CHAPTER 4

SIMULTANEOUS DETERMINATION AND VALIDATION OF HPLC METHOD FOR ESOMEPRAZOL MAGNESIUM, FENOFIBRATE AND VENLAFAXINE HCl

VALIDATION OF AN ANALYTICAL METHOD

The principal purpose of analytical method validation is to ensure that a selected analytical procedure will give reproducible and reliable results that are adequate for the intended purpose. It is thus necessary to define properly, both the conditions in which the procedure is to be used and the purpose for which it is intended.

CHARACTERISTICS OF ANALYTICAL PROCEDURES

Important characteristics that need to be specific for analytical procedures are listed below and defined, with an indication of how they may be determined. Not all the characteristics are applicable to every test procedure or to every material. Much depends on the purpose for which the procedure is required.

Accuracy

The accuracy of the procedure is the closeness of the results obtained by the procedure to the true value. Accuracy may be determined by applying the procedure to samples of the material to be examined that have been prepared with quantitative accuracy. Wherever possible, these samples should contain all the components of the material including the analyte. Samples in which the analyte has been incorporated in quantities some 10% above the expected range of the values should be prepared. Accuracy may also be determined by comparing the results with those obtained using an alternative procedure that has already been validated.

Precision

The precision of the procedure is the degree of agreement among individual test results. It is measured by scatter of individual results from the mean and it is usually expressed as the standard deviation or as the coefficient of variation (relative standard deviation) when the complete procedure is applied repeatedly to separate, identical samples drawn from the same homogenous batch of material.

Repeatability

This is the precision of the procedure when repeated by an analyst under the same set of conditions (same reagents, equipments, settings, and laboratory) and within a short interval of time. The repeatability of a procedure is assessed by carrying out complete separate determinations on separate identical samples of the same homogenous batch of material and thus provides a measure of the precision of the procedure under normal operating conditions.

Reproducibility

This is the precision of the procedure when it is carried out under different conditions usually in different laboratories, on separate, identical samples taken form the same homogenous batch of material. Comparisons of results obtained by different analyst, by the use of different equipment, or by carrying out the analysis at different times can also provide valuable information.

Linear Dynamic Range

The detector response is said to be linear if the difference in response for two concentrations of a given compound is proportional to the difference in concentration of the two samples. Such response appears as a straight line in the calibration curve. The linear dynamic range is that concentration over which the detector output is linearly related to the solute concentration. The linear dynamic range extends from the minimum detectable to that concentration where the response index is greater or less than the defined linearity limits.

Selectivity

The selectivity of a procedure is its ability to measure an analyte in a manner that is free from interference from other components in the sample being examined. Selectively may be expressed in terms of the bias of the assay results obtained when the procedure is applied to the analyte in the presence of expected levels of other components.

Limit of detection (LOD)

The limit of detection is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. Such a limit is usually expressed in terms of concentration of analyte in the sample. Where the final measurement is based on an instrumental reading due account will be needed to be taken of the background response (the signal – to – noise characteristics of the response observed). In several cases, visual inspections of the results is also used for determining LOD.

Limit of quantification (LOQ)

The limit of quantification is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analysing samples containing diminishing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. Where the final assessment is based on an instrumental reading the magnitude of background response (the signal – to – noise ratio) may be needed to be assessed and taken into account. In many cases the limit of quantification is approximately twice the limit of detection.

METHOD DEVELOPMENT FOR DETERMINATION OF ESOMEPRAZOLE MAGNESIUM, VENLAFAXINE HCl AND FENOFIBRATE IN MIXUTRE

According to the information collected from literature there is no reported method for simultaneous determination of esomeprazole, venlafaxine HCl and fenofibrate using HPLC which can be applied for detection of these drugs present in water at low concentrations. In the present work we report development and validation of a new HPLC method for simultaneous determination of esomeprazole, venlafaxine HCl and fenofibrate in a synthetic mixture. For recovery studies, treated sewage water collected from a Sewage Treatment Plant (STP), Vadodara, India was used. The new method is simple and sensitive HPLC method with total run time less than twenty minutes for the simultaneous determination of esomeprazole, venlafaxine

HCl and fenofibrate. The method has been validated and can be applied to quality control and for other analytical purposes.

EXPERIMENTS

Materials and Reagents

Same as mentioned in Chapter 3 except A.R grade formic acid and ammonium acetate were purchased from Qualigens and used as such.

Instrumentation

For HPLC *(Validation method)*

The LC system used was a Shimadzu LC 2010 C_{HT} series 200 binary pump equipped with auto sampler and UV detector. The output signal was monitored and processed using Empower software.

For MS *(Identification of API)*

Water – Micro Mass Quattro Detectors.

For LC – MS *(Identification of the target drugs environmental water sample)*

Water Alliance 2695 with PDA (996) Detector. Waters Micro Mass ZQ Mass Detector.

Conditions

For HPLC *(Validation method)*

Separation was carried out on a C18 column (150cm x 4.6mm, 3.5µm particle size), from Agilent. Mobile phase **A** contained a mixture of buffer and acetonitrile in the ratio 75:25 (*v/v*). Mobile phase **B** consisted of buffer and acetonitrile in the ratio of 30:70 (*v/v*). The buffer consists of 0.3% formic acid. The mobile phase was premixed, filtered through a 0.45µm nylon filter and degassed. The flow rate was kept at 1.1mL min^{-1} throughout. The LC gradient was time (min) / mobile phase: 0.00 / **A**,

6.01 / **B** and 15.01 / **A**. The detection was monitored at 230nm. The injection volume was 10µL.

For MS *(Identification of API)*

Mass range: 110 – 1000amu. Mode: Direct Injection with Electro Spray Ionisation (+ve ion mode). Diluent: Water: Acetonitrile (30:70).

For LC – MS *(Identification of environmental water sample)*

BDS Hypersil C8 column (250 x 4.6mm, 5µ particle size) using a mixture of acetonitrile: buffer (0.13% formic acid, 15.50% 0.1 mol L^{-1} ammonium acetate) in the ratio 25:75 (v/v) (pH 3.8) as mobile phase **A** and acetonitrile as mobile phase **B** with flow rate 1.0mL min^{-1} Gradient time table is given in Table 4.1. Mass range: 110 – 1000amu. Mode: Electro Spray Ionisation (+ve ion mode) through HPLC.

Table4.1. Validation: LC – MS gradient

Time	Mobile Phase A%	Mobile Phase B%
0	100.0	0.0
15	100.0	0.0
35	30.0	70.0
40	30.0	70.0
41	100.0	0.0
45	100.0	0.0

Environmental Sample Preparation

Environmental water samples treated as mentioned in *Chapter 2*.

Preparation of standard stock solution

Preparation of esomeprazole standard stock solution

A quantity of 41.68mg esomeprazole standard was weighed into a volumetric flask of 10mL capacity, dissolved in 5mL methanol and the volume was made upto the mark with methanol. Solution concentration was 4147.16mg L^{-1} **(Stock Solution A)**. A volume of 2.5mL **Stock Solution A** was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile. Solution

concentration was 103.79mg L^{-1} **(Solution A1).** A volume of 1.0mL **Standard Solution A1** was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile. The actual concentration of solution was 103.68mg L^{-1} **(Standard Solution A2).**

Preparation of venlafaxine HCl standard stock solution

A quantity of 40.92mg venlafaxine HCl standard was weighed into a volumetric flask of 10mL capacity, dissolved in 5mL methanol and the volume was made upto the mark with methanol. Solution concentration was 4017.54mg L^{-1} **(Stock Solution B).** A volume of 2.5mL **Stock Solution B** was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile. Solution concentration was 1017.89mg L^{-1} **(Standard solution B1).** A volume of 1.0mL **Standard Solution B1** was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile. Solution concentration was 101.79mg L^{-1} **(Standard solution B2).**

Preparation of fenofibrate standard stock solution

A quantity of 42.0mg fenofibrate standard was weighed into a volumetric flask of 10mL capacity, dissolved in 5mL methanol and the volume was made upto the mark with methanol. Solution concentration was 40179mg L^{-1} **(Stock Solution C).** A volume of 2.5mL **Stock Solution C** was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile. Solution concentration was 1044.75 **(Standard Solution C1).** A volume of 1.0mL standard solution was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile. Solution concentration was 104.48mg L^{-1} **(Standard Solution C2).**

Preparation of Mixture standard solution

A quantity of 2.5mL each of above three **Stock Solutions A, B and C,** into a volumetric flask of 10mL capacity and volume was made upto the mark with acetonitrile. The solution is called **Standard Solution (ABC).** Solution concentration

was 1036.79, 1017.89 and 1044.75mg L^{-1} for esomeprazole, venlafaxine and fenofibrate respectively.

Preparation of standard solutions for LDR

A volume of 2.5, 1.0, 0.5, 0.5 and 0.1mL **Standard Solution (ABC)** was transferred into separate volumetric flasks of 5, 5, 5, 10 and 10mL capacity individually and the volume of each flask was made upto the mark with acetonitrile.

Solutions concentration were 518.40, 207.63, 103.68, 51.84 and 10.37mg L^{-1} **(Solution D, E, F, G and H)**, respectively for esomeprazole.

Solutions concentration were 508.94, 203.58, 101.79, 50.89 and 10.18mg L^{-1} **(Solution D, E, F, G and H)**, respectively for venlafaxine.

Solutions concentration were 522.38, 208.95, 104.48, 52.24 and 10.45mg L^{-1} **(Solution D, E, F, G and H)**, respectively for fenofibrate.

Preparation of standard solution for LOD / LOQ

A volume of 1.0mL and 0.5mL **Standard Solution (ABC)** was transferred into separate volumetric flasks of 10mL capacity and the volume of each flask was made upto the mark with acetonitrile. Solution concentration were 103.68, 101.79 and 104.48mg L^{-1} for esomeprazole, venlafaxine and fenofibrate **(Solution I)** and 51.84, 50.89 and 52.24mg L^{-1} for esomeprazole, venlafaxine and fenofibrate **(Solution J)** respectively.

A volume of 0.5mL and 1.0mL of **Standard Solution (J)** were transferred into separate volumetric flasks of 25mL and 10mL capacity and the volume of each flask was made upto the mark with acetonitrile. Solutions were called **Solution (K), Solution (L)**. Solution concentrations were 1.02, 1.02 and 1.05mg L^{-1} for esomeprazole, venlafaxine and fenofibrate **(K)** and 5.18, 5.09 and 5.22mg L^{-1} for esomeprazole, venlafaxine and fenofibrate **(L)**.

Preparation of solution for precision and accuracy (fortification in environmental water sample)

Precision: Six replicates of solution **(E)** and **(H)** were injected in to HPLC and %RSD was calculated.

Esomeprazole: A quantity of 10.42mg references substances was weighed into a volumetric flask of 10mL capacity and dissolved in to methanol, sonicated for two minutes and the volume was made upto the mark with methanol. **[Stock solution (RE1), 1036.79mg L^{-1}]**

Venlafaxine: A quantity of 10.23mg reference substance was weighed into a volumetric flask of 10mL capacity and dissolved in to methanol, sonicated for two minutes and the volume was made upto the mark with methanol. **[Stock solution (RE2), 1017.885mg L^{-1}]**

Fenofibrate: A quantity of 10.50mg reference substance was weighted into a volumetric flask of 10mL capacity and dissolved in to methanol, sonicated for two minutes and the volume was made upto the mark with methanol. **[Stock solution (RE3), 1044.75mg L^{-1}]**

Thereafter a volume of 1.0mL each of Solution **(RE1, RE2, RE3)** was transferred into a volumetric flask of 10mL capacity and the volume was made upto the mark with acetonitrile **[Solution (RE4)**, concentration 103.68, 101.79, 104.48mg L^{-1} for esomeprazole, venlafaxine and fenofibrate respectively].

Fortification and preparation of sample solution

Fortification was performed at two levels, 5mg L^{-1} and 50mg L^{-1}.

5mg L^{-1} level: A volume of 0.5mL of each of **Solution (RE4)** was transferred into a volumetric flask of 10mL capacity and mixed into environmental water sample, sonicated for two minutes and the volume was made upto the mark with environmental water sample. The solution concentrations were 5.18, 5.09 and 5.22mg $L^{-1}$for esomeprazole, venlafaxine and fenofibrate respectively. **[Solution (RW1)]**.

50mg L^{-1} level : A volume of 2.5mL of **Solution (RE4)** was transferred into a volumetric flask of 5mL capacity and dissolved into water, sonicated for two minutes and the volume was made upto the mark with environmental water sample. The solution concentration were 51.84, 50.89 and 52.24mg L^{-1} for esomeprazole, venlafaxine and fenofibrate respectively. **[Solution (RW2)]**

Preparation of system suitability solution

A volume of 1.0mL **Standard Solution (ABC)** was transferred into separate volumetric flasks of 10mL capacity and the volume of each flask was made upto the mark with acetoitrile. Solution concentration was 103.68mg L^{-1} for esomeprazole, 101.79mg L^{-1} for venlafaxine, 104.48mg L^{-1} for fenofibrate **[Solution (SS)]** respectively.

Analytical Method Validation

The method was validated for specificity, precision, LOD, LOQ, Linearity dynamic range, accuracy, robustness and system suitability. The validated analytical method satisfies International Conference on Harmonisation guideline. (ICH Topic Q2 R1)

Specificity

The specificity of the method for esomeprazole, venlafaxine and fenofibrate was studied by injecting acetonitrile (solvent used for standard and sample solutions preparation), mobile phase, methanol, esomeprazole standard, venlafaxine standard and fenofibrate standard.

System Suitability

The solution (SS) was injected on to HPLC in six replication and %RSD was calculated for retention time and peak area of esomeprazole, venlafaxine and fenofibrate separately.

Linear dynamic range (LDR)

The **Standard Solutions (D, E, F, G and H)** were injected onto the HPLC in two replications and the mean areas were plotted against concentration (mg L^{-1}). The correlation co – efficient (r), slope (b) and intercept (a) were calculated.

Limit of detection (LOD) and Limit of Quantification (LOQ)

The solution **(K and L)** were injected onto HPLC in three replications to determine limit of detection and limit of Quantification. The minimum concentration, which could be detected by the HPLC with S/N ratio of 3 ± 0.5, was calculated as limit of detection (LOD). The minimum concentration, which could be quantified by the HPLC with S/N ratio between 5 to 10, was calculated as limit of quantification (LOQ).

Limit of Detection and Limit of Quantification

For calculating the LOD and LOQ values, solutions with known decreasing concentrations of analytes were injected into the HPLC system. The limit of detection (LOD) and quantification (LOQ) were then measured by calculating the minimum level at which the analytes can be readily detected (signal to noise ratio of 3:1) and quantified (signal to noise ratio of 10:1) with accuracy, respectively.

