A VALIDATED LC-MS/MS BIOANALYTICAL METHOD DEVELOPMENT FOR THE SIMULTANEOUS DETERMINATION OF NEBIVOLOL, HYDROCHLOROTHIAZIDE, METOPROLOL AND AMLODIPINE IN HUMAN PLASMA

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ABSTRACT

For the development and validation of a bioanalytical approach that uses liquid chromatography tandem mass spectrometry to simultaneously measure Nebivolol, hydrochlorothiazide, Metoprolol, and amlodipine in human plasma (LC-MS),where amlodipine was used as an internal standard, a simple, rapid, sensitive, and specific method has been proposed (IS). Acetonitrile recovered the following analytes: ammonium acetate of nebivolol 142-2840ng/ml (r^2 value 0.9994), hydrochlorothiazide 12.1-22ng/ml (r^2 value 0.9993), and metoprolol 8.09-161.8ng/ml (r^2 value 0.9992) using amlodipine as internal standard. LOD and LOQ of nebivolol 46.86ng/ml & 142ng/ml, hydrochlorothiazide 3.4ng/ml & 12.1ng/ml and metoprolol 2.67ng/ml & 8.09ng/ml were found. Drugs were extracted by Liquid -Liquid extraction method. According to USFDA guidelines the method was established and validated.

Keywords: Bio-analytical, Human Plasma, Nebivolol, Hydrochlorothiazide, Metoprolol, LC-MS/MS.

1. INTRODUCTION

This thesis is based on the writer's research in this laboratory for the past year on the development of bio analytical methods in human plasma for nebivolol, hydrochlorothiazide, metoprolol, and amlodipine. Before addressing the experimental findings, a quick introduction to bio-pharmaceutical analysis, drug biological sample analysis, biological sample preliminary treatment, and Anti-hypertensive drugs is a substance that is effective against high blood pressure. There are many different types of anti-hypertensive agents and they work in different ways to lower blood pressure. Angiotensin-converting enzyme (ACE) inhibitors and diuretics are two of the most well-known antihypertensive medications, and they've long been used as first-line therapies for hypertension. Reduced blood pressure by 5mmHg reduces the risk of stroke by 34% and ischemic heart disease by 21%, as well as the risk of dementia, heart failure, and cardiovascular disease mortality. Nebivolol is a selective antagonist of the 1 receptor. The activation of 1 receptors by adrenaline causes a rise in heart rate and blood pressure, as well as an increase in oxygen consumption by the heart. Nebivolol inhibits these receptors, reversing epinephrine's impact of reducing blood pressure & heart rate. Beta blockers also prevent the production of renin, a hormone generated by the kidneys that causes an increase in blood pressure.

Diuretics in the thiazide class include hydrochlorothiazide. It works on the kidneys to limit convoluted (Na+) reabsorption in the distal convoluted tubule, lowering blood volume. By contending for the chloride position on the transporter, the key site of an electro neutral Nacl Co-transporter appears to be involved in nephron activity. In the distal convoluted tubule, hydatid disease inhibits Na+ transport. tubule.

Metoprolol is beta1inhibitors of adrenergic receptors that are exclusive to however, they have no effect on beta 2 receptors in cardiac cells. This inhibition lowers cardiac output by exerting negative chronotopic and inotropic effects without affecting membrane stability or functioning as a natural sympatholytic.Amlodipine is a peripheral artery vasodilator that works by acting directly on vascular smooth muscle cells to reduce peripheral vascular resistance and lower blood pressure. Amlodipine is a calcium antagonist (also known as a calcium ion antagonist or a

slow channel blocker) that works by preventing calcium ions from entering vascular smooth muscle and cardiac muscle.The objective of the work is to Developing and Validating of Bio-analytical LC-MS/MS method for estimating Nebivolol, Hydrochlorothiazide, and Metoprolol in human plasma simultaneously. The method is developing as per USFDA guidelines and predicted to be accurate, specific and precise. **(Fig1a-1d) 1a: hydrochlorothiazide,**

1b: nebivolol, 1c: metoprolol, 1d: amlodipine

FIGURE1a:

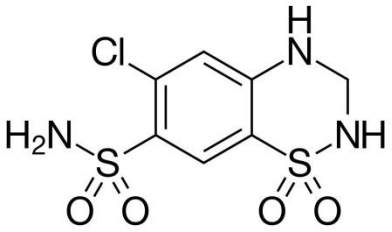


FIGURE1b:

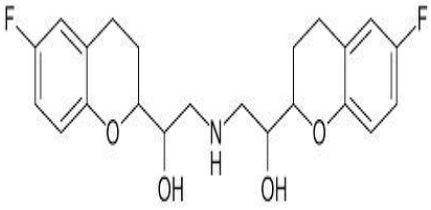
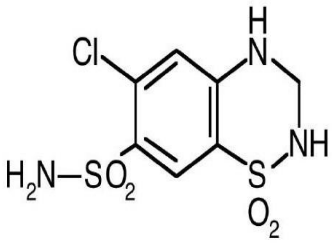


FIGURE1c:



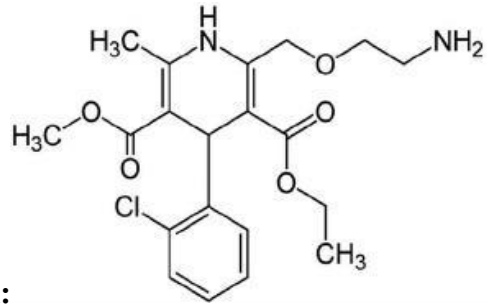


FIGURE1d:

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents Used

Cyanomethane of S.D. Fine Chemicals Ltd provided HPLC grade and AR grade ammonium acetate, while Milli-Q RO system provided water HPLC grade. Nebivolol, Hydrochlorothiazide, Metoprolol taken as a working standard and Amlodipine used as an internal standard obtained from Indian Pharmacopoeia, New Delhi.

2.2 Instruments Used

The Isobestic point was predicted by Shimadzu (UV-1700) Spectrophotometer. The analysis of the drugs were accomplished on a Shimadzu gradient HPLC system with LC-10 AT-VP solvent

supply system (pump), SPDM-10 AVP photo diode array detector, and class-VP data station configuration. The procedure was followed and optimized in Shimadzu LC-MS/MS 8030 was defined with Lab solution data solution, Triple quadrupole analyzer, ESI interface. Zorbax SB C_{18} (5µ, 50 x 3.6mm i.d.) used as the analytical columns.

3. OPTIMIZATION OF MS AND CHROMATOGRAPHIC CONDITION

3.1 LC/MS PARAMETERS

Fixed loop injection (10µl) were distributed on an LC-MS/MS Zorbax SB C_{18} (5µ, 50 x 3.6mm i.d.) and the mobile phase used Ethyl nitrile: Ammonium Acetate (80:20v/v) and the pH 6. Total flow rate was 0.5ml/min temperature in the column oven was ambient. Mass detector was used and run time was found to be 5.0min.

