1004.8
R5	130	2.6	50.07	50	13.135	13.187	1001.4

ii) Injected 20 µl of standard solution, prepared as mentioned 3.6, in triplicate and recorded the chromatograms upto 42 min. Calculate the mean area counts of the standard.

iii) Injected 20 µl each of the recovery solution R1, R2, R3, R4 and R5 into the chromatograph in triplicate and recorded the chromatograms upto 42 min.

vi) Calculated the mean and RSD (%) of the detector responses for each set.

vii) Calculated the amount of each spiked impurity in each set of the recovery sample and calculated the percentage recovery.

Acceptance limit :

a) Percentage recovery not more than 95.0 % and not less than 105.0 %.

b) RSD (%) of detector response for each component is not more than 5.0 %.

9.4.11 Minimum Quantitation level and Minimum Detection level

i) Prepared a stock solution for this study by pipetting out 1 ml of Solution 1 & Solution 2 (About 250 µg/ml each of impurity and cephalexin as mentioned under section 3.6) into a 50ml volumetric flask. Diluted to volume with diluent [about 5 µg/ml each component]-MDL stock solution.

Prepared subsequent diluted solutions as shown in the Table 9.4.11.1 and injected them in triplicate and recorded the chromatograms upto 42 min.

Table 9.4.11.1 Dilutions & concentrations for Minimum Quantitation level and Minimum Detection level

Level	*Vol. of MDL stock soln (ml)*	*Final Dilution (ml)*	*Final Concentrations(µg/ml)*		
			α-Phenyl glycine	*7-ADCA*	*Δ^2-7-ADCA*
D1	10	10	5.012	5 028	5.002
D2	5.0	10	2 506	2.514	2.501
D3	2.5	10	1.253	1.257	1.251
D4	1.20	10	0.601	0.603	0.600
D5	0.6	10	0.301	0.302	0.300
D6	0.3	10	0.150	0 151	0.150
D7	0.1	10	0 050	0.050	0.050

Contd....

Level	*Vol. of soln L (ml)*	*Final Dil. (ml)*	*Final Concentrations(µg/ml)*		
			Phenyl acetic acid	*7-PADCA*	*Cephalexin*
D1	10	10	5.052	5 072	5 004
D2	5 0	10	2 526	2.536	2 502
D3	2.5	10	1.263	1 268	1.251
D4	1 20	10	0 606	0 609	0 600
D5	0.6	10	0.303	0.304	0.300
D6	0.3	10	0.152	0 152	0.150
D7	0 1	10	0 051	0.051	0 050

iii) Injected 20 µl each of the into the chromatograph in triplicate and recorded the chromatograms upto 42 min

iv) Calculated the RSD (%) of the triplicate injections for each level

Acceptance limit :

The MQL of each component is the lowest concentration at which the RSD (%) of the triplicate injections not more than 5.0 %. The MDL of each component is that concentration at which the detector shows a positive response.

9.4.12 Ruggedness of the method :

A sample previously analysed for related substances was re-analyzed by another analyst independently by this method and the results are compared.

Acceptance limit :The difference in the results of the two analysis is not more than 5.0 % of the impurity limit.

9.5 Results & Discussions :

9.5.1 Identification :20 µl of solution A to solution I are injected individually and the chromatograms recorded upto 42 min.

Results & discussion :

Figure-182 to Figure-187 shows typical chromatograms of individual components.

Component		Approx. RT
α-Phenyl glycine	:	5 min
7-ADCA	:	8 min
Δ^2-7-ADCA	.	20 min
Phenyl Acetic acid	.	23 min
Cephalexin	.	31 min
7-PADCA	:	36 min

The above results show that all the components are clearly separated and identifiable.

9.5.2 System suitability :

Before starting a set of analysis, the system suitability solution is injected in duplicate. The resolution between the Δ^2-7-ADCA and Phenyl acetic acid is calculated.

Results & discussion :

Figure-181 shows a typical system suitability chromatogram. Figure-180 represents the typical blank chromatogram.

The Resolution factor R between Δ^2-7-ADCA and Phenyl acetic acid = 5.08

[Limit: NLT 4.0]

As the resolution meets the system suitability requirements the chromatographic system was used for further studies

9.5.3 Instrument precision :

System Precision Solution :

i) Set up the system as mentioned under the chromatographic conditions.

ii) Injected 20 µl of the system precision solution six times and recorded the chromatograms upto 42 min.