Precision

Precision of the developed method was determined at two levels, 10mg L^{-1} and 200mg L^{-1} of three drugs. For evaluating the within-day precision, results of six replicate analyses of two different concentrations of samples were used on a single day. The between – day precision was calculated from results obtained from the same samples analyzed on five different days.

Accuracy

Method accuracy was determined by fortifying known amounts of esomeprazole, venlafaxine HCl and fenofibrate to the pre – analysed environmental

water sample at the LOQ level (5.0mg L^{-1}) and 10 times LOQ level (50mg L^{-1}) and then comparing the added concentration with the found concentration. The concentration of three drugs in each replicate were calculated using the following formula:

$$\text{Concentration (ppm)} = \frac{Y - a}{b} \times D$$

where,

Y = Peak area of the sample
a = Constant
b = Regression coefficient for Y on X
D = Dilution factor

The %RSD was calculated using the following formula:

$$\text{Precision (\% RSD)} = \frac{\text{Standard Deviation}}{\text{Mean Concentration}} \times 100$$

The accuracy (%Recovery) was calculated using the following formula:

$$\text{\% Recovery} = \frac{\text{Recovered concentration}}{\text{Fortified concentration}} \times 100$$

Identification of Esomeprazole, Venlafaxine HCl and Fenofibrate by LC – MS

Esomeprazole, Venlafaxine HCl and Fenofibrate were identified by MS. Environmental water samples were analysed by LC – MS.

RESULTS AND DISCUSSION

To develop the method different stationary phases (C18, C8), mobile phases containing buffers like formic acid, ammonium acetate and organic modifiers like acetonitrile in the mobile phase were used.

At the beginning of method development a chromatographic condition was set for the separation of esomeprazole, venlafaxine HCl and fenofibrate individually by BDS Hypersil C8 column (250 x 4.6mm, 5µ particle size) using a mixture of acetonitrile: buffer (0.13% formic acid, 15.50% 0.1mol L^{-1} ammonium acetate) in the ratio 25:75 (v/v) (pH 3.8) as mobile phase A and acetonitrile as mobile phase B at a wavelength of 302nm with flow rate 1.0mL min^{-1} with run time 45min. The gradient

LC conditions are mentioned in Table 4.1. *(Page No. 138.).*

To reduce the run time chromatographic conditions were changed. This was achieved on a C18 (150cm x 4.6mm, 3.5µm particle size) column and mixture of acetonitrile: buffer (0.3% formic acid) in the ratio 25:75 (v/v) as mobile phase A and in the ratio 30:70 (v/v) as mobile phase B. At the wavelength of 230nm all the three drugs gave a good response. Under these conditions, sharp peaks that belong to Esomeprazole, Venlafaxine HCl and Fenofibrate were obtained at retention time 3.25, 4.77 and 13.12 minutes respectively as shown in Figure 4.1.

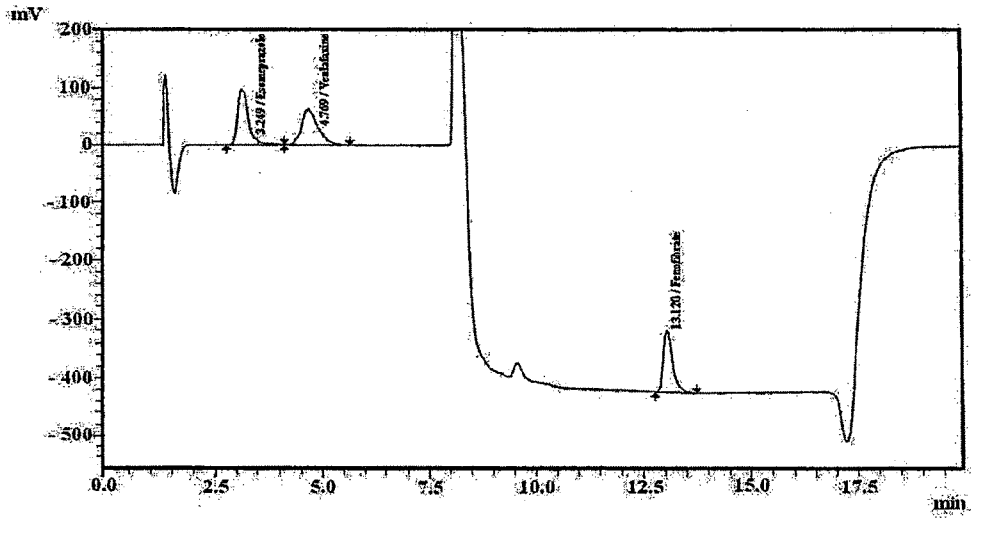

Figure 4.1 Chromatogram for esomeprazole, venlafaxine HCl and fenofibrate

The tailing factor for esomeprazole, venlafaxine HCl and fenofibrate was 1.288, 1.478 and 1.290 respectively.

Method Validation

Specificity

Since there was no interference of peaks of esomeprazole, venlafaxine HCl and fenofibrate standard, in to each other, as well as no interfering peaks appeared at retention time of above compounds, the method was considered to be specific for the each of analyte. The representative chromatograms of 100mg L^{-1} esomeprazole,

100mg L^{-1} venlafaxine HCl, 100mg L^{-1} fenofibrate, methanol, acetonitrile and mobile phase obtained for the specificity study are given in Figure 4.2, 4.3, 4.5, 4.6, 4.7 and 4.8 respectively.