Ideal Electrospray Ionization Negative Mode was used as Ion source. In polarity the Nebivolol observed in Positive mode, Hydrochlorothiazide Negative mode, Metoprolol Positive mode by using ion source. Probe temperature was maintained ambient and Desolvation Line (DL) as 250ºc and Detector Voltage 1.3Kv. Retention time was found to be (2.339, 1.219, 2.227 and 3.045) for Nebivolol, Hydrochlorothiazide, Metoprolol and Amlodipine. MRM (Multiple Reactions Monitoring) was used as scan type. Multiple Reactions Monitoring value was obtained to be (406.00/ 44.15 (m/z), 295.65/ 268.85 (m/z), 268.05/ 56.10 (m/z) and 409.00/ 238.05 (m/z)) for Nebivolol, Hydrochlorothiazide, Metoprolol and Amlodipine.

3.2 PREPARATION OF MOBILE PHASE AND STOCK SOLUTION

3.2.1 Mobile Phase Buffer (10MM Ammonium Acetate)

Weigh approximately 0.7708gm of 1L ammonium acetate is transferred to standard flask and diffused the ammonium acetate makeup the volume with milli Q water with and volume mixed the buffer solution well, mark the solution and keep at ambient temperature.

3.2.2 Mobile Phase Preparation

Solvent A: Acetonitrile

Solvent B: Mobile phase buffer (10Mm Ammonium acetate with pH 6)

Mobile phase ratio: Solvent A: Solvent B (80:20v/v)

Auto sampler rinsing injection: 10MM Ammonium acetate (80 :20v/v) 800ml of Acetonitrile and 200ml of buffer was continued and carried into a relevant pre-sized vessel using graduated measuring cylinder, combined well, marked the mobile phase solutions and reserved at ambient temperature.

3.2.3 Preparation of Standard Solution of Nebivolol (1.0mg/ml)

Nebivolol was weighed around 10.0mg and transferred to 10.0ml volumetric flask by using a calibrated digital balance. In a limited amount of DMSO diffuse the Nebivolol and makeup the volume with acetonitrile solvent and homogenized well by vibrate the volumetric flask, mark the container and keep at 2-8°C.

3.2.4 Preparation of Standard Solution of Hydrochlorothiazide (1.0mg/ml)

10.0mg of hydrochlorothiazide put in the balance and shifted in 10.0ml of volumetric flask by using a calibrated digital balance suspend the hydrochlorothiazide with cyanomethane and made up the volume with same solvent and integrated well by vibrate the volumetric flask, mark the container stored at 2-8°C.

3.2.5 Preparation of Standard Solution of Metoprolol (1.0mg/ml)

Measured 10.0mg of Metoprolol by using a calibrated digital balance and transferred into a 10.0ml of calibrated flask. Metoprolol is diffused with cyanomethane and volume is made up with the same solvent and integrated well by vibrate the volumetric flask, mark the container kept at 2-8°C.

3.2.6 Preparation of Standard Solution of Internal Standard (1.0mg/ml)

Using a calibrated digital balance, 10.0mg of Amlodipine was measured and shifted into a 10.0ml volumetric flask. Deliquesced the amlodipine with Cyanomethane and make up the volume with the same reagent and homogenized well by shaking the volumetric flask, labelled and stored at 2-8^{o}c.

3.2.7 Preparation of Working Standard Solution of Nebivolol

10.0ml of each of 142.0, 1384 and 2840ng/ml of Nebivolol standard solutions using the Nebivolol mobile phase and stock solution and kept at -20± 2ºc until analysis.

3.2.8 Preparation of Working Standard Solution of Hydrochlorothiazide

Prepared standard solutions of hydrochlorothiazide for each 10.0ml of 12.1, 121 and 242ng/ml using the hydrochlorothiazide mobile phase and stock solution and kept at -20± 2ºc til study.

3.2.9 Preparation of Working Standard Solution of Metoprolol

Metoprolol standard solution is contributed for each 10.0ml of 8.09, 80.9 and 161.8ng/ml using the Metoprolol mobile phase and stock solution and kept at -20± 2ºc til study.

3.2.10 Preparation Stock Calibration Curve Samples (CC)

Nebivolol standard stock solution provided 10.0ml each of 142, 284, 568, 1136, 1384, 1952, 2236, 2520 and 2840ng/ml of Nebivolol calibration curve and mobile phase and reserved at -20± 2ºc till study.

Contributed 10.0ml each of 12.1, 24.2, 48.4, 96.8, 121, 169.4, 193.6, 217.8 and 242ng/ml of hydrochlorothiazide calibration curve sample using hydrochlorothiazide mobile phase and standard stock solution and kept at 20±2ºc til analysis.

Using standard stock solution of Metoprolol prepared 10.0ml each of 8.09, 16.18, 32.36, 64.72, 80.9, 113.26, 129.44, 14562, 161.8ng/ml of Metoprolol calibration curve samples and mobile phase stored at 20± 2ºc til analysis.

Concepts and procedures for sample preparation will be explored, as well as the conditions that must be met in order for a good sample to be prepared High-throughput sample preparation procedures are described for the extraction and concentration of analytes from biological matrices. Finally, the importance of selective extraction technologies such molecular imprinted phases is emphasized.

(A) Liquid-Liquid Extraction

(B) Solid-Phase Extraction

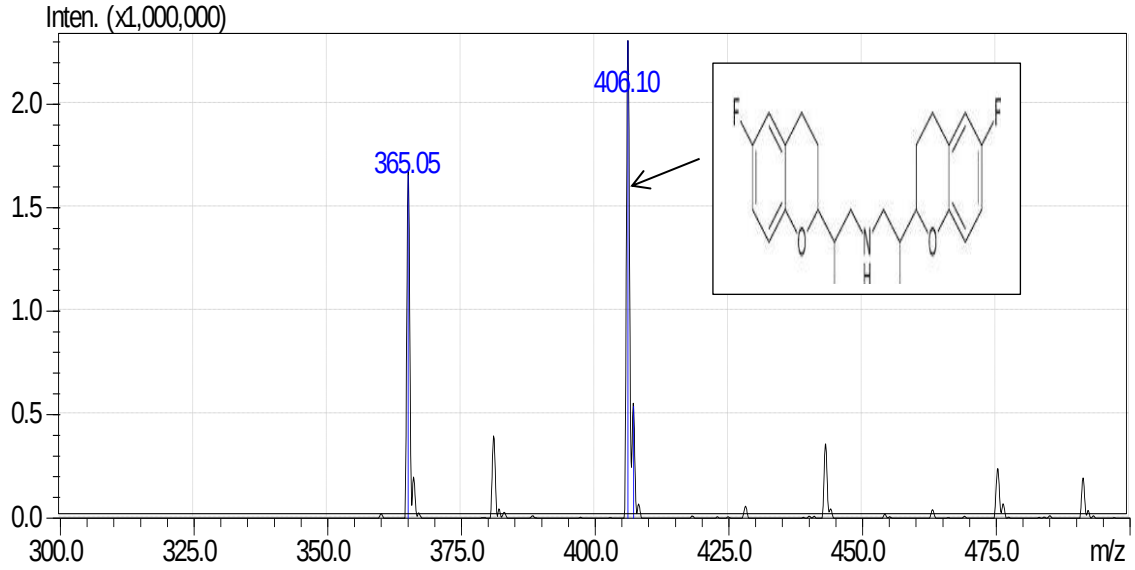
(C) Protein precipitation

But in this three, precipitation of the plasma proteins is taken as an extracting method. It is used to concentrate proteins and purify them from various impurities during the downstream processing of biological products.