Individual area counts and RSD (%) values are shown in Table 9.5.3.1:

Results and Discussion :

Figure-191 shows a typical system precision chromatogram.

Table 9.5.3.1: Instrument precision

Injection	*Detector Response (Area counts)*		
	α-Phenyl glycine	*7-ADCA*	*Δ^2-7-ADCA*
1	229878	248898	312282
2	226633	244669	309276
3	228784	246285	311170
4	230990	248757	314102
5	229409	246315	311985
6	229972	246461	312500
Mean	229278	246898	311886
SD (±)	1485	1633	1599
RSD (%)	0.65	0.66	0.51

Contd.

Injection	*Detector Response (Area counts)*		
	Phenyl acetic acid	*7-PADCA*	*Cephalexin*
1	317097	348366	18838179
2	320093	345058	18597662
3	314030	347658	18768116
4	326978	352196	18684481
5	313150	350439	18629999
6	315007	351622	18705106
Mean	317726	349223	18703924
SD (±)	5169	2706	88635
RSD (%)	1.63	0.77	0.47

Acceptance limit :

RSD not more than 5.0 %.

Solution stability study :

Solution stability preparation:

Prepared as mentioned under section 9.3.10.

i) Injected 20 µl of the stability study solution in duplicate, periodically and recorded the chromatograms upto 42 min

Calculated the relative standard deviation for the detector response for each component over the period.Individual area counts and RSD (%) values are shown in Table below:

Time (in Hours)	*Detector Response (Area counts)*		
	α-Phenyl glycine	*7-ADCA*	*Δ^2-7-ADCA*
Initial	2518775	3035249	3698415
17 hrs.	2609571	3073948	3757267
30 hrs.	2513906	3036847	3574995
Mean	2547417	3048681	3676892
SD (±)	53882	21896	93023
RSD (%)	2.11	0.72	2.53

Contd.

Time (in Hours)	*Detector Response (Area counts)*		
	Phenyl acetic acid	*7-PADCA*	*Cephalexin*
Initial	3575197	3086498	3560901
17 hrs.	3650861	3027002	3609845
30 hrs.	3540365	2815953	3530268
Mean	3567004	2976484	3567005
SD (±)	40138	142171	40138
RSD (%)	1.13	4 78	1.13

Figure-129 represents solution stability study chromatogram

Acceptance limit:

RSD (%) of detector response for each component is not more than 5.0 %

The above results are well within the acceptance limits and indicates instrument precision. Figure-192 represents method precision chromatogram

[Limit : RSD % impurities calculated from the six sets is NMT 5.0 %]

9.5.5 Linearity and range :

The linearity of detector (UV) response for impurities was determined by preparing and injecting solutions in the concentration range of 50-150 % of limit conc. are of α-Phenyl glycine, 7-ADCA, Δ^2-7-ADCA, 7-PADCA, Phenyl acetic acid and Cephalexin.

Linearity solutions were prepared as under described under Section 9.4.12 (Table 9.4.12 1)

Results and discussion :

Figure-193 represents Linearity chromatogram, Figure-197 to 202 shows typical linearity plots for various impurities. The results of individual impurities is shown in Table 4.6.1 to 4.6.6

Acceptance limits:

RSD (%) of area counts at for each level for individual components not more than 5.0 % Plot of concentration versus detector response for each component is linear. The regression correlation coefficient (r) not less than 0.99

Table 9.5.5.1 : Linearity of α-Phenyl glycine

Level	*Detector response (area counts)*				
	Inj. 1	*Inj. 2*	*Inj. 3*	*Mean*	*RSD (%)*
L1	111510	115309	113928	113582	1.69
L2	171985	173017	171678	172227	0.41
L3	226640	229804	225878	227441	0.92
L4	268902	277992	283404	276766	2.65
L5	323955	340444	341082	335160	2.90
Slope				21847	
Intercept				6044.6	
Correlation coefficient (r)				0.9992	

Table 9.5.52 : Linearity of 7-ADCA

Level	*Detector response (area counts)* 5				
	Inj. 1	*Inj. 2*	*Inj. 3*	*Mean*	*RSD (%)*
L1	118896	123409	122496	121600	1.96
L2	182491	183737	182830	183019	0.35
L3	239917	244034	240923	241625	0.89
L4	288148	297120	301524	295597	2.31
L5	346134	364490	363130	357918	2.86
Slope				23287	
Intercept				5773.6	
Correlation coefficient (r)				0.9995	