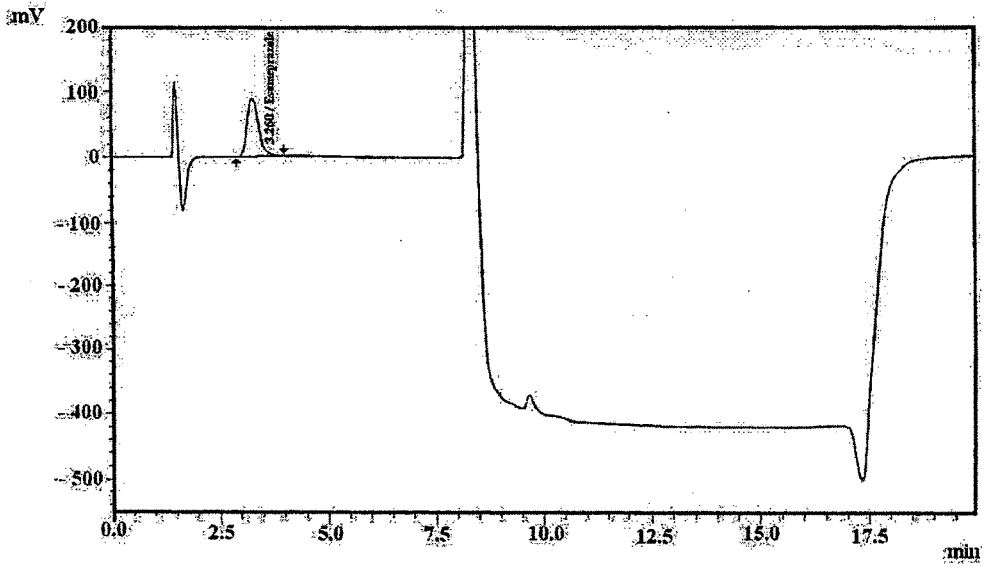

Figure 4.2. Specificity – Chromatogram for 100mg L^{-1} esomeprazole

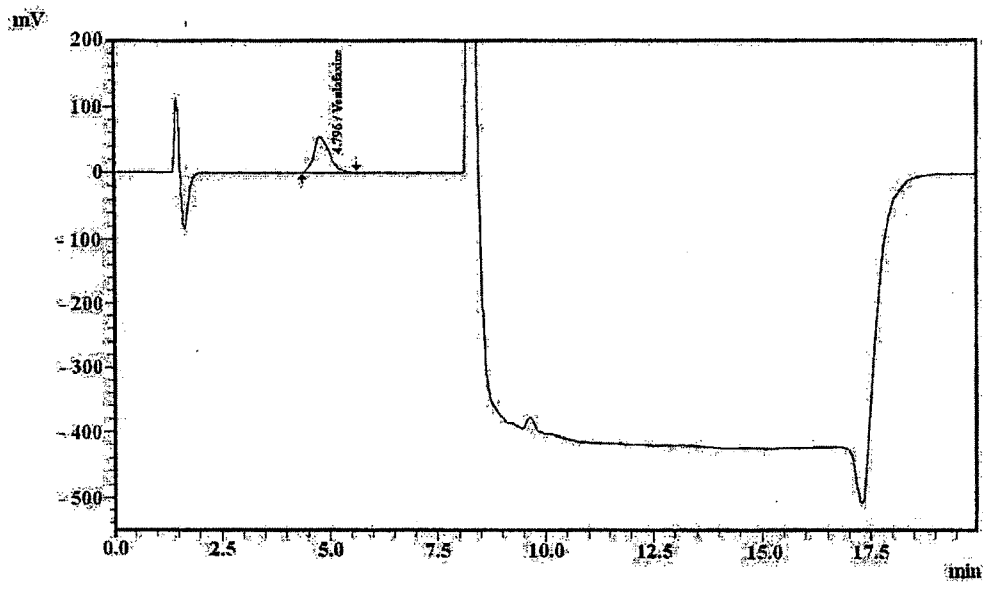

Figure 4.3. Specificity – Chromatogram for 100mg L^{-1} venlafaxine HCl

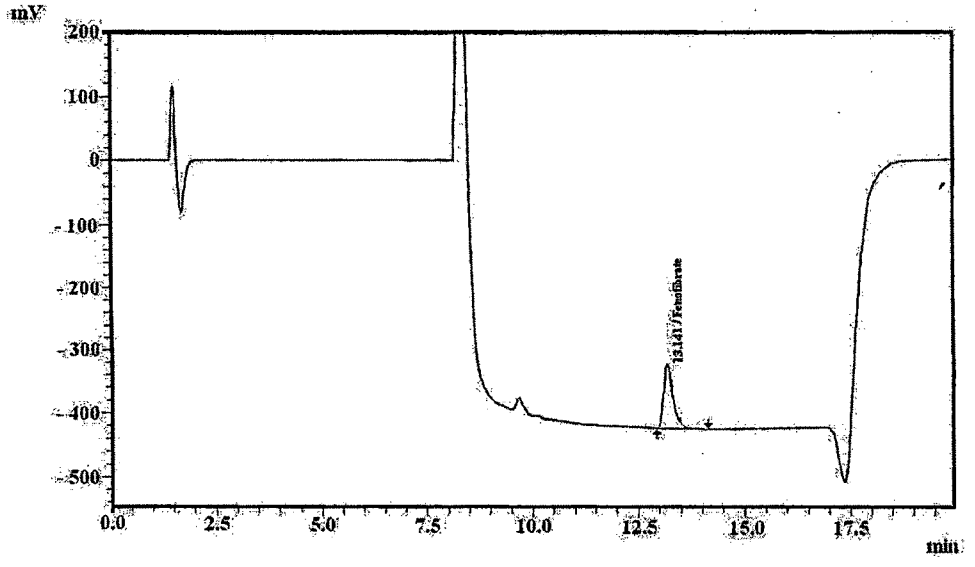

Figure 4.4. Specificity – Chromatogram for 100 mg L^{-1} fenofibrate

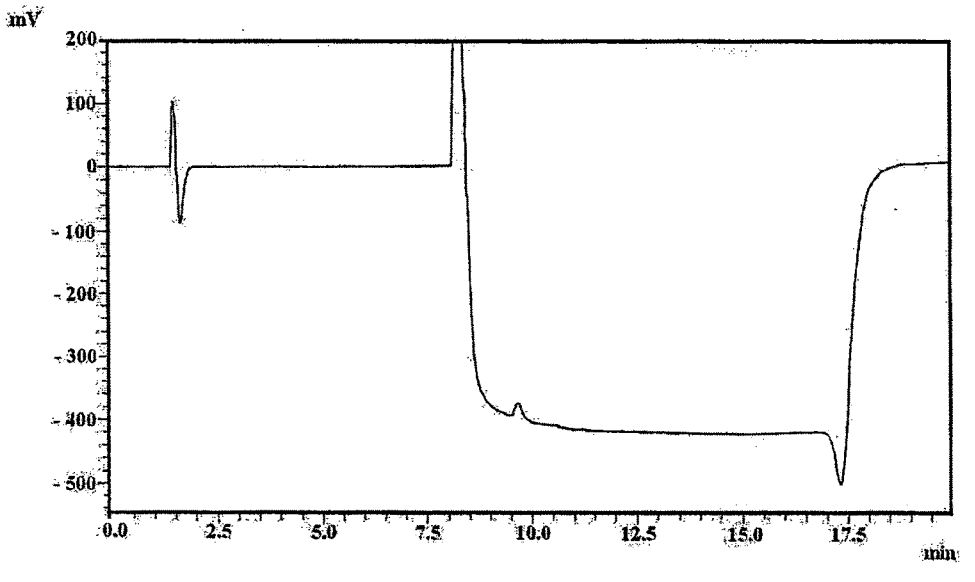

Figure 4.5. Specificity – Chromatogram for acetonitrile

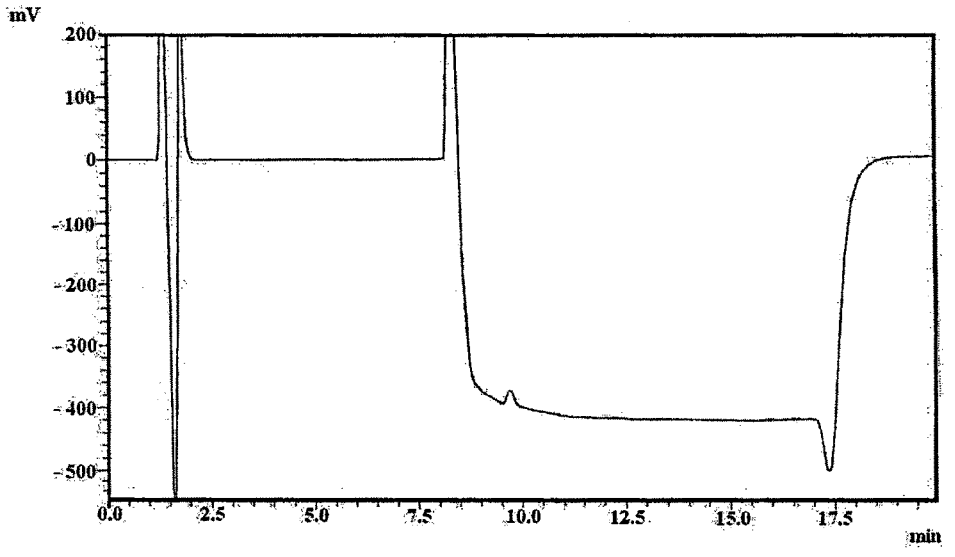

Figure 4.6. Specificity – Chromatogram for methanol

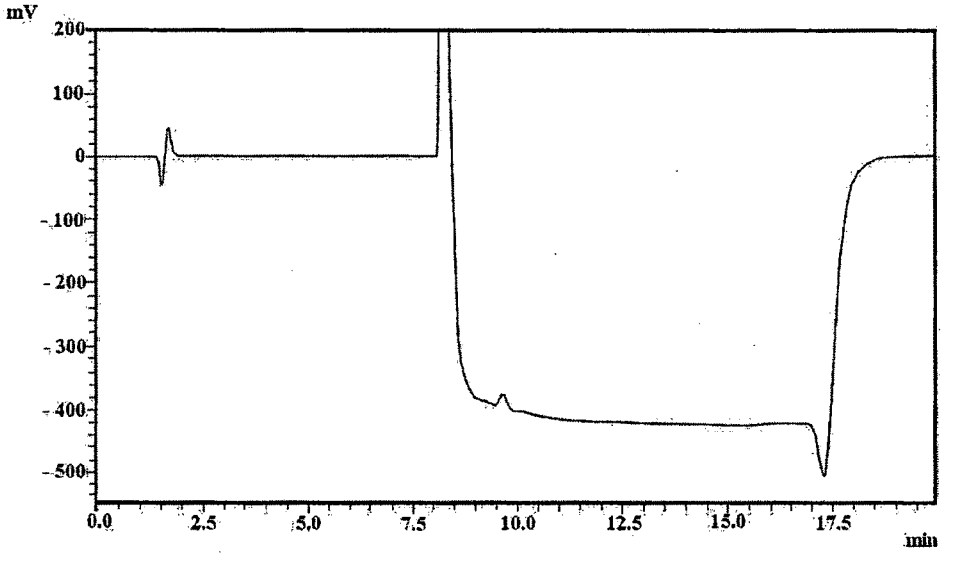

Figure 4.7. Specificity – Chromatogram for mobile phase

System Suitability

The %RSD for retention times were 0.03, 0.02 and 0.08 for esomeprazole, venlafaxine HCl and fenofibrate respectively. The %RSD for peak area were 1.16, 1.16 and 0.88 for esomeprazole, venlafaxine HCl and fenofibrate respectively. The results are shown in Table 4.2, 4.3 and 4.4.

The representative chromatograms of 100mg L^{-1} R1, R2, R3, R4, R5 and R6 obtained for the system suitability study are given in Figure 4.8, 4.9, 4.10, 4.11, 4.12 and 4.13 respectively.

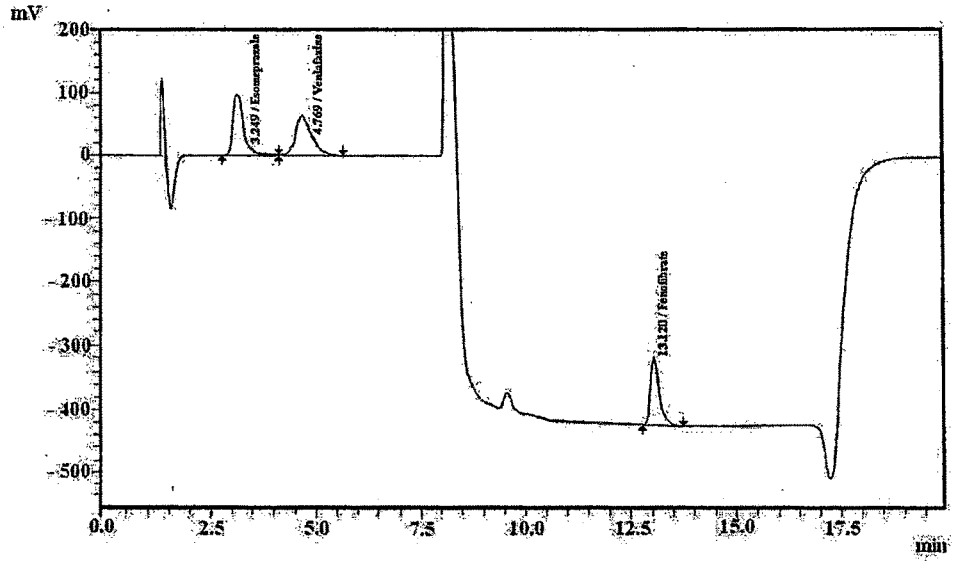

Figure 4.8. System suitability – Chromatogram for 100mg L^{-1} R1

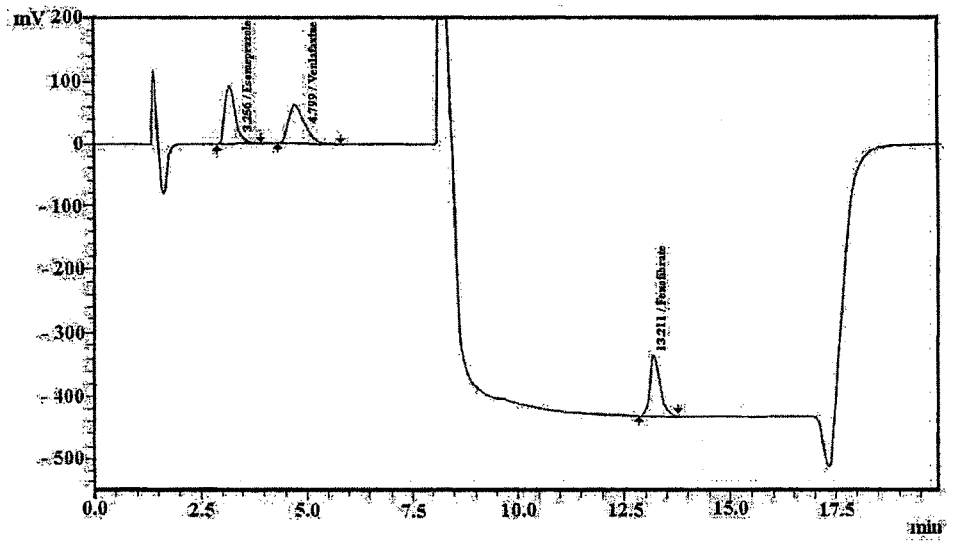

Figure 4.9. System suitability – Chromatogram for 100mg L^{-1}R2

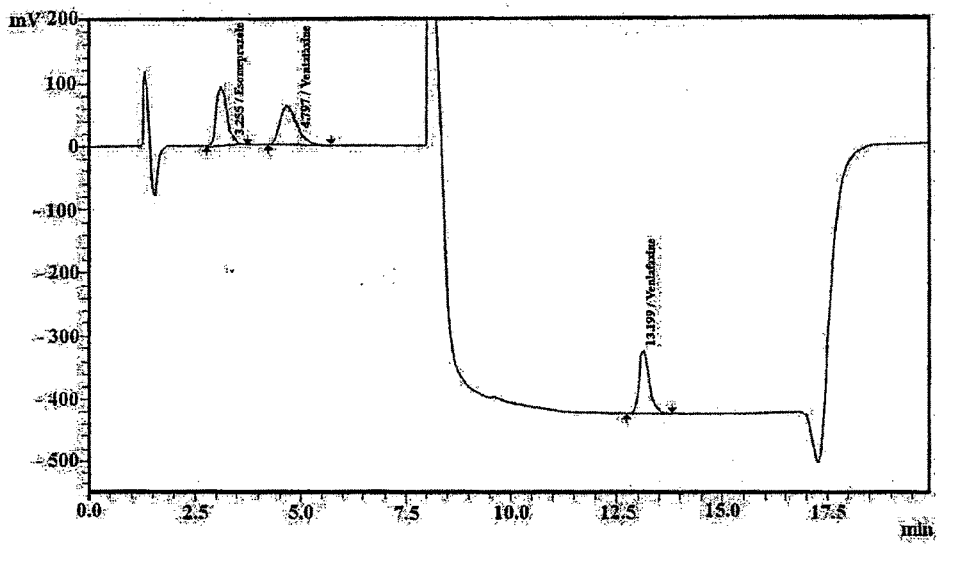

Figure 4.10. System suitability – Chromatogram for 100mg L^{-1} R3