3.2.11 Preparation of Quality Control (QC) Samples

In centrifuge tube (2.0ml), add 0.3ml of precipitating agent, add 0.1ml of 100ng/ml of internal standard solution, add plasma 0.6ml of vortex the solution for 30 sec and add 1ml of methyl alcohol again vortex for 20 sec and centrifuged at 4000RPM for 10 minutes, distributed the supernatant layer and save it and centrifuge at 3000RPM for 10mins, protect the sample for the analysis.

fig.2: Typical standard ms -spectrum of Nebivolol



4. RESULTS AND DISCUSSION

4.1 Method Optimization

We are exposed to millions of new pollutants that are undetectable by the devices now in use. While many chemists may be familiar with LC-MS/MS for routine analysis method creation

with compound optimization required, the mode of ionization is the most important decision in many LC-MS/MS methods. It serves as a quick reference for LC-MS/MS optimization.

1. Dilution of the chemical standard
2. MS/MS optimization
3. Chromatography optimization
4. Verification with a calibration curve

To method development and measure the each category like resolution, run time, peak and symmetry in the requirements of Liquid Chromatography-MS/MS can be nearly new for the quantification of hydrochlorothiazide, Nebivolol, amlodipine and Metoprolol**(fig2-15)**.The optimised condition was used to inject a blank sample, a standard sample, and a test sample, and chromatograms were recorded. As a result, chemists must determine and optimise instrument parameters, so they can be identified.LC-MS/MS (liquid chromatography-tandem mass spectrometry/mass is used to quantify and identify trace amounts of chemicals with high senstivity.Optimization of MS/MS settings and LC conditions. The other usual LC-MS/MS settings were applied for each MS/MS parameter adjustment, including ion source parameters (collision das, curtain gas, 25 psi, gas 1, 55 psi, gas 2, 55 psi, ion source voltage, 5.5 kv, temperature, 550 oc). Finally, the test's optimal parameters and signal intensity were integrated.