Table 9.5.5.3 : Linearity of Δ^2-7-ADCA

Level	*Detector response (area counts)*				
	Inj. 1	*Inj. 2*	*Inj. 3*	*Mean*	*RSD (%)*
L1	153740	158755	159207	157234	1.93
L2	234526	235082	234072	234560	0.22
L3	306932	312090	308468	309163	0.86
L4	369531	381916	389356	380268	2.63
L5	443248	468151	470099	460499	3.25
Slope				30053	
Intercept				7692.70	
Correlation coefficient (r)				0.9997	

Table 9.5.5.4 : Linearity of Phenyl acetic acid

Level	*Detector response (area counts)*				
	Inj. 1	*Inj. 2*	*Inj. 3*	*Mean*	*RSD (%)*
L1	145526	153846	154156	151176	3.24
L2	225404	227163	227316	226628	0.47
L3	294006	301658	298041	297902	1 28
L4	355758	345783	374170	358570	4.02
L5	427288	448578	441858	439241	2.48
Slope				28020	
Intercept				11585	
Correlation coefficient (r)				0.9985	

Table 9.5.5.5 : Linearity of 7-PADCA

Level	*Detector response (area counts)*				
	Inj. 1	*Inj. 2*	*Inj. 3*	*Mean*	*RSD (%)*
L1	134636	137627	135507	135923	1.13
L2	199162	200241	197266	198890	0.76
L3	255676	258079	260608	258121	0.96
L4	320146	330596	333558	328100	2.15
L5	404493	405926	407616	406012	0.39
Slope				26386	
Intercept				-2247.9	
Correlation coefficient (r)				0.997	

Table 9.5.5.6 : Linearity of Cephalexin

Level	*Detector response (area counts)*				
	Inj. 1	*Inj. 2*	*Inj. 3*	*Mean*	*RSD (%)*
L1	156637	160526	153987	157050	2.09
L2	225079	224417	222877	224124	0.50
L3	293994	299446	293962	295801	1.07
L4	350656	361937	366435	359676	2.26
L5	418031	439570	438258	431953	2.80
Slope				27392	
Intercept				19578	
Correlation coefficient (r)				0.9997	

9.5.6 Accuracy :

Recovery study was performed at 70, 85, 100, 115 and 130 % levels of 1 % each of α-Phenyl glycine, 7-ADCA, Δ^2-7-ADCA, Phenyl acetic acid, 7-PADCA and Cephalexin).

Table 9.5.6.1 : Recovery of α-Phenyl glycine from Cephalexin

Sr. No.	*Level (%)*	*Actual amount added (µg/ml)*	*Amount recovered (µg/ml)*	*% Recovery = Amt found / Amt. added x 100*
R1	70	7.017	7.132	101.64
R2	85	8.520	8.577	100.66
R3	100	10 024	10.124	101.00
R4	115	11.528	11.729	101.74
R5	130	13.031	13.305	102.10
Mean				101.43
RSD (%)				0.58

Table 9.5.6.2 : Recovery of 7-ADCA from Cephalexin

Sr. No.	*Level (%)*	*Actual amount added (µg/ml)*	*Amount recovered (µg/ml)*	*% Recovery = Amt found ---------- x 100 Amt. Added*
R1	70	7.039	7.150	101.58
R2	85	8 548	8.611	100.75
R3	100	10.056	10.111	100.55
R4	115	11.564	11.758	101.67
R5	130	13.073	13.136	100.48
Mean				101.00
RSD (%)				0.57

Table 9.5.6.3 : Recovery of Δ^2-7-ADCA from Cephalexin

Sr. No.	*Level (%)*	*Actual amount added (µg/ml)*	*Amount recovered (µg/ml)*	*% Recovery = Amt found ---------- x 100 Amt. added*
R1	70	7.003	7.071	100.98
R2	85	8.503	8.566	100.74
R3	100	10.004	10 046	100.42
R4	115	11 505	11.535	100.26
R5	130	13.005	13.109	100.80
Mean				100.64
RSD (%)				0.29