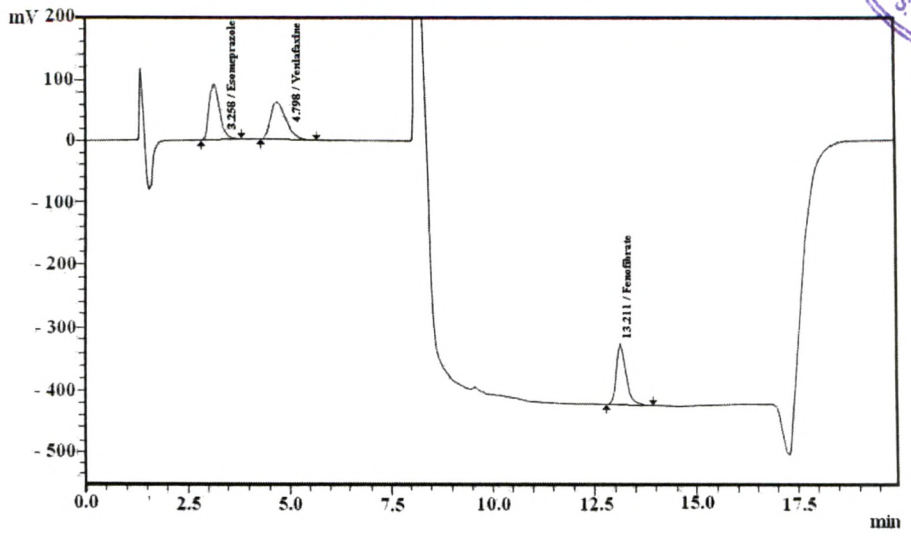

Figure 4.11. System suitability – Chromatogram for 100mg L^{-1} R4

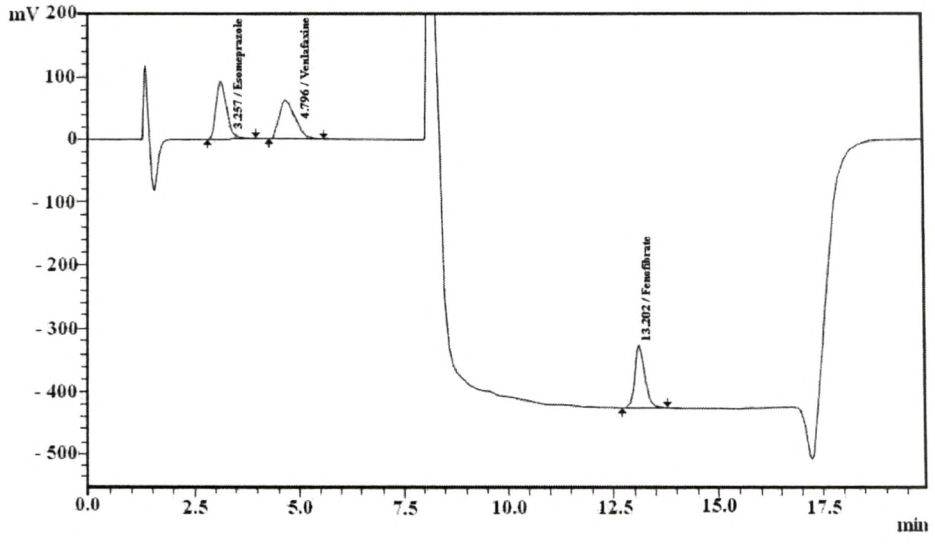

Figure 4.12. System suitability – Chromatogram for 100mg L^{-1} R5

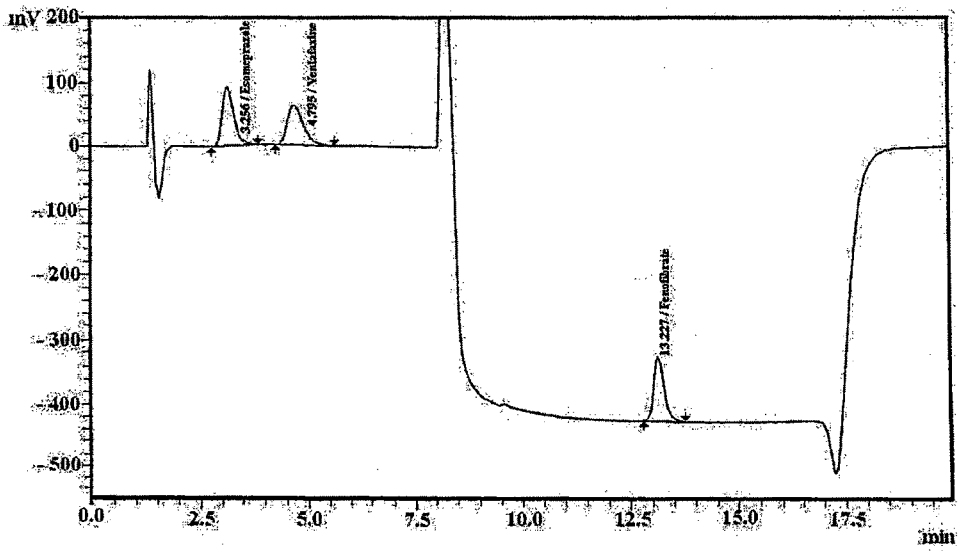

Figure 4.13. System suitability – Chromatogram for 100mg L^{-1} R6

Table 4.2. System suitability study for esomeprazole.

Esomeprazole (103.68mg L^{-1})		
Replication	Retention Time	Peak Area
R1	3.255	1627480
R2	3.256	1619394
R3	3.255	1589943
R4	3.258	1622639
R5	3.257	1648607
R6	3.256	1623318
Mean	3.256	1621897
SD	0.001	18839
%RSD	0.03	1.16

Table 4.3. System suitability study for venlafaxine HCl.

Venlafaxine HCl (101.79mg L^{-1})		
Replication	Retention Time	Peak Area
R1	4.798	1651785
R2	4.799	1640829
R3	4.797	1647049
R4	4.798	1613478
R5	4.796	1612408
R6	4.795	1611894
Mean	4.797	1629574
SD	0.001	18930
%RSD	0.02	1.16

Table 4.4. System suitability study for fenofibrate.

Fenofibrate (104.48mg L^{-1})		
Replication	Retention Time	Peak Area
R1	13.197	1641687
R2	13.211	1605120
R3	13.199	1629048
R4	13.211	1613823
R5	13.202	1623625
R6	13.227	1606558
Mean	13.208	1619977
SD	0.011	14182
%RSD	0.08	0.88

Linear Dynamic Range (LDR)

The computed equations of the calibration curve for the three drugs are: esomeprazole: $y = 16375.54x - 3513.49$, for venlafaxine HCl: $y = 15400.66x + 30904.46$, and for fenofibrate: $y = 15356.84x + 15485.60$. The results shown in Table 4.5, 4.6 and 4.7.