fig.3: Typical standard ms -spectrum of Hydrochlorothiazide

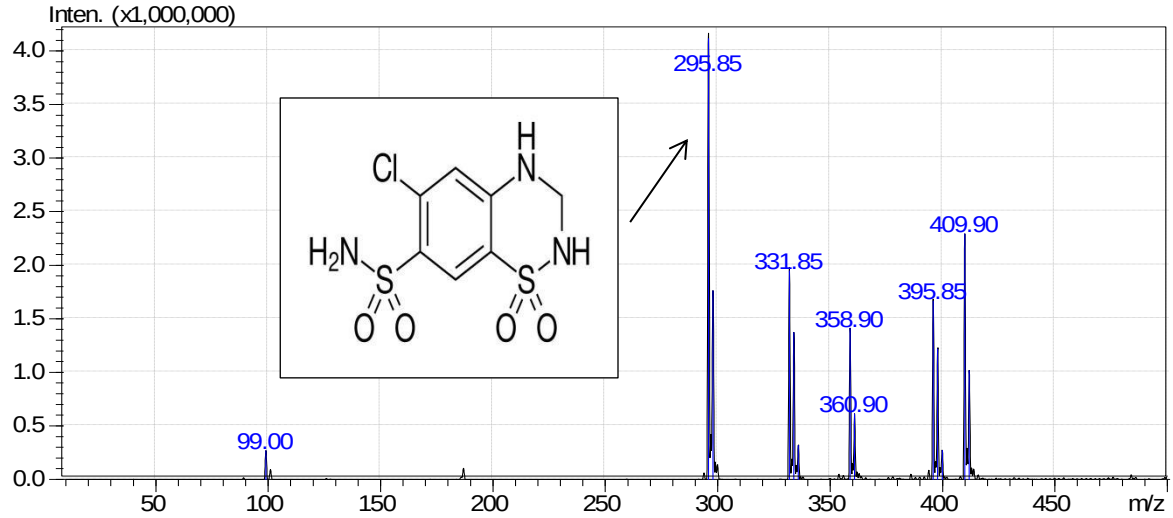


fig.4: Typical standard ms -spectrum of Metoprolol

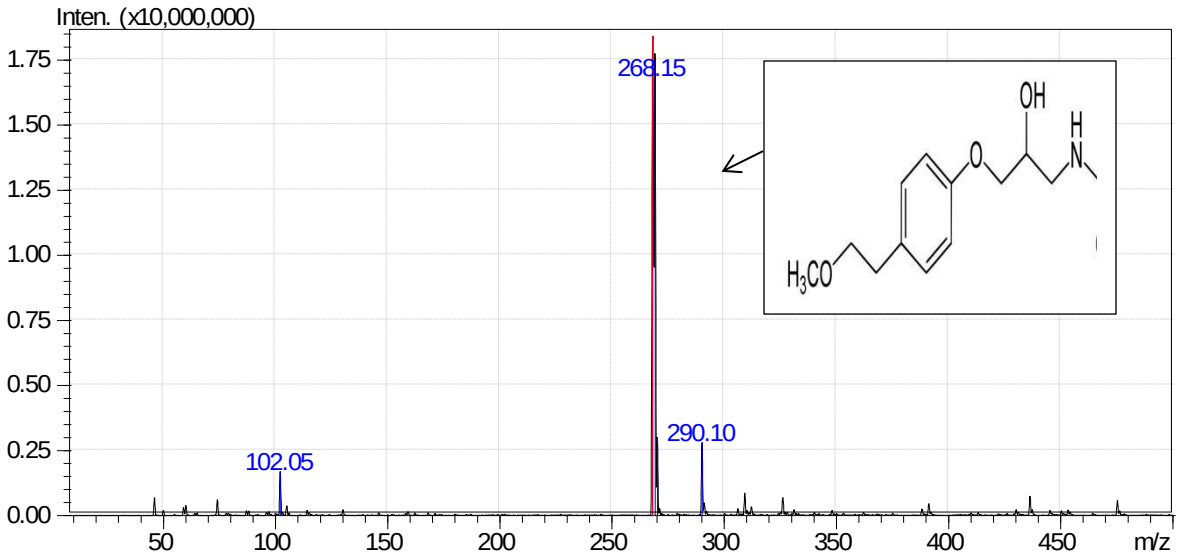


fig.5: Typical standard ms -spectrum of Amlodipine

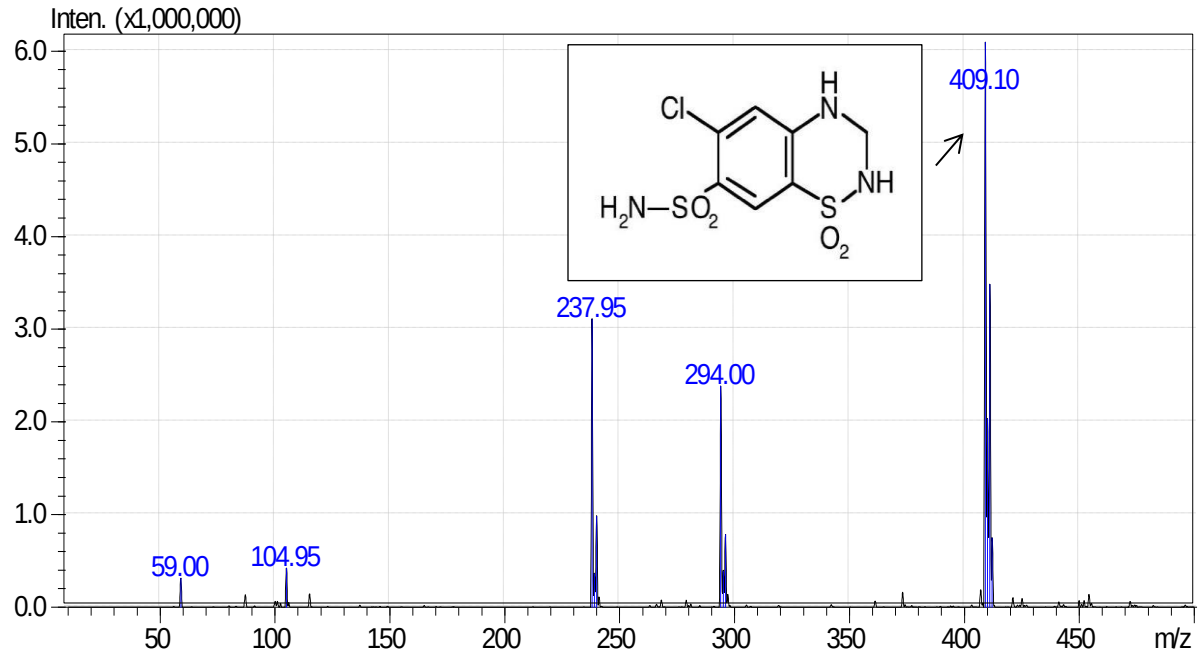