Table 9.5.6.4 : Recovery of Phenyl acetic acid from Cephalexin

Sr. No.	*Level (%)*	*Actual amount added (µg/ml)*	*Amount recovered (µg/ml)*	*% Recovery = Amt found ---------- x 100 Amt. added*
R1	70	7 073	7.110	100.53
R2	85	8 588	8.308	96.74
R3	100	10.104	10.407	103.00
R4	115	11.666	11.181	96.22
R5	130	13.187	12.900	98.21
Mean				98.94
RSD (%)				2.85

Table 9.5.6.5 : Recovery of 7-PADCA from Cephalexin

Sr. No.	*Level (%)*	*Actual amount added (µg/ml)*	*Amount recovered (µg/ml)*	*% Recovery = Amt found ---------- x 100 Amt. added*
R1	70	7.101	7.283	102.56
R2	85	8.622	8.689	100.77
R3	100	10.144	10.067	99.24
R4	115	11.666	11.510	98.67
R5	130	13.187	13.127	99.54
Mean				100.16
RSD (%)				1.55

[Limit : Recovery - 95.0 % - 105.0 %]

Figure-133 represents recovery experimen

9.5.7 Limit of Detection and Quantitation :

1.0 ml each of solution 1 and solution 2 was taken into a 50 ml volumetric flask, diluted to volume with diluent. This was used as MDL stock solution. Further dilutions were made and injected in triplicate into the chromatography.

Table 9.5.7.1 to 9.5.7 7 shows the results of the study.

Results & discussion :

Table 9.5.7.1 : Limit of Detection and Limit of Quantitation Study (α-Phenyl glycine)

Level	*Conc. (µg/ml)*	*Inj. 1*	*Inj. 2*	*Inj. 3*	*Mean area*	*RSD (%)*
D1	5.012	112169	113831	114466	113489	1.05
D2	2.506	56472	55631	55812	55972	0.79
D3	1.253	28944	29744	29017	29235	1.51
D4	0.601	14505	14409	14220	14378	1.01
	0.301	7032	7259	7392	7228	2.52
	0.150	4238	3085	3226	3516	17.89
D7	0.050	-	-	-	-	-

Table 9.5.7.2 : Limit of Detection and Limit of Quantitation Study (7-ADCA)

Level	*Conc. (µg/ml)*	*Inj. 1*	*Inj. 2*	*Inj. 3*	*Mean area*	*RSD (%)*
D1	5.028	123422	125133	125688	124748	0.95
D2	2.514	61724	60605	60664	60997	1.03
D3	1 257	31020	31796	31841	31552	1.46
D4	0.603	15143	15361	15070	15191	0.99
	0.302	6949	6910	7101	6987	1.44
	0.151	2798	2424	2715	2645	7.42 *
D7	0.050	-	-	-	-	-

Table 9.5.7.3 : Limit of Detection and Limit of Quantitation Study (Δ^2-7-ADCA)

Level	*Conc. (µg/ml)*	*Inj. 1*	*Inj. 2*	*Inj. 3*	*Mean area*	*RSD (%)*
D1	5.002	148572	150386	151281	150080	0.92
D2	2 501	74018	72967	73428	73471	0.72
D3	1.251	37886	37979	35156	37007	4.33
D4	0.600	18426	18625	18228	18426	1.08
	0.300	8447	7842	6959	7749	9.65 *
	0.150	3418	3942	3746	3702	7.15
D7	0.050	-	-	-	-	-

Table 9.5.7.4 : Limit of Detection and Limit of Quantitation Study (Phenyl acetic acid)

Level	*Conc. (µg/ml)*	*Inj. 1*	*Inj. 2*	*Inj. 3*	*Mean area*	*RSD (%)*
D1	5.052	143946	144866	138919	142577	2.24
D2	2.526	63752	63856	59978	62529	3.53
D3	1.263	10750	20812	14343	15302	33.32*
D4	0.606	-	-	-	-	-

Table 9.5.7.5 : Limit of Detection and Limit of Quantitation Study (7-PADCA)

Level	*Conc. (µg/ml)*	*Inj. 1*	*Inj. 2*	*Inj. 3*	*Mean area*	*RSD (%)*
D1	5.072	130243	131404	132696	131448	0.93
D2	2.536	66246	65330	65968	65848	0.71
D3	1.268	28180	29269	29072	28840	2.01
D4	0.609	14140	14076	11760	13325	10.17*
	0.304	7674	7835	8112	7873	2.81
	0.152	-	-	-	-	-