The results show that an excellent correlation existed between the peak area and concentration. The correlation coefficient (r^2) was 0.999, 0.999 and 0.999 for esomeprazole, venlafaxine HCl and fenofibrate respectively.

The representative chromatograms of 500mg L^{-1}, 200mg L^{-1}, 100mg L^{-1}, 50mg L^{-1} and 10mg L^{-1} of esomeprazole, venlafaxine HCl and fenofibrate respectively with two sets R1 and R2 are shown in Figures 4.14 to 4.23.

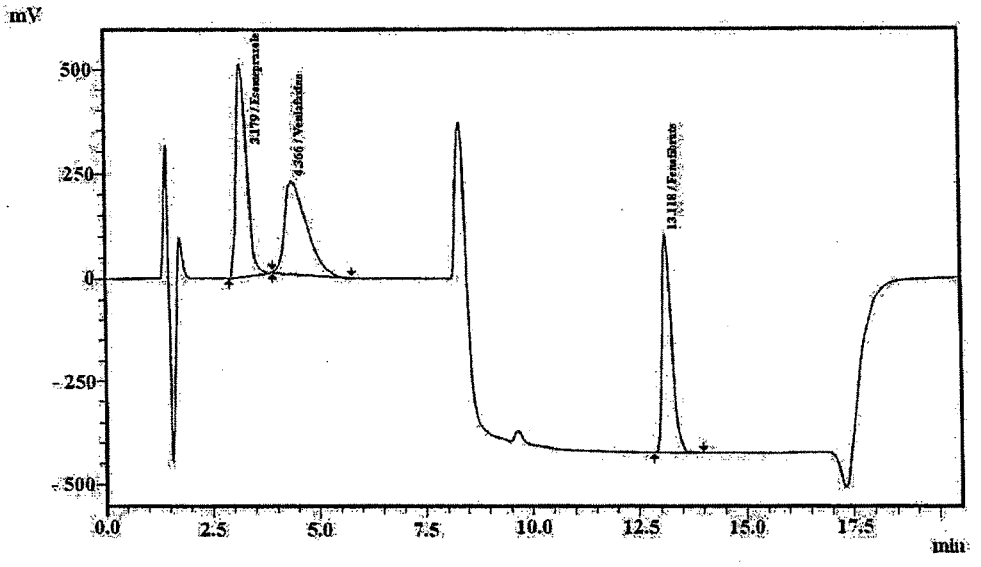

Figure 4.14. Linear dynamic range – Chromatogram for 500mg L^{-1}R1

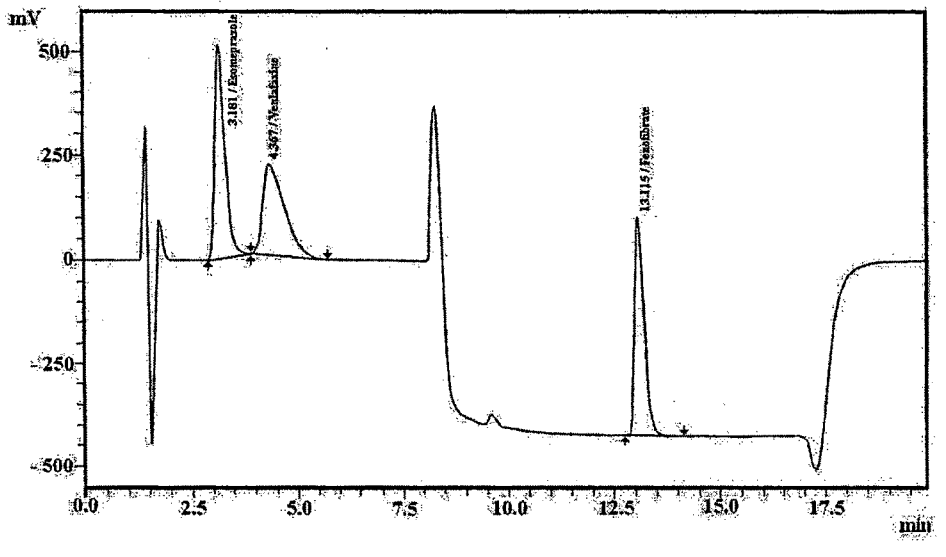

Figure 4.15. Linear dynamic range – Chromatogram for 500mg L^{-1}R2

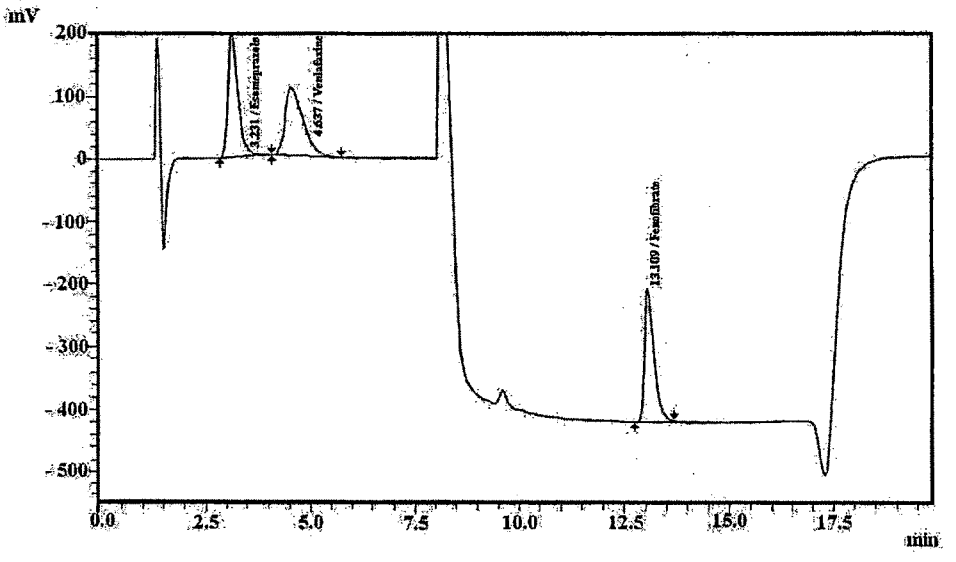

Figure 4.16. Linear dynamic range – Chromatogram for 200mg L^{-1}R1

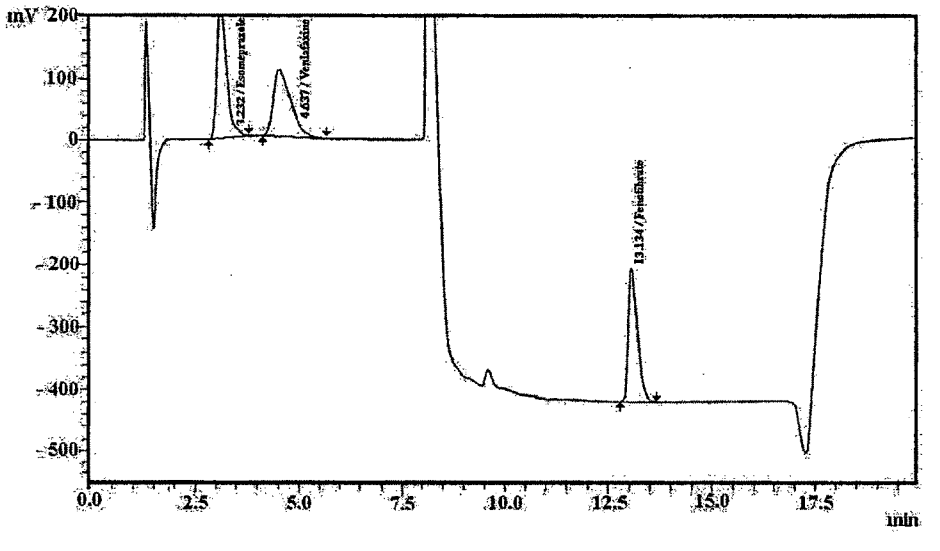

Figure 4.17. Linear dynamic range – Chromatogram for 200mg L^{-1}R2

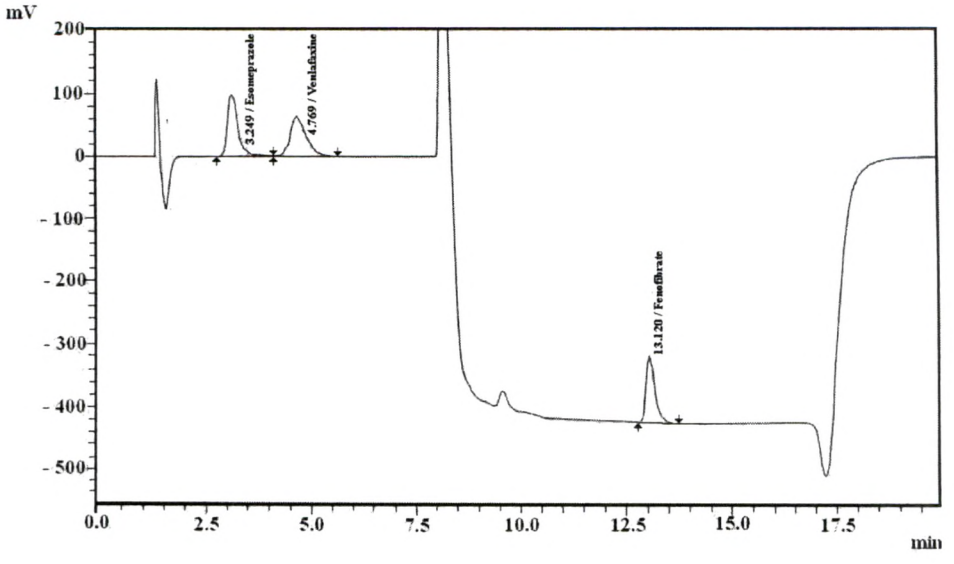

Figure 4.18. Linear dynamic range – Chromatogram for 100mg L^{-1}R1

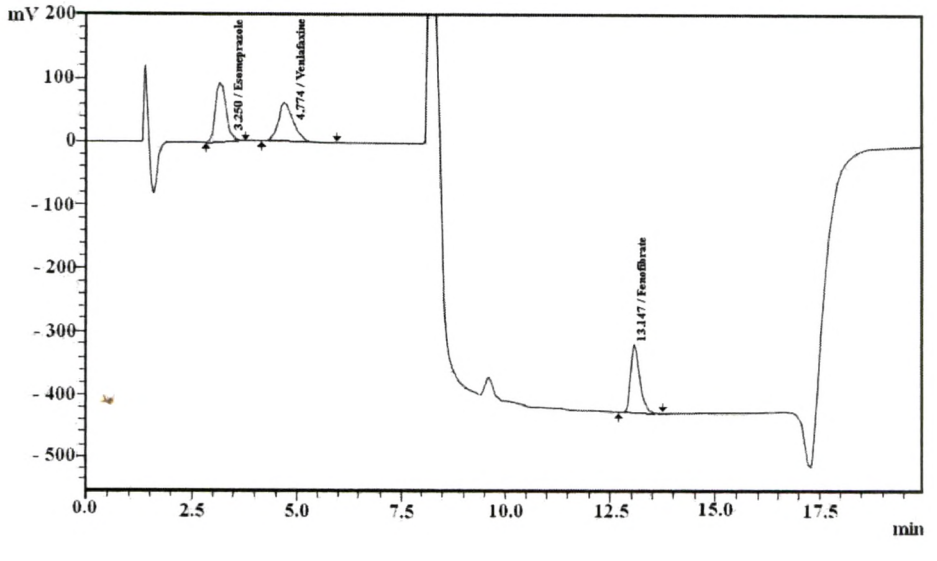

Figure 4.19. Linear dynamic range – Chromatogram for 100mg L^{-1}R2

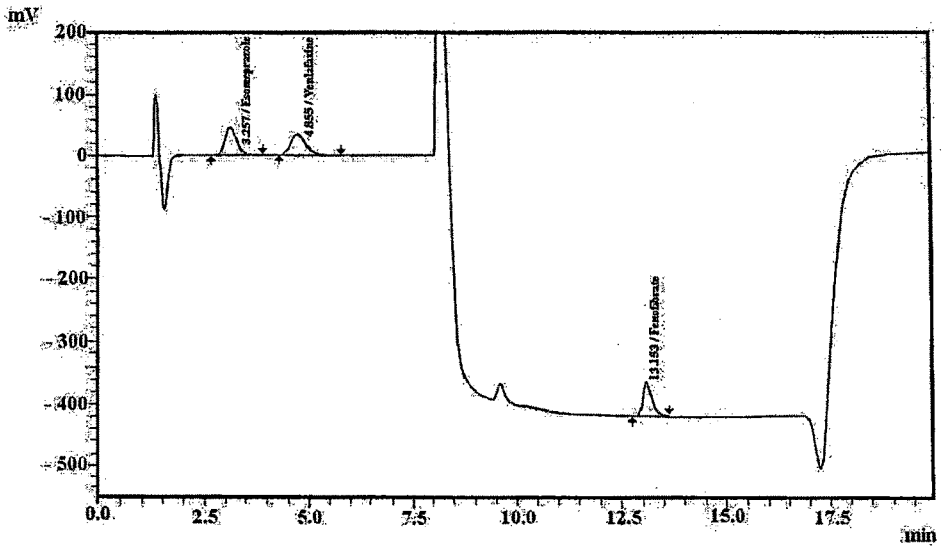

Figure 4.20. Linear dynamic range – Chromatogram for 50mg L^{-1}R1

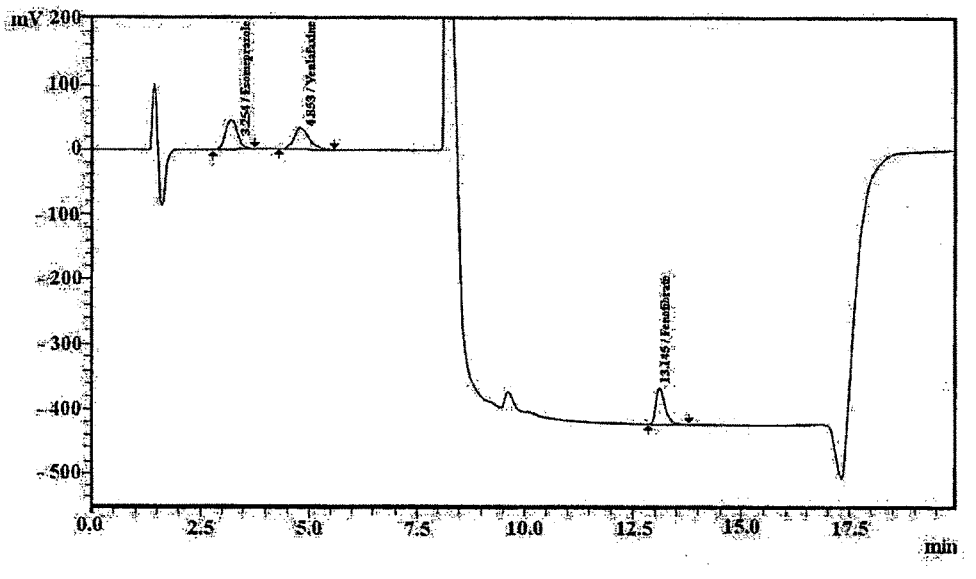

Figure 4.21. Linear dynamic range – Chromatogram for 50mg L^{-1}R2

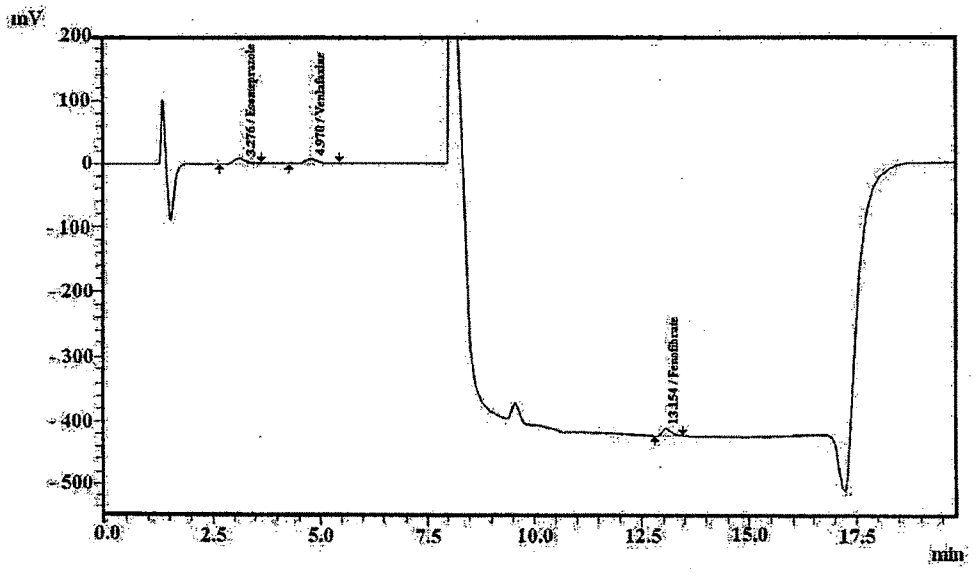

Figure 4.22. Linear dynamic range – Chromatogram for 10mg L^{-1}R1

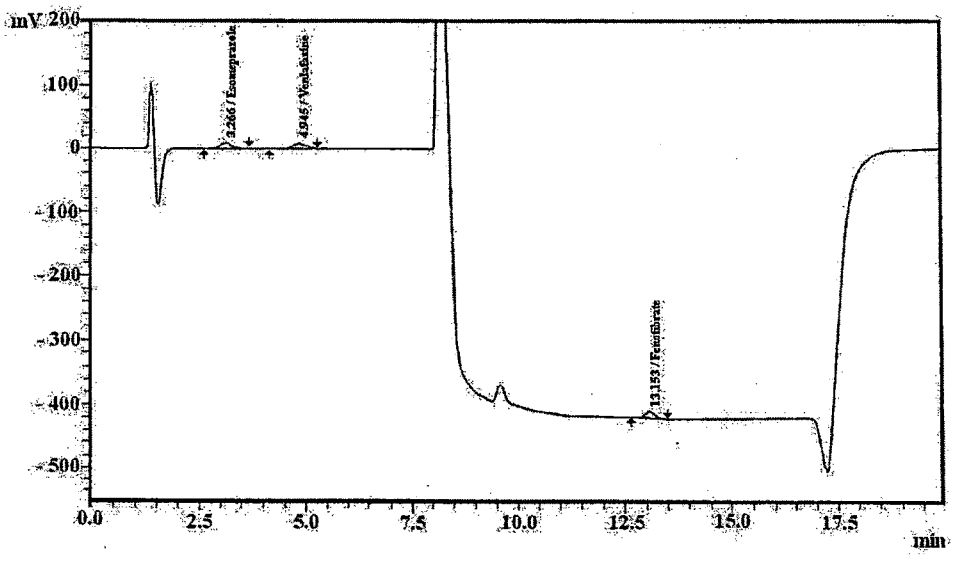

Figure 4.23. Linear dynamic range – Chromatogram for 10mg L^{-1}R2

Table 4.5 Linear Dynamic Range Data for Esomeprazole Standard

Concentration(mg L^{-1})	Replications	Peak Area Counts	Mean Peak Area Counts	%Variation
10.37	R1	181684	182442.50	- 0.83
	R2	183201		
51.84	R1	838509	829888.50	2.06
	R2	821268		
103.68	R1	1700148	1682775.00	2.04
	R2	1665402		
207.36	R1	3435061	3404385.00	1.79
	R2	3373709		
518.40	R1	8486354	8484196.00	0.05
	R2	8482038		
Typical Calculation				
$\%\ \text{Variation} = \frac{\text{Maximum Area} - \text{Minimum Area}}{\text{Maximum Area}} \times 100$			$= \frac{181684 - 183201}{183201} \times 100$	$= -0.83$

Table 4.6. Linear Dynamic Range Data for Venlafaxine HCl Standard

Concentration(mg L^{-1})	Replications	Peak Area Counts	Mean Peak Area Counts	%Variation
10.18	R1	164175	164956.5	- 0.95
	R2	165738		
50.89	R1	825901	823896.5	0.49
	R2	821892		
101.79	R1	1591977	1595884.5	- 0.49
	R2	1599792		
203.58	R1	3213848	3192950.5	1.3
	R2	3172053		
508.94	R1	7861435	7858265	0.08
	R2	7855095		
Typical Calculation				
$\%\ \text{Variation} = \frac{\text{Maximum Area} - \text{Minimum Area}}{\text{Maximum Area}} \times 100$			$= \frac{164175 - 165738}{165738} \times 100$	$= -0.95$

Table 4.7. Linear Dynamic Range Data for Fenofibrate Standard

Concentration(mg L^{-1})	Replications	Peak Area Counts	Mean Peak Area Counts	%Variation
10.45	R1	170748	170033.5	0.84
	R2	169319		
52.24	R1	823158	824412.5	- 0.3
	R2	825667		
104.48	R1	1586211	1603156	- 2.14
	R2	1620101		
208.95	R1	3257370	3246370.5	0.68
	R2	3235371		
522.38	R1	8017173	8031572.5	- 0.36
	R2	8045972		
Typical Calculation				
$\%\ \text{Variation} = \frac{\text{Maximum Area} - \text{Minimum Area}}{\text{Maximum Area}} \times 100$			$= \frac{170748 - 169319}{170748} \times 100$	$= 0.84$

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection of esomeprazole was 1.02mg L^{-1} with signal to noise ratio of 2.75. The lowest quantifiable concentration for esomeprazole with signal to noise ratio of 7.8 was 5.18mg L^{-1}. Results are shown in Table 4.8.

The limit of detection of venlafaxine was 1.02mg L^{-1} with signal to noise ratio of 3.46. The lowest quantifiable concentration for venlafaxine with signal to noise ratio of 8.34 was 5.09mg L^{-1}. Results are shown in Table 4.9.

The limit of detection of fenofibrate was 1.05mg L^{-1} with signal to noise ratio of 2.66. The lowest quantifiable concentration for fenofibrate with signal to noise ratio of 7.77 was 5.22mg L^{-1}. Results are shown in Table 4.10.

The representative chromatograms of LOD and LOQ studies for esomeprazole, venlafaxine HCl and fenofibrate are given in Figures 4.24 to 4.29.