fig.6: Typical standard chromatogram of Nebivolol

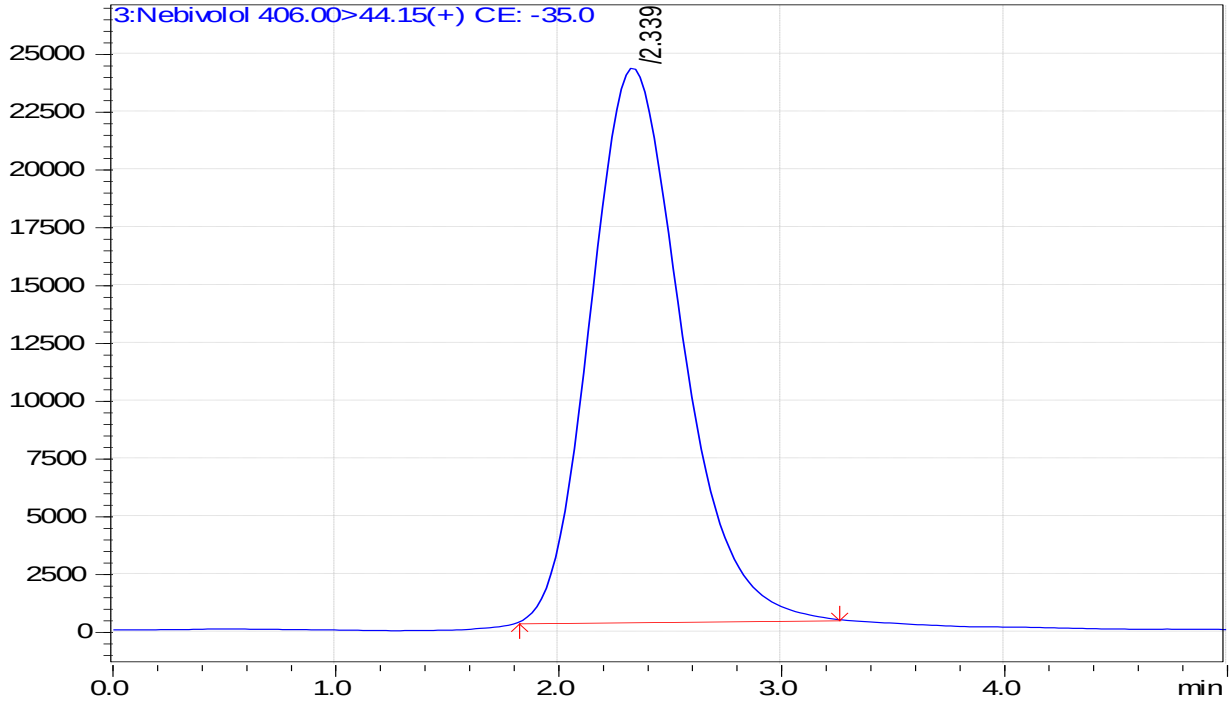


fig.7: Typical standard chromatogram of Metoprolol

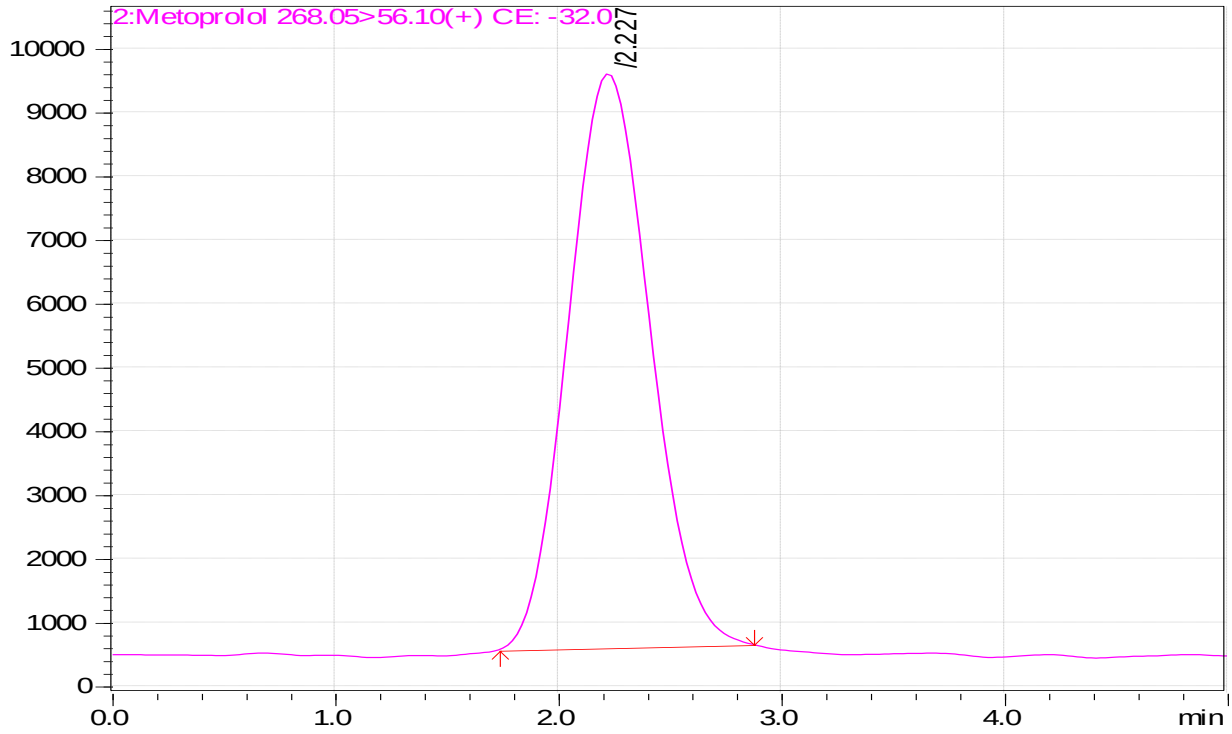


fig.8: Typical standard chromatogram of Hydrochlorothiazide

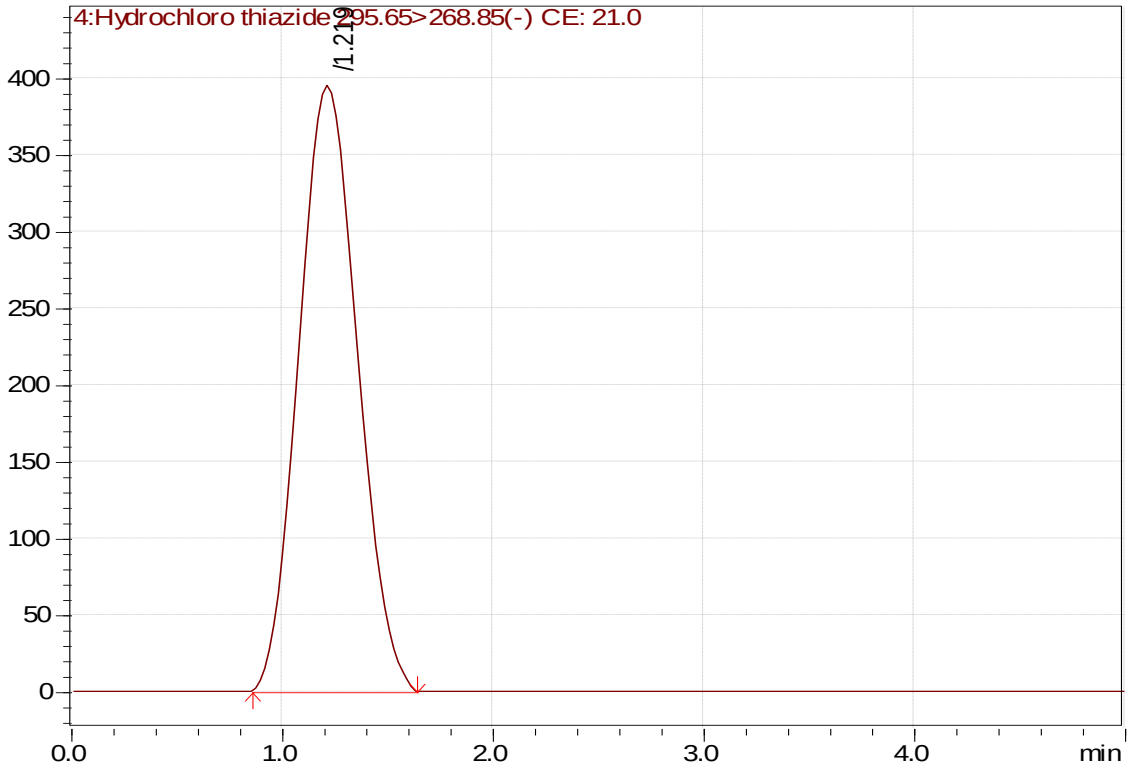