Table 9.5.7.6 : Limit of Detection and Limit of Quantitation Study (Cephalexin)

Level	*Conc. (µg/ml)*	*Inj. 1*	*Inj. 2*	*Inj. 3*	*Mean area*	*RSD (%)*
D1	5.004	158005	159162	158718	158628	0.37
D2	2.502	81065	80120	80395	80527	0.60
D3	1.251	45730	46461	41175	44455	6.44*
D4	0.600	26179	26467	26439	26362	0.60
	0.300	11969	16433	16082	14828	16.74

The limit of quantitation and detection for each impurity is as follows :

Table 9.5.7.7 : Limit of Detection and Limit of Quantitation Summary

	Limit of quantitation		*Limit of detection*	
	(µg/ml)	*(%)*	*(µg/ml)*	(%)
α-Phenyl glycine	0.301	0.03	0 15	0.02
7-ADCA	0.302	0.03	0.15	0.02
Δ^2-7-ADCA	0.600	0.06	0.15	0.02
Phenyl acetic acid	2.526	0.25	1.26	0.13
Cephalexin	2.502	0.25	0.30	0.03
7-PADCA	1.268	0.13	0.30	0.03

Figure-195 shows typical chromatogram for LOD/LOQ study.

9.5.8 Ruggedness :

Method ruggedness is established by a sample [Spiked with impurities (1% of sample concentration 1000 µg/ml i.e 10 µg/ml each of impurities)] previously analyzed for

related substances is re analyzed by another analyst independently by this method and the results are compared. Figure-196 shows typical chromatograms of ruggedness

Ruggedness-I
Analyst : NRP

Ruggedness-II
Analyst : ALP

	Cephalexin B.No:ws(04)	*Cephalexin B.No:ws(04)*
	By calculation	***By calculation***
α-Phenyl glycine	0.999 %	1.010
7-ADCA	0.974 %	0.999
Δ^2-7-ADCA	0.986 %	1.016
Phenyl acetic acid	1.024 %	1.021
7-PADCA	0.971 %	0.998

[Limit.: Difference between two sets of results should not be more than 0.05%]

9.6SUMMARY AND CONCLUSIONS :

Acceptance limit		**Actual results**		
System suitability Resolution between Delta ADCA and Phenyl acetic acid.	NLT 4.0	4.98 to 6.4		
		α-Phenyl glycine	7-ADCA	Δ^2-7-ADCA
Precision Instrument - RSD (%) of Detector Response for each impurity	≤ 5.0 %	0.65	0.66	0.51
Method - RSD (%) of each Impurity %	≤ 5.0 %	1.35	1.34	1.56
Linearity and Range Correlation coefficient (r)	≥ 0.99	0.9992	0.9995	0.9997
RSD (%) of detector responses	≤ 5.0 %	2.90	2.86	3.25
Accuracy Percentage recovery	95.0 % - 105.0 %	101.43 %	101.00 %	100.64 %
Minimum quantitation level (in %)		0.03 %	0.03 %	0.06 %
RSD (%) at MQL	≤ 5.0 %			

Contd..

		Phenyl acetic acid	7-PADCA	Cephalexin
Precision				
Instrument - RSD (%) of Detector Response for each impurity	≤ 5.0 %	1.63	0.77	0.47
Method - RSD (%) of each Impurity %	≤ 5.0 %	3 78	1.78	-
Linearity and Range				
Correlation coefficient (r)	≥ 0.99	0.9985	0.997	0 9997
RSD (%) of detector responses	≤ 5 0 %	4.02	2.15	2.80
Accuracy				
Percentage recovery	95 0 % - 105.0 %	98 94 %	100.16 %	-
Minimum quantitation level		0.25 %	0.13 %	0.25 %
RSD (%) at MQL	≤ 5.0 %			

The results of the study indicate that this method for related substances and process impurities in cephalexin is precise, accurate, linear in detector response and rugged.

9.7 Recommendations and Limitations

1.This method is recommended for the analysis of cephalexin samples for the related substances and process impurities .

2 This method shows precision, linearity and accuracy for all known impurities like α-Phenyl glycine, 7-ADCA, Δ^2-7-ADCA, Phenyl acetic acid and 7-PADCA.