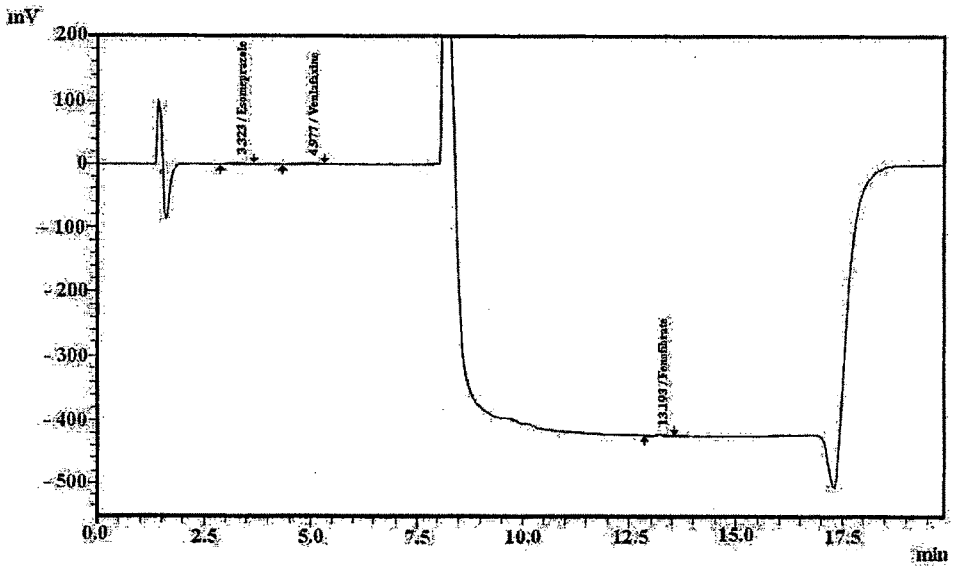

Figure 4.24. LOD – LOQ – Chromatogram for 1mg L^{-1} R1

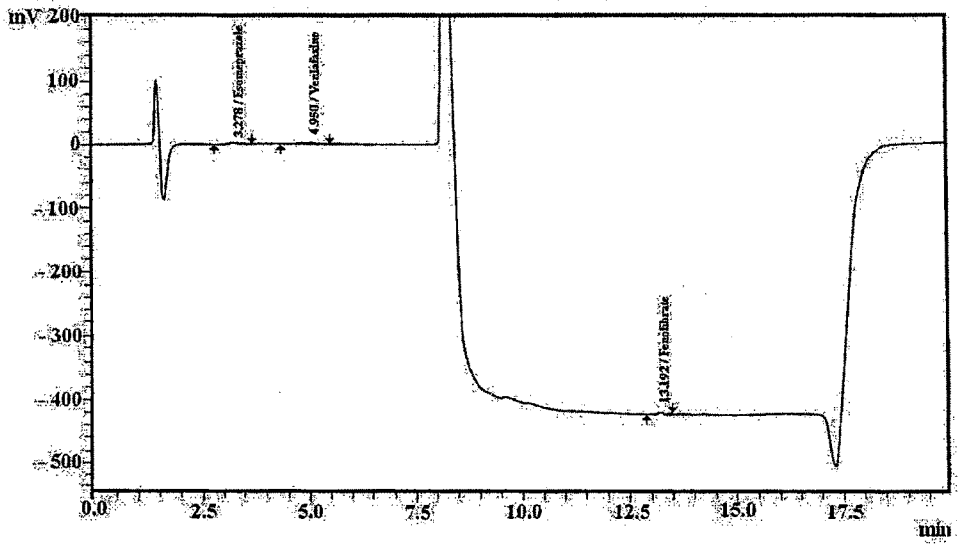

Figure 4.25. LOD – LOQ – Chromatogram for 1mg L^{-1} R2

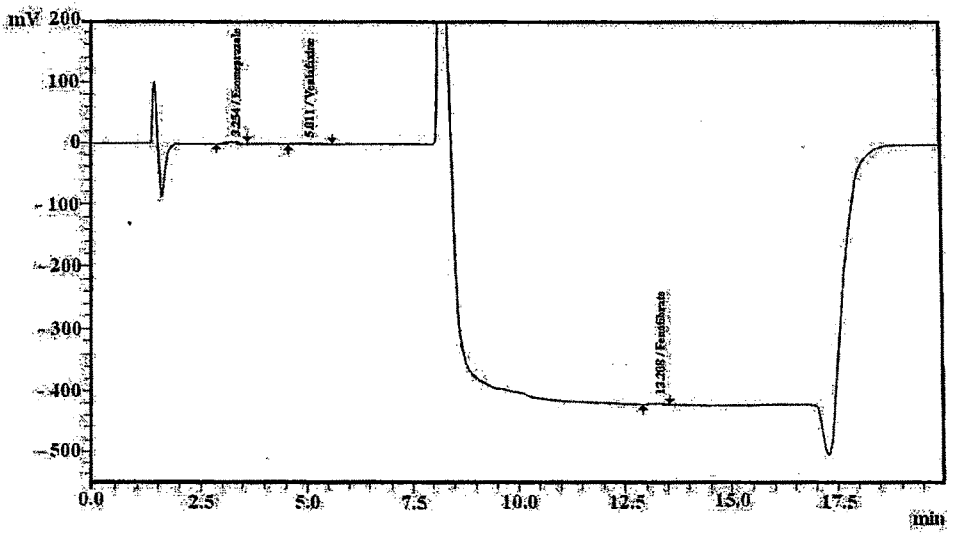

Figure 4.26. LOD – LOQ – Chromatogram for 1mg L^{-1}R3

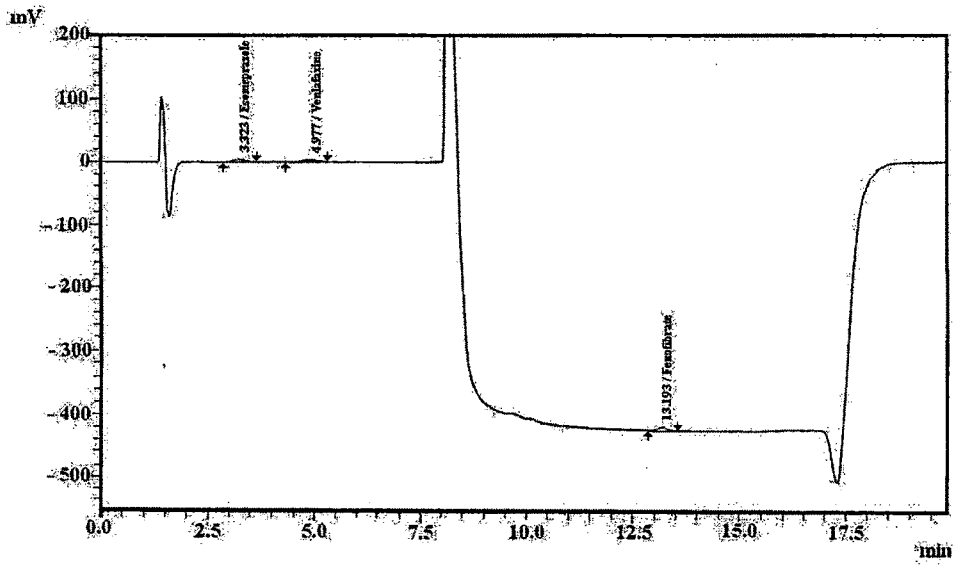

Figure 4.27. LOD – LOQ – Chromatogram for 5mg L^{-1} R1

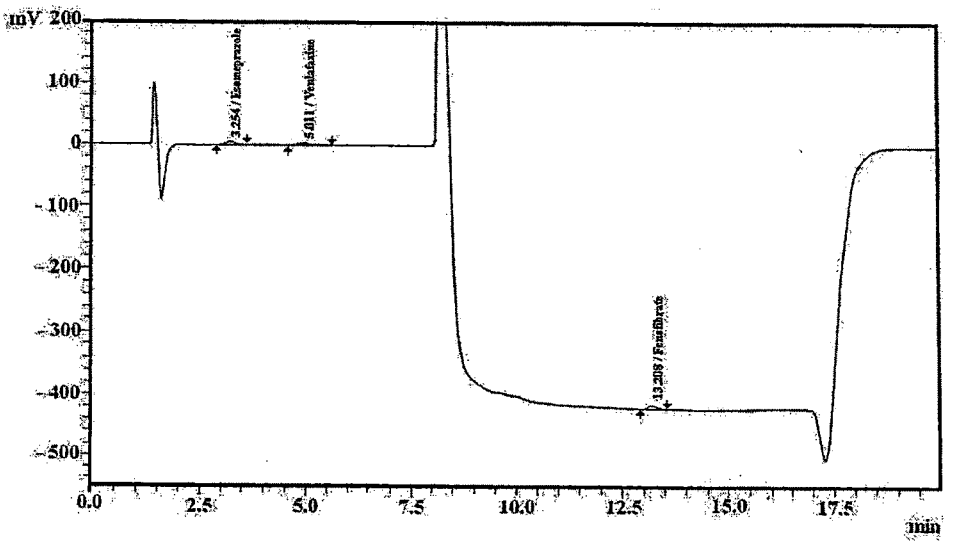

Figure 4.28. LOD – LOQ – Chromatogram for 5mg L^{-1} R2

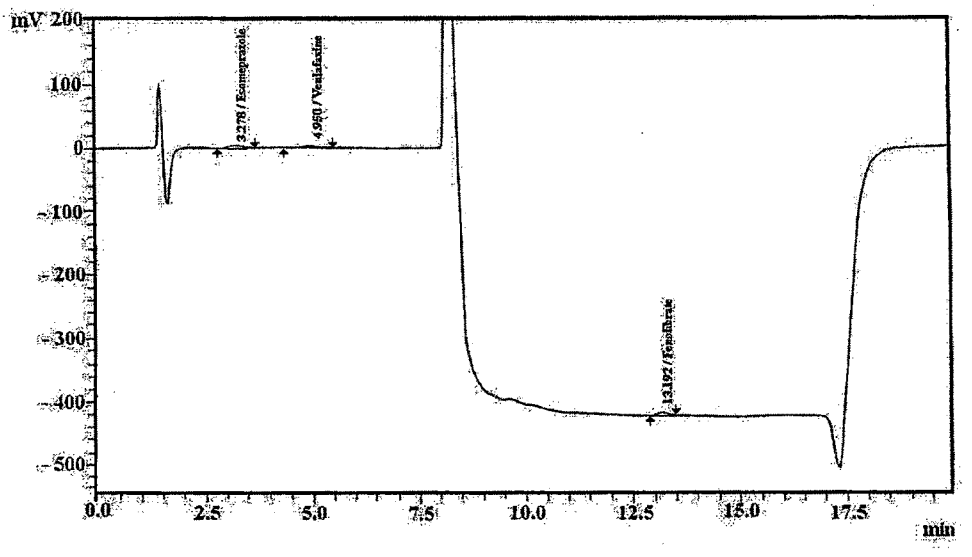

Figure 4.29. LOD – LOQ – Chromatogram for 5mg L^{-1} R3

Table 4.8. LOD and LOQ for esomeprazole

Solution Concentration (mg L^{-1})	Replication	Peak Area Count	Mean Peak Area	Mean Noise	Signal to Noise Ratio (S/N)	LOD	LOQ
1.02	R1	29558	29061	10531.83	2.75	LOD	
	R2	28082					
	R3	29543					
5.18	R1	89142	82213		7.8		LOQ
	R2	68945					
	R3	88552					

Replication	Total Peak Area of Noise in Blank (a)	N° of Noise Peak in Blank (b)	Average = a / b
I	31077	3	10359
II	32114	3	10704.66
Average Noise Peak Area of Blank			10531.83

Typical Calculation

$$\text{Signal to Noise Ratio} = \frac{\text{Mean Peak Area}}{\text{Average Noise Area}}$$

Limit of Detection	Limit of Quantification
$\frac{29061}{10531.83} = 2.75$	$\frac{82213}{10531.83} = 7.8$
1.02mg L^{-1}	5.18mg L^{-1}

Table 4.9. LOD and LOQ for venlafaxine HCl

Solution Concentration ($mg\ L^{-1}$)	Replication	Peak Area Count	Mean Peak Area	Mean Noise	Signal to Noise Ratio (S/N)	LOD	LOQ
1.02	R1	27842	28018	10531.83	2.66	LOD	
	R2	32113					
	R3	24099					
5.09	R1	87512	83625		8.34		LOQ
	R2	80588					
	R3	82776					

Replication	Total Peak Area of Noise in Blank (a)	N° of Noise Peak in Blank (b)	Average = a / b
I	31077	3	10359
II	32114	3	10704.66
Average Noise Peak Area of Blank			10531.83

Typical Calculation

$$\text{Signal to Noise Ratio} = \frac{\text{Mean Peak Area}}{\text{Average Noise Area}}$$

Limit of Detection	Limit of Quantification
$\frac{28018}{10531.83} = 2.66$	$\frac{83625}{10531.83} = 8.34$
$1.02 mg\ L^{-1}$	$5.09 mg\ L^{-1}$

Table 4.10. LOD and LOQ for fenofibrate

Solution Concentration ($mg\ L^{-1}$)	Replication	Peak Area Count	Mean Peak Area	Mean Noise	Signal to Noise Ratio (S/N)	LOD	LOQ
1.05	R1	36641	36475	10531.83	3.46	LOD	
	R2	44924					
	R3	28489					
5.22	R1	76593	76923		7.77		LOQ
	R2	81116					
	R3	73059					

Replication	Total Peak Area of Noise in Blank (a)	N° of Noise Peak in Blank (b)	Average = a / b
I	31077	3	10359
II	32114	3	10704.66
Average Noise Peak Area of Blank			10531.83

Typical Calculation

$$\text{Signal to Noise Ratio} = \frac{\text{Mean Peak Area}}{\text{Average Noise Area}}$$

Limit of Detection	Limit of Quantification
$\frac{36475}{10531.83} = 3.46$	$\frac{76923}{10531.83} = 7.77$
$1.05 mg\ L^{-1}$	$5.22 mg\ L^{-1}$

Precision (%RSD)

The precision (%RSD) of solutions of esomeprazole, venlafaxine HCl and fenofibrate at 10mg L^{-1} level were 0.79, 0.73 and 0.62% respectively. The corresponding precisions (%RSD) at 200mg L^{-1} level were 0.39, 0.91 and 0.35% respectively. Results are shown in Table 4.11. Representative chromatograms for esomeprazole, venlafaxine HCl and fenofibrate at 10mg L^{-1} is shown in Figure 4.27 and at 200mg L^{-1} is shown in Figure 4.28.

Table 4.11. Precision study at 10mg L^{-1} and 200mg L^{-1}

Precision (10mg L^{-1}level)			
Replication	Esomeprazole	Venlafaxine	Fenofibrate
	(10.37mg L^{-1})	(10.18mg L^{-1})	(10.45mg L^{-1})
R1	170272	162825	162418
R2	172358	160483	161302
R3	172587	162984	161535
R4	172709	161482	163487
R5	174530	160892	163801
R6	172875	160254	162163
Mean	172555	161487	162451
SD	1362.25	1175.89	1013.81
%RSD	0.79	0.73	0.62
Precision (200mg L^{-1})			
Replication	Esomeprazole	Venlafaxine	Fenofibrate
	(207.36 mg L^{-1})	(203.58mg L^{-1})	(208.95mg L^{-1})
R1	3429268	3224735	3245100
R2	3457973	3209076	3234179
R3	3426742	3160318	3251789
R4	3433912	3227045	3252902
R5	3418623	3172329	3227377
R6	3435213	3222352	3255675
Mean	3433622	3202643	3244504
SD	13318.02	29064.36	11390.2
%RSD	0.39	0.91	0.35

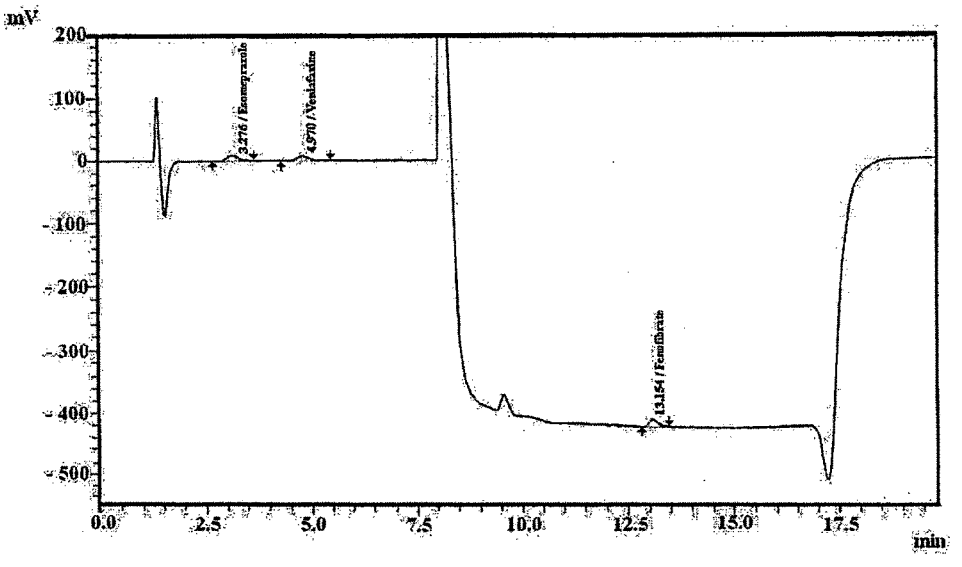

Figure 4.27. Precision – Chromatogram for 10mg L^{-1}

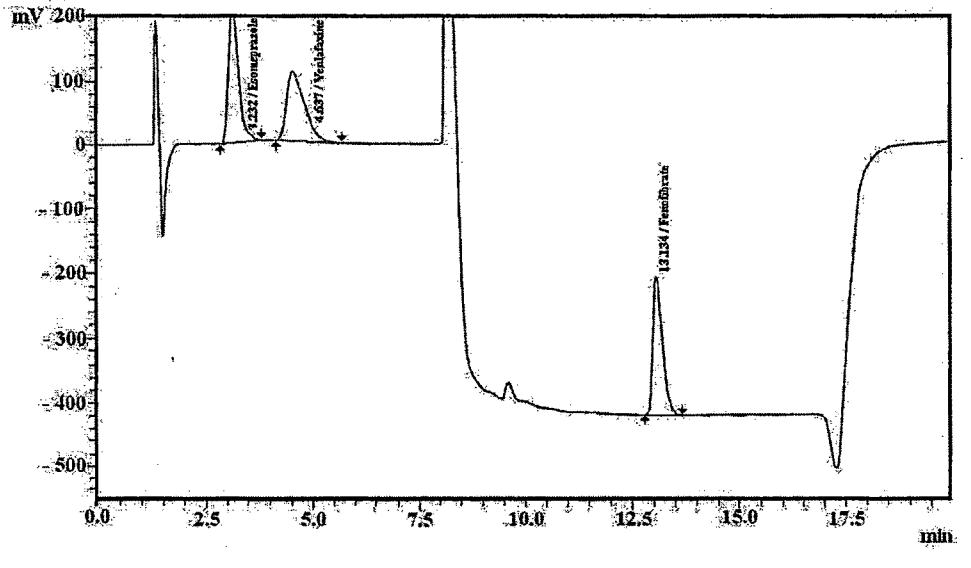

Figure 4.28. Precision – Chromatogram for 200mg L^{-1}

Accuracy (%Recovery)

The mean accuracies (%recovery) of esomeprazole, venlafaxine and fenofibrate in environmental water samples at LOQ level were 95.21, 73.28 and 71.07% respectively.

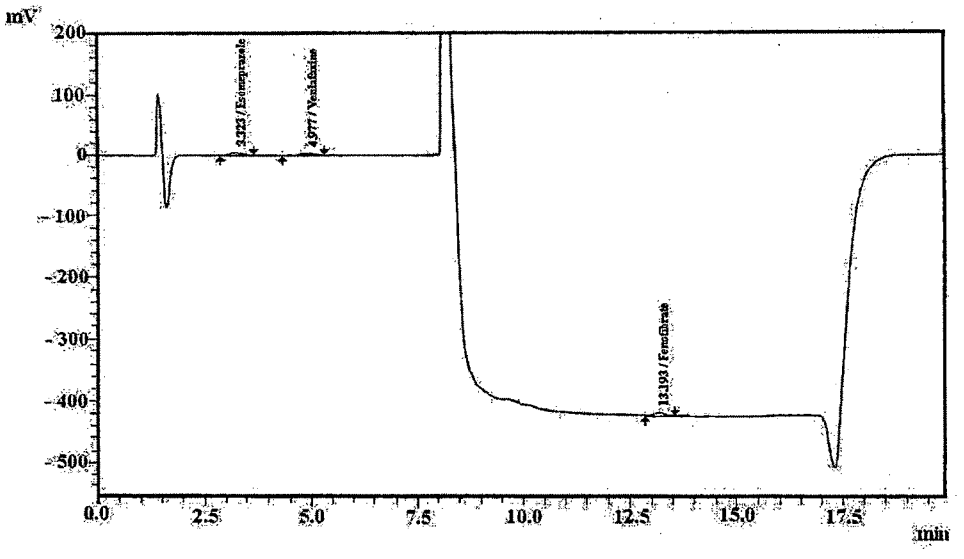

Figure 4.29. Accuracy – Chromatogram 5mg L^{-1} R1

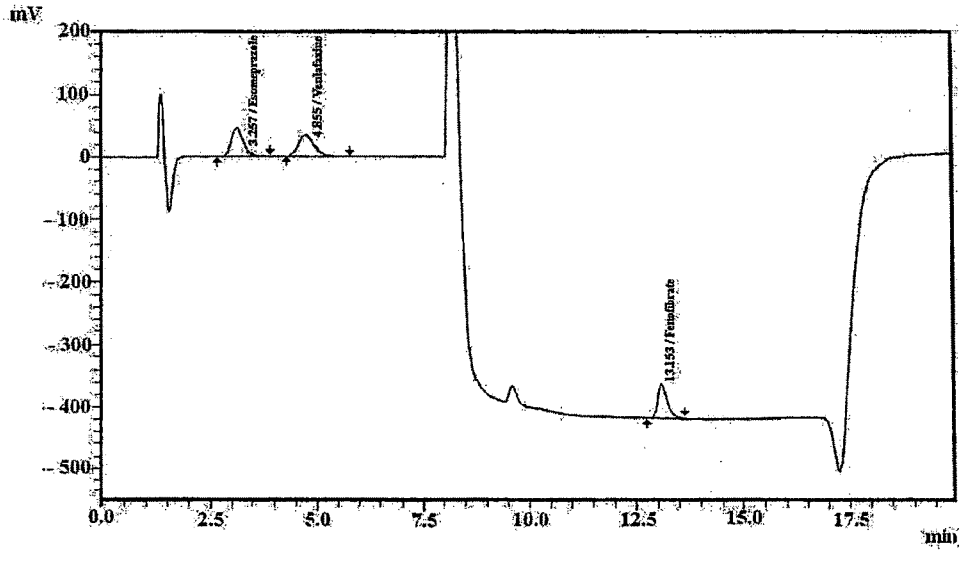

Figure 4.30. Accuracy – Chromatogram 50mg L^{-1} R1

The corresponding mean accuracies (%recovery) at 10 times LOQ level were 73.1, 75.36 and 73.72% for esomeprazole, venlafaxine and fenofibrate respectively. Results shown in Table 4.12, 4.13 and 4.14. The representative chromatograms of accuracy study are shown in Figure 4.29 to 4.30.