fig.8:Typical standard chromatogram of Amlodipine

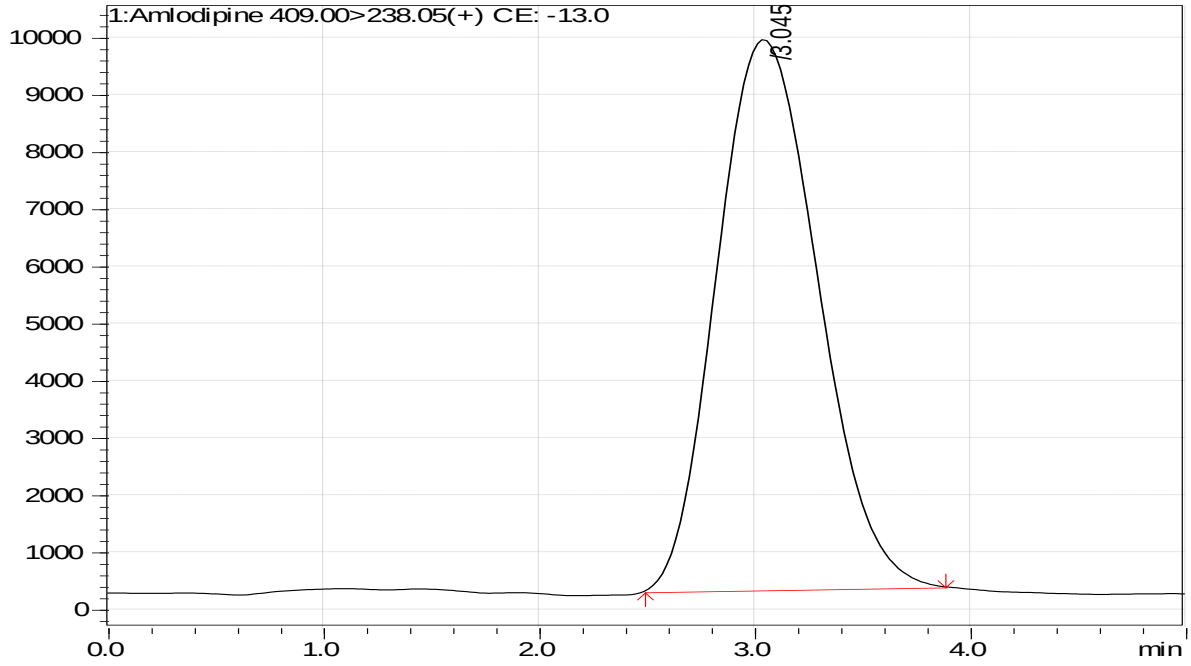


fig.9: Overlay Of Standard Chromatogram of Four Drugs

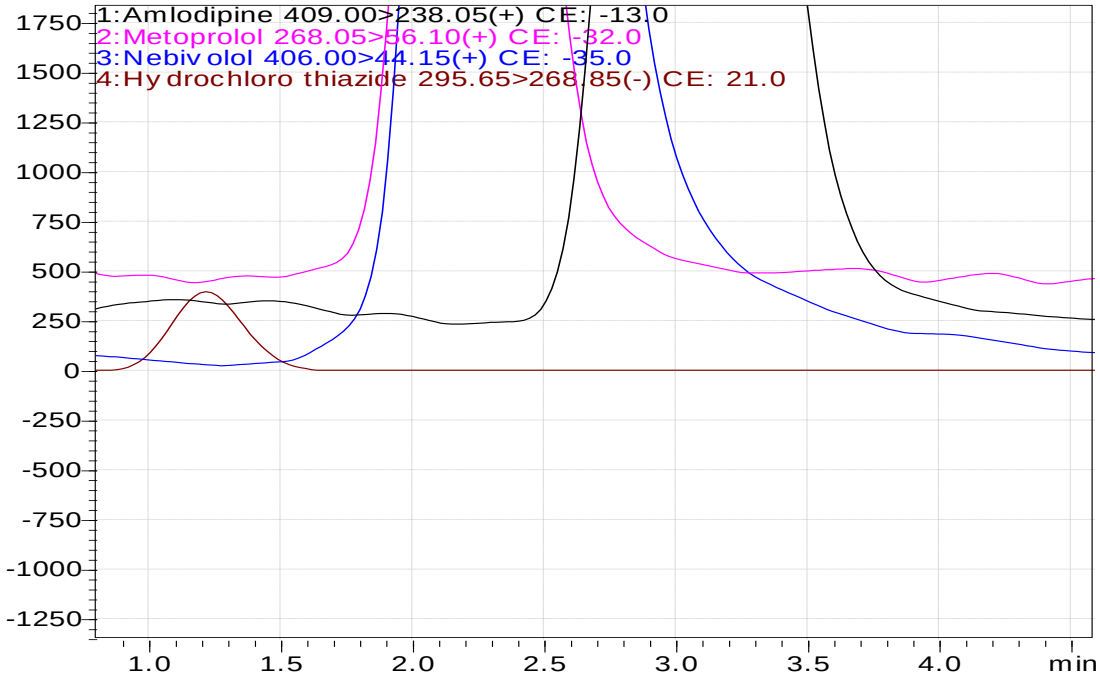


fig.10: Typical chromatogram of blank plasma

fig11 : Product ion scan of Nebivolol, Hydrochlorothiazide, Metoprolol and amlodipine (IS)