Table 4.12. Acuracy study for esomeprazole

Fortification Level (mg L^{-1})	Replication	Peak Area of Sample	Recovered (mg L^{-1})	Recovery (%)	Mean Conc.	Mean Recovery (%)	Standard Deviation	% RSD
Control	R1	ND	ND	-	-	-	-	-
	R2	ND	ND	-				
5.18	R1	80995	5.16	99.61	4.93	95.21	4.25	4.46
	R2	79294	5.06	97.68				
	R3	75890	4.85	93.63				
	R4	79891	5.09	98.26				
	R5	71206	4.56	88.03				
	R6	76291	4.87	94.02				
51.84	R1	599559	36.83	71.05	37.89	73.1	2.00	2.73
	R2	624209	38.33	73.94				
	R3	606541	37.25	71.86				
	R4	63611	39.06	75.35				
	R5	600478	36.88	71.14				
	R6	635240	39.01	75.25				

Typical Calculation

Intercept with y – axis (a)	- 3513.49	Slope of the line (b)	16375.54
Correlation of coefficient (r)	0.999	Dilution Factor (D)	-

Concentration (mg L^{-1})	Precision (%RSD)	%Recovery
$= \frac{Y - a}{b} \times D$	$= \frac{\text{Standard Deviation}}{\text{Mean Recovery}} \times 100$	$= \frac{\text{Quantity Recovered}}{\text{Quantity Fortified}} \times 100$
$= \frac{80995 - (-3513.49)}{16375.54}$	$= \frac{4.25}{95.21} \times 100$	$= \frac{5.16}{5.18} \times 100$
= 5.16mg L^{-1}	= 4.46%	= 99.61%

ND = Not detected

Table 4.13 Acuracy study for venlafaxine HCl

Fortification Level (mg L^{-1})	Replication	Peak Area of Sample	Recovered (mg L^{-1})	Recovery (%)	Mean Conc.	Mean Recovery (%)	Standard Deviation	% RSD
Control	R1	ND	ND	-	-	-	-	-
	R2	ND	ND	-				
5.09	R1	89470	3.8	74.66	3.73	73.28	7.00	9.56
	R2	96331	4.25	83.50				
	R3	91150	3.91	76.82				
	R4	80563	3.22	63.26				
	R5	84292	3.47	68.17				
	R6	88378	3.73	73.28				
50.89	R1	611302	37.69	74.06	38.35	75.36	1.68	2.23
	R2	623447	38.48	75.61				
	R3	640479	39.58	77.78				
	R4	628523	38.8	76.24				
	R5	602696	37.13	72.96				
	R6	622729	38.43	75.52				

Typical Calculation			
Intercept with y – axis (a)	30904.46	Slope of the line (b)	15400.66
Correlation of coefficient (r)	0.999	Dilution Factor (D)	-

Concentration (mg L^{-1})	Precision (%RSD)	%Recovery
$= \frac{Y - a}{b} \times D$	$= \frac{\text{Standard Deviation}}{\text{Mean Recovery}} \times 100$	$= \frac{\text{Quantity Recovered}}{\text{Quantity Fortified}} \times 100$
$= \frac{89470 - (30904.46)}{15400.66}$	$= \frac{7.00}{73.28} \times 100$	$= \frac{3.8}{5.09} \times 100$
= 3.8mg L^{-1}	= 9.56%	= 74.66%

ND = Not detected

Table 4.13 Acuracy study for fenofibrate

Fortification Level (mg L^{-1})	Replication	Peak Area of Sample	Recovered (mg L^{-1})	Recovery (%)	Mean Conc.	Mean Recovery (%)	Standard Deviation	% RSD
Control	R1	ND	ND	-	-	-	-	-
	R2	ND	ND	-				
5.22	R1	74970	3.87	74.14	3.71	71.07	5.62	7.9
	R2	80175	4.21	80.65				
	R3	69675	3.53	67.62				
	R4	70573	3.59	68.77				
	R5	67612	3.39	64.94				
	R6	71396	3.64	69.73				
52.24	R1	605787	38.44	73.58	38.51	73.72	1.50	2.03
	R2	595464	37.77	72.30				
	R3	601726	38.17	73.07				
	R4	608102	38.59	73.87				
	R5	629623	39.99	76.55				
	R6	60004	38.07	72.88				

Typical Calculation

Intercept with y – axis (a)	15485.60	Slope of the line (b)	15356.84
Correlation of coefficient (r)	0.999	Dilution Factor (D)	-

Concentration (mg L^{-1})	Precision (%RSD)	%Recovery
$= \frac{Y-a}{b} \times D$	$= \frac{\text{Standard Deviation}}{\text{Mean Recovery}} \times 100$	$= \frac{\text{Quantity Recovered}}{\text{Quantity Fortified}} \times 100$
$= \frac{74970-(15485.60)}{15356.84}$	$= \frac{5.62}{71.07} \times 100$	$= \frac{3.87}{5.22} \times 100$
= 3.87mg L^{-1}	= 7.9%	= 74.14%

ND = Not detected

Identification of Esomeprazole, Venlafaxine HCl and Fenofibrte by LC – MS

The MS spectrum of esomeprazole in methanol is shown in Figure 4.31.

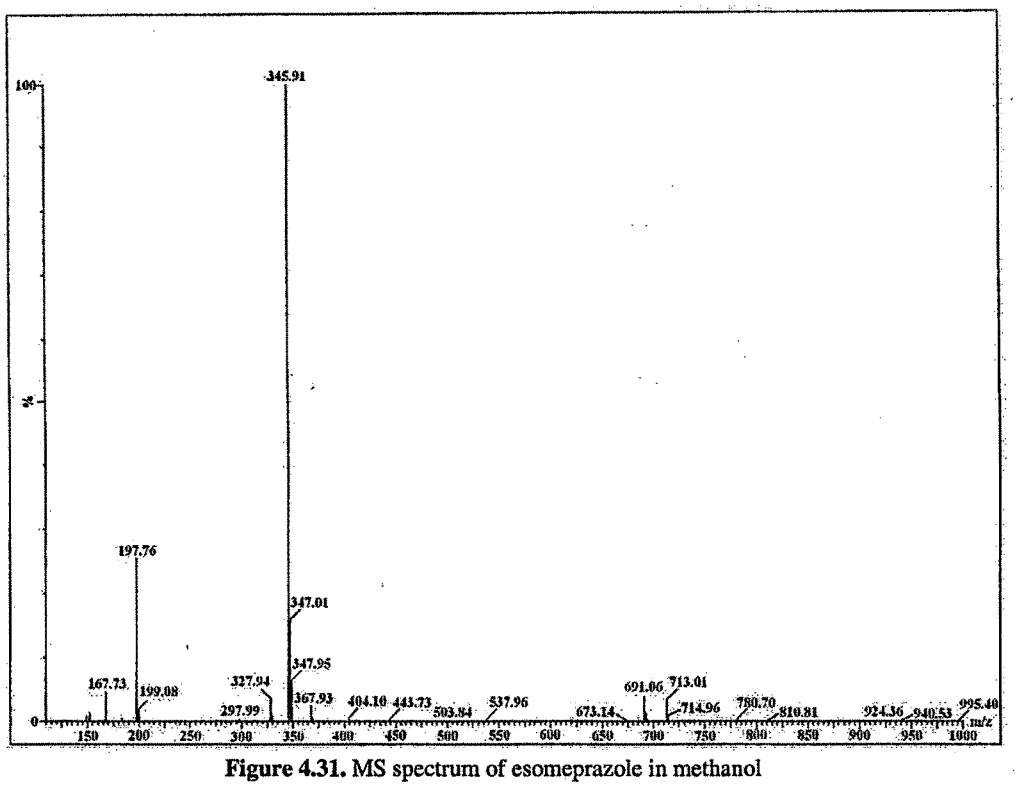

Figure 4.31. MS spectrum of esomeprazole in methanol

The MS spectrums of venlafaxine HCl and fenofibrate in methanol are shown in Figure 4.31 and Figure 4.32.

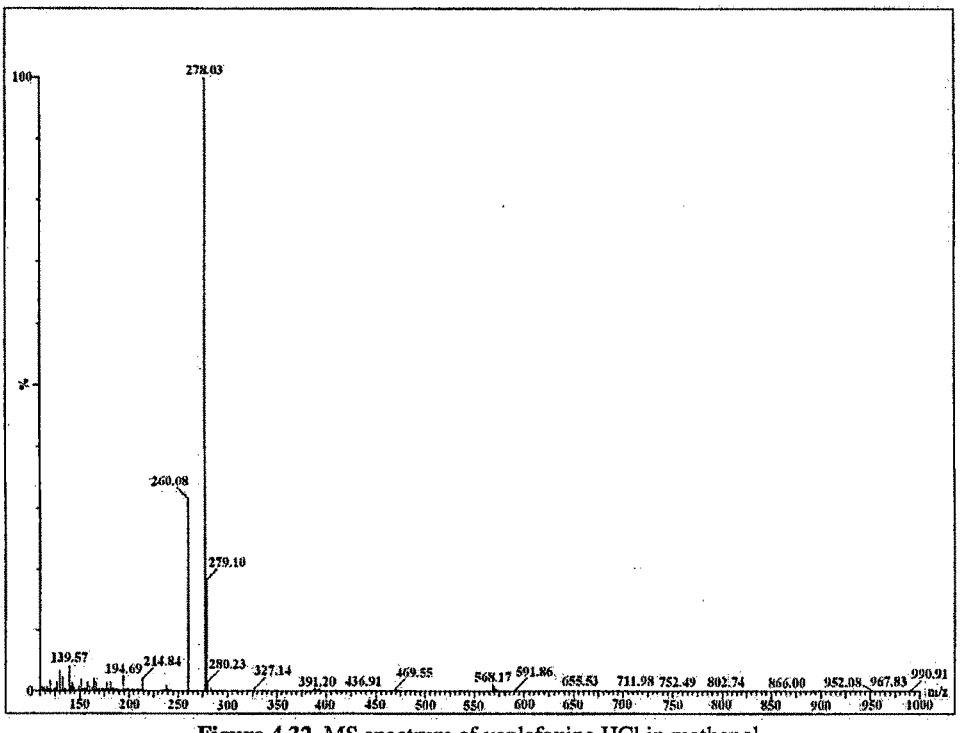

Figure 4.32. MS spectrum of venlafaxine HCl in methanol

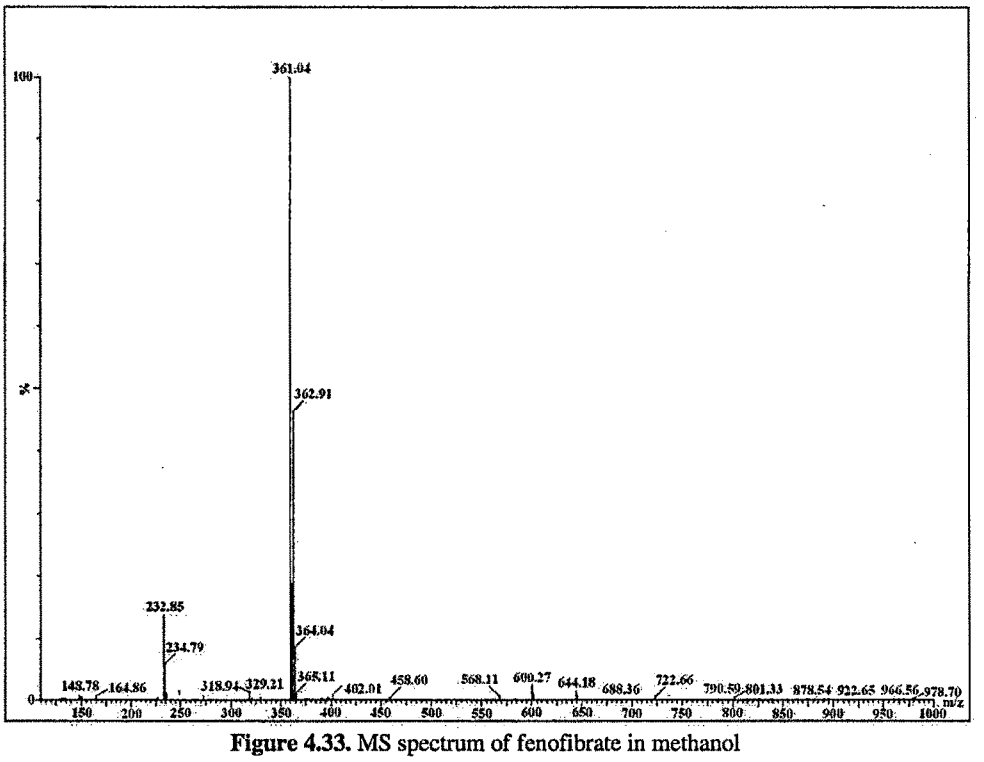

Figure 4.33. MS spectrum of fenofibrate in methanol

Separation and Identification of Esomeprazole, Venlafaxine HCl and Fenofibrate by LC – MS.