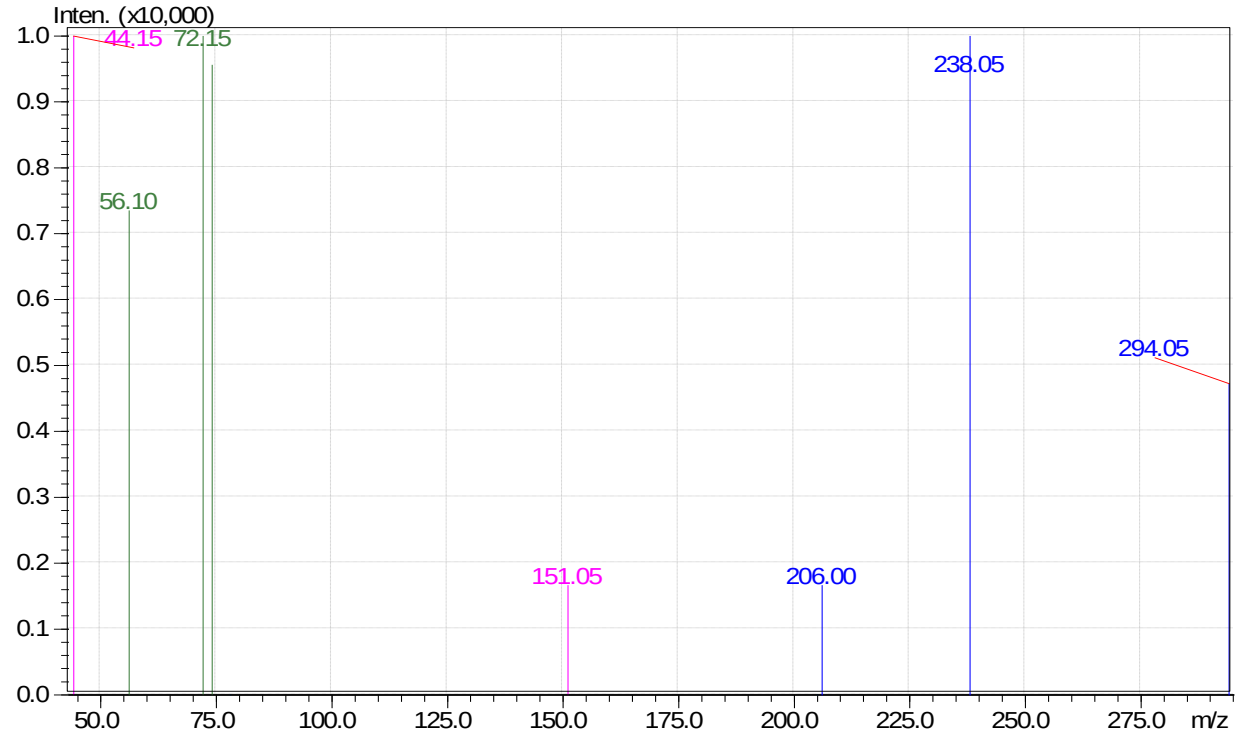


fig.12: Typical standard chromatogram of Nebivolol in plasma

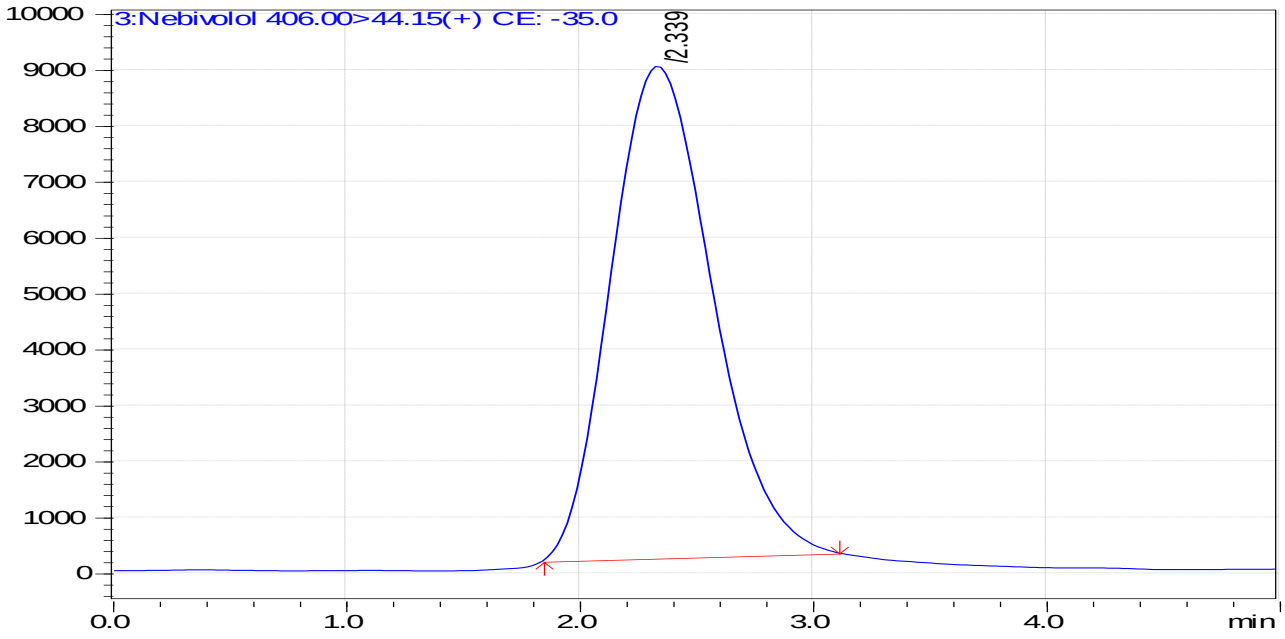


fig.13: Typical standard chromatogram of Hydrochlorothiazide in plasma

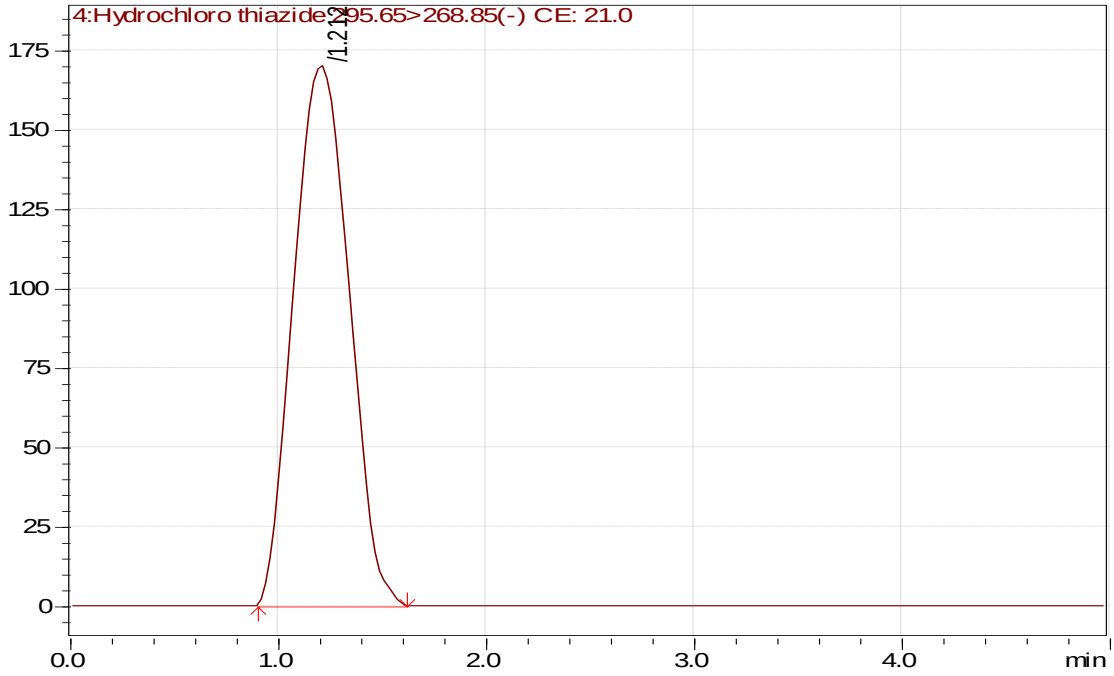


fig.14 : Typical standard chromatogram of Metoprolol in plasma

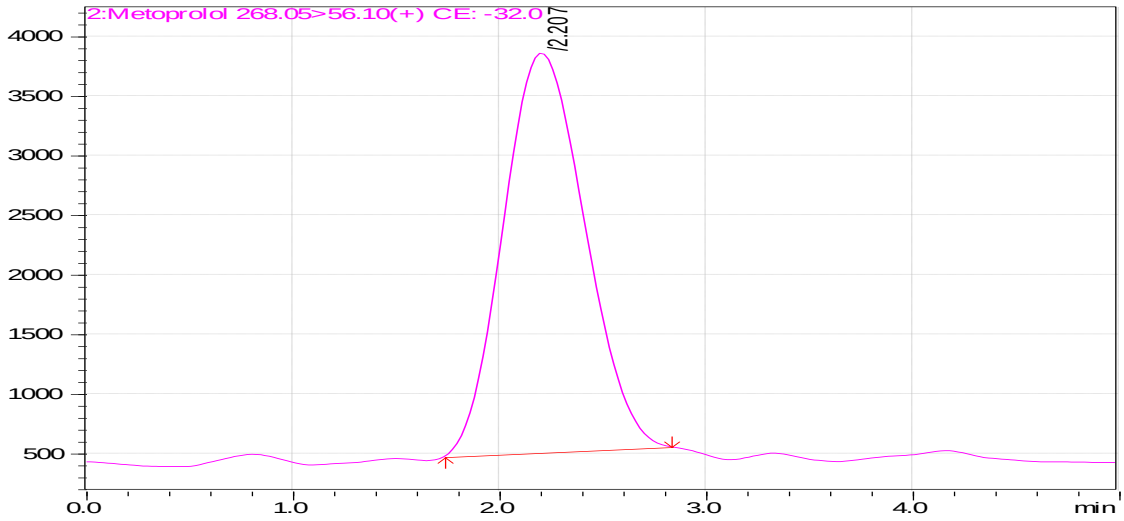
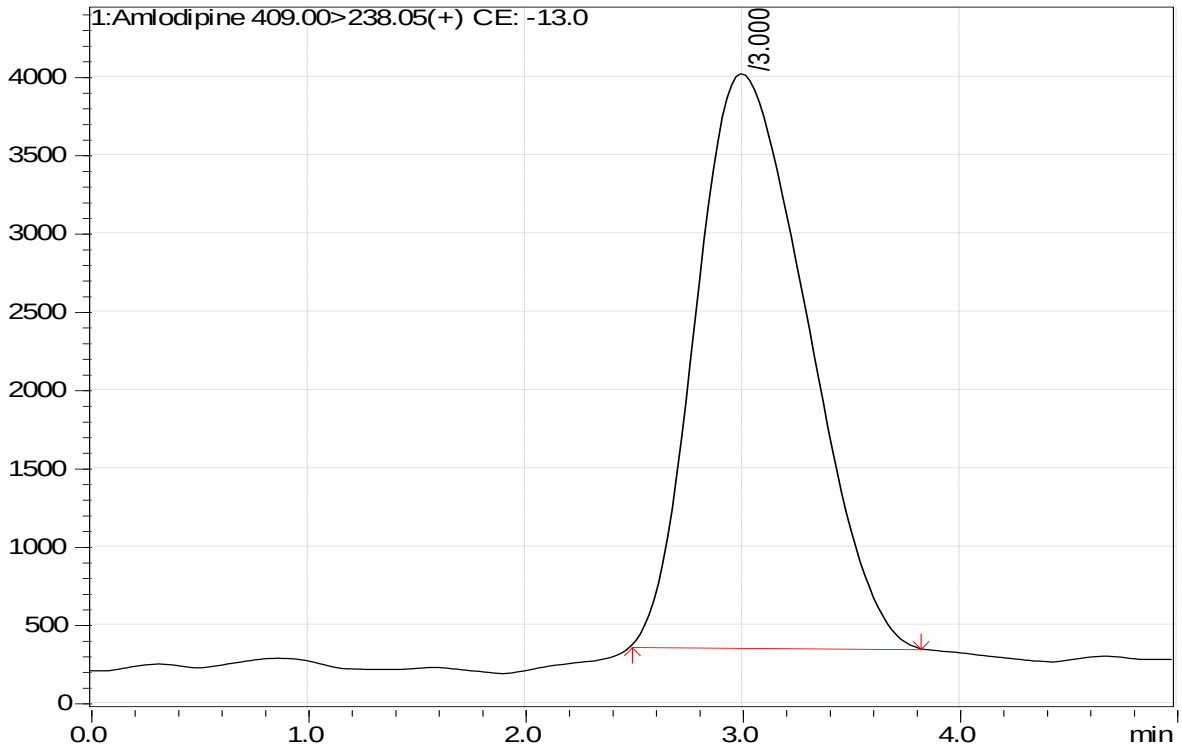


fig.15: Typical standard chromatogram of Amlodipine in plasma



4.2 Method Validation

The method's selectivity, linearity, precision, accuracy, and recovery were all tested. In chemical analysis, method validation is a critical step in obtaining trustworthy results. The LC-MS/MS method was validated in accordance with guidelines for Bio analytical method validation published by US-FDA (Food and Drug Administration) and the European Dug Administration guidelines on Bio analytical method validation.

Development and validation of analytical methods,Any pharmaceutical development programme must include the creation, validation, and transfer of analytical methods. Method development that is effective guarantees that laboratory resources are maximized while techniques achieve the objectives set out at each stage of drug development. Validation of analytical methods is now a critical component of successful drug development and characterization.

Relative and absolute recovery studies were used to determine the accuracy of the optimised approach. Percent of a return values for Nebivolol in plasma were arranged from 93.18 to 99.48 %**.** Percent of a return values for Hydrochlorothiazide in plasma arranged from 91.87 to 98.87%. Percent of a return results for Metoprolol in plasma arranged from 88.66 to 98.18 %. As a result, it can be concluded that the created procedures are accurate and dependable **(table 1 and table 2).**Estimation of drugs were optimized and it was found to be precise. For coefficient of variation importance proof is there, that is within the limit of concentrations.

Table 1: RECOVERY STUDIES OF NEBIVOLOL, HYDROCHLOROTHIAZIDE AND METOPROLOL

Concentration(ng/ml)	**NEBIVOLOL**		
	Mean(ng/ml) ± SD	**CV (%)**	**Accuracy (%)**
LQC(142)	132.31±6.28	4.74	93.18
MQC(1384)	1355.68± 5.70	0.42	97.95
HQC(2840)	2825.31± 5.47	0.19	99.48
Concentration(ng/ml)	**HYDROCHLOROTHIAZIDE**		
	Mean(ng/ml) ± SD	**CV (%)**	**Accuracy (%)**
LQC(12.1)	11.11± 0.55	4.96	91.87
MQC(121)	117.56± 2.40	2.40	97.15
HQC(242)	234.53± 0.59	0.24	98.87
Concentration(ng/ml)	**METOPROLOL**		
	Mean(ng/ml) ± SD	**CV (%)**	**Accuracy (%)**

LQC(8.09)	7.17± 0.32	4.54	88.66
MQC(80.9)	77.02± 1.26	1.63	95.20
HQC(161.8)	158.86± 1.30	0.77	98.18

Table 2: PRECISION STUDIES OF NEBIVOLOL, HYDROCHLOROTHIAZIDE AND METOPROLOL (Inter- day and Intra- day)

Analyte	**QCs(ng/ml)**	**Mean Concentration found (ng/ml) ± SD**	**Intra-day**	
			Accuracy (%)	**Precision (%)**
Nebivolol	LQC(142)	129.98± 5.34	91.53	4.11
	MQC(1384)	1348.54± 5.0	97.43	0.37
	HQC(2840)	2820.20±4.81	99.30	0.17
Hydrochlorothiazide	LQC(12.1)	10.61± 0.51	87.74	4.84
	MQC(121)	116.84±2.35	96.56	2.01
	HQC(242)	234.26±0.52	96.80	0.22
Metoprolol	LQC(8.09)	7.03±0.31	86.89	4.44
	MQC(80.9)	76.41±1.15	94.44	1.51
	HQC(161.8)	157.57±1.03	97.38	0.65
Analyte	**QCs(ng/ml)**	**Mean Concentration found (ng/ml)± SD**	**Inter-day**	
			Accuracy (%)	**Precision (%)**

Nebivolol	LQC(142)	127.±5.10	89.79	4.09
	MQC(1384)	1343.59±4.80	97.08	0.35
	HQC(2840)	2818±4.80	99.25	0.15
Hydrochlorothiazide	LQC(12.1)	10.61±0.49	87.68	4.62
	MQC(121)	114.84±2.21	94.91	1.93
	HQC(242)	232.96±0.50	96.26	0.21
Metoprolol	LQC(8.09)	7.01±0.31	86.73	4.43
	MQC(80.9)	75.27±1.10	93.04	1.47
	HQC(161.8)	156.60±0.9	96.79	0.59

Blank plasma as were injected for six times and examined and chromatograms were documented. Particular selected drugs and internal standards were injected and in retention time there is no endogenous inferences observed. In chromatograms no extra peaks were finded out.

Retention time of Nebivolol, Hydrochlorothiazide, Metoprolol were 2.399min, 1.219min, 2.277min and respectively and Internal standard Retention time was 3.045 min.

The enhanced approaches were found to be linear within a specific concentration range for various drugs. Calibration curves were plotted between the response factor and the concentration of the standard solutions.

Linearity for Nebivolol was found to be 142, 284, 568, 1136, 1384, 1952, 2236, 2520 and 2840ng/ml, Hydrochlorothiazide was found to be 12.1, 24.2, 48.4, 96.8, 121, 169.4, 193.6, 217.8 and 242ng/ml and Metoprolol was found to be 8.09, 16.18, 32.36, 64.72, 80.9, 113.26, 129.44, 14562, 161.8ng/ml **(table 3)** The R^2value for Nebivolol 0.9994, Hydrochlorothiazide 0.993 and Metoprolol 0.9992**(fig 16 – 18)**.

Table 3: LINERITY & RANGE

S. No	Nebivolol (ng/ml)	HCTZ (ng/ml)	Metoprolol (ng/ml)	Internal Standard conc (ng/ml)	Response Factor (Nebivolol)	Response Factor (HCT)	Response Factor (Metoprolol)
1	142	12.1	8.09	100	0.00886	0.02635	0.30240
2	284	24.2	16.18	100	0.02083	0.05421	0.60410
3	568	48.4	32.36	100	0.03541	0.11197	1.20005
4	1136	96.8	64.72	100	0.07202	0.21505	2.41665
5	1384	121	80.9	100	0.08847	0.27083	3.00321
6	1952	169.4	113.26	100	0.12630	0.38295	4.39574
7	2236	193.6	129.44	100	0.14588	0.42152	4.99388
8	2520	217.8	145.62	100	0.16187	0.47978	5.48109
9	2840	242	161.8	100	0.18619	0.52455	6.06495

Fig16 : CALIBRATION CURVE OF NEBIVOLOL