HPLC of environmental water sample with PDA detector did not show any peaks correspond to the three drugs when the environmental water sample was analysed without pre – concentration. LC – MS for the Environmental water sample without pre – concentration did not show presence of the three drugs. The representative chromatograms of 100mg L^{-1} esomeprazole, venlafaxine HCl and fenofibrate in methanol obtained by HPLC, PDA detector are shown in Figure 4.34, 4.35 and 4.36 respectively.

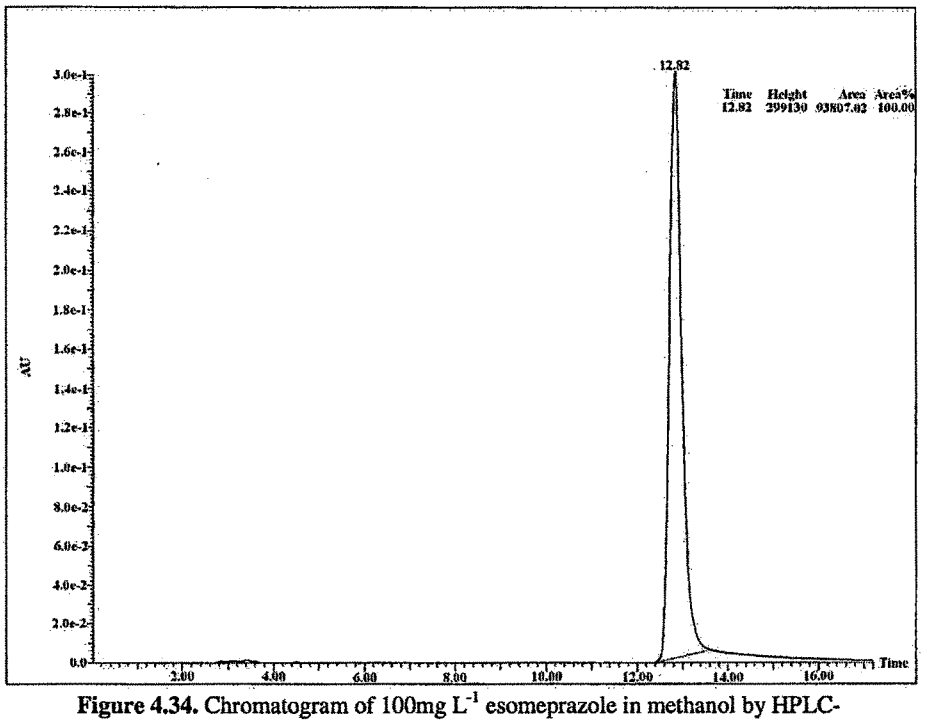

Figure 4.34. Chromatogram of 100mg L^{-1} esomeprazole in methanol by HPLC-

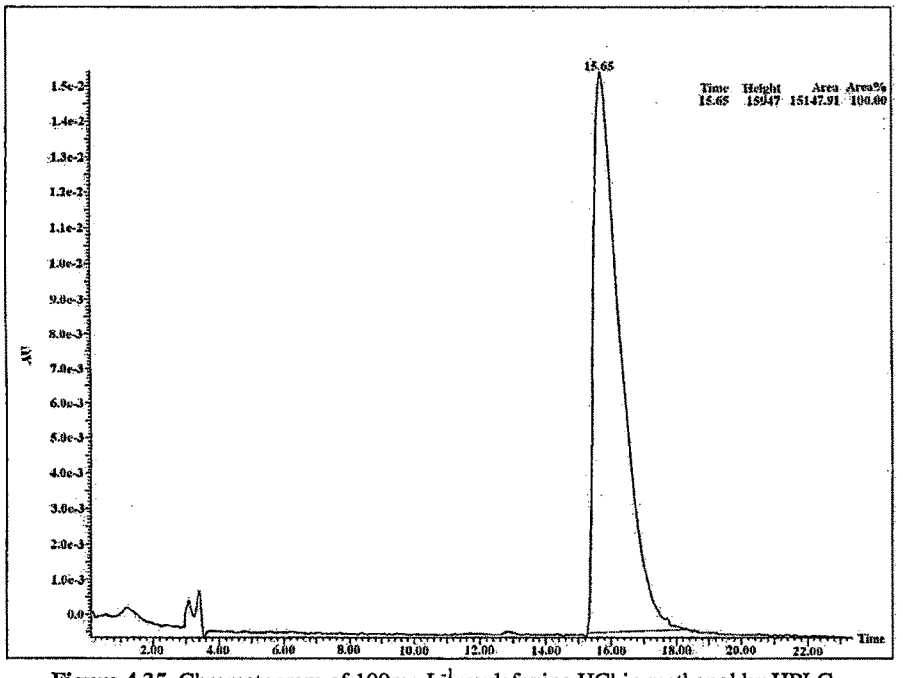

Figure 4.35. Chromatogram of 100mg L^{-1} venlafaxine HCl in methanol by HPLC-

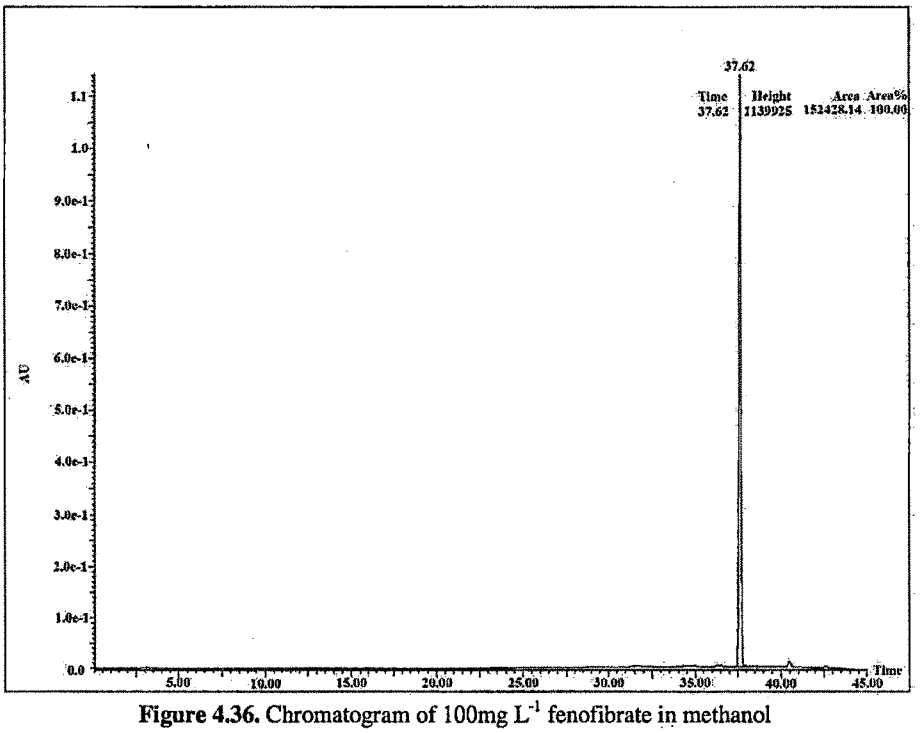

Figure 4.36. Chromatogram of 100mg L^{-1} fenofibrate in methanol

The representative chromatograms of 5, 10 and 15µL of environmental water sample analyzed by HPLC – PDA detector are shown in Figure 4.37, 4.38 and 4.39.

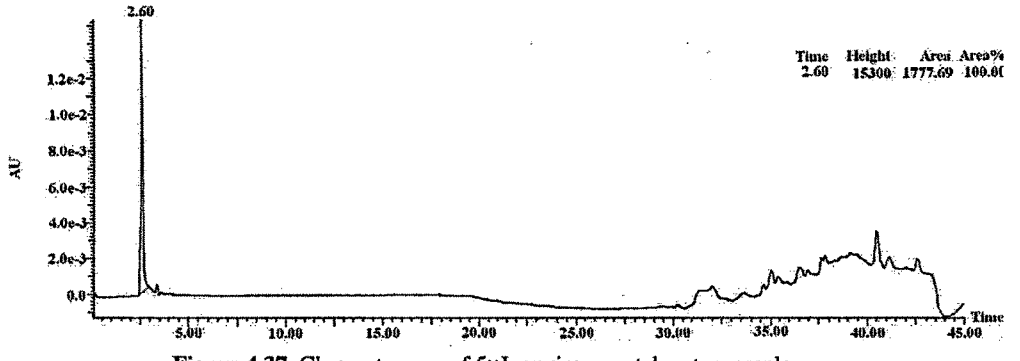

Figure 4.37. Chromatogram of 5µL environmental water sample

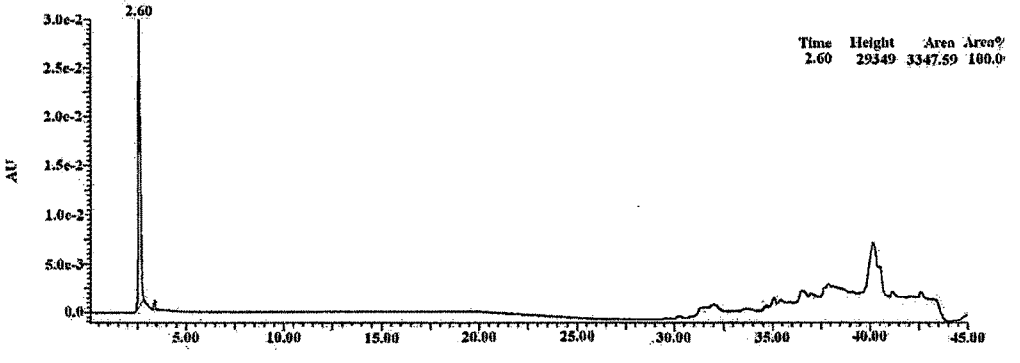

Figure 4.38. Chromatogram of 10µL environmental water sample

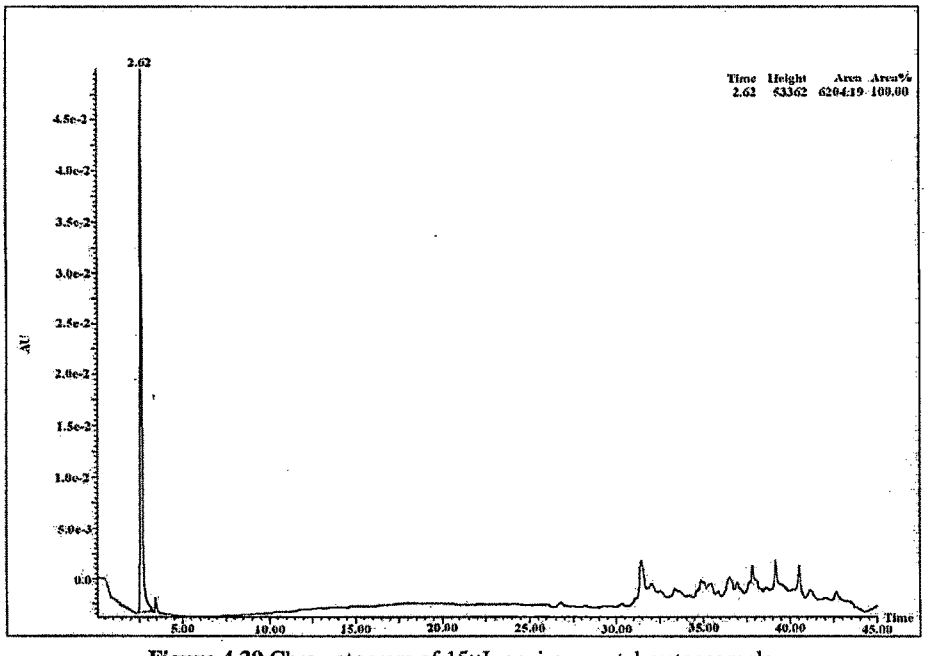

Figure 4.39 Chromatogram of 15µL environmental water sample

The MS spectrums of environmental water sample is shown in Figure 4.40.

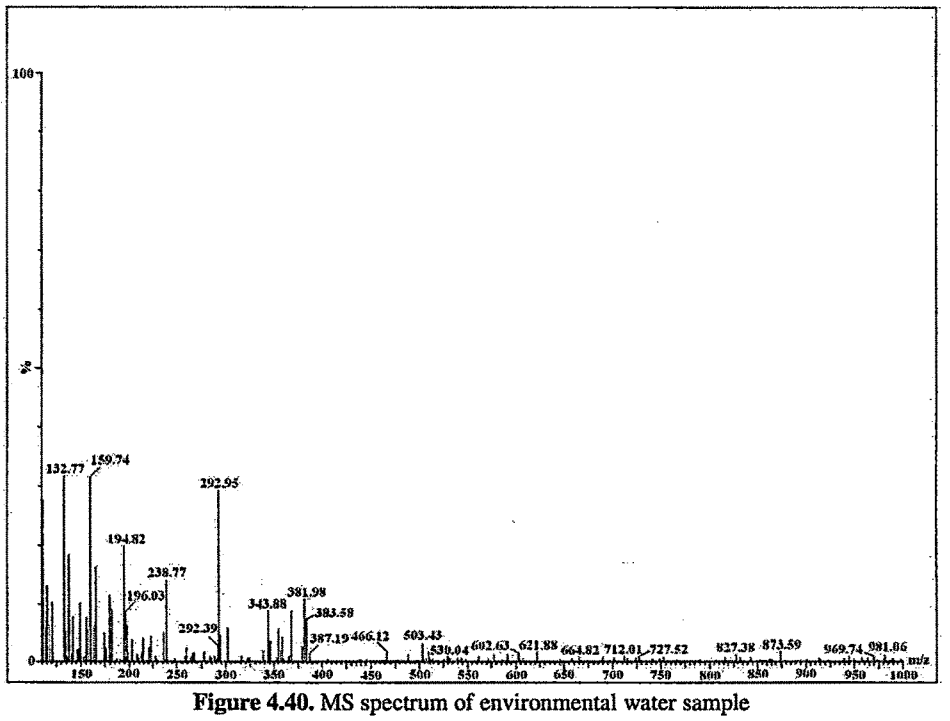

Figure 4.40. MS spectrum of environmental water sample

CONCLUSION

The gradient RP – LC method developed for determination of esomeprazole, venlafaxine HCl and fenofibrate is precise, accurate and specific. The developed, validated method could separate esomeprazole, venlafaxine HCl and fenofibrate with good resolution. The method can be used for routine analysis.

From LC – MS, results does not show presence of esomeprazole, venlafaxine HCl or fenofibrate in environmental water sample. This indicates water samples collected from STP (Vadodara – India) after treatment does not show presence of esomeprazole, venlafaxine HCl and fenofibrate. This may be due to extensive dilution occurring during the treatment process or the STP is efficient in removing the drug effectively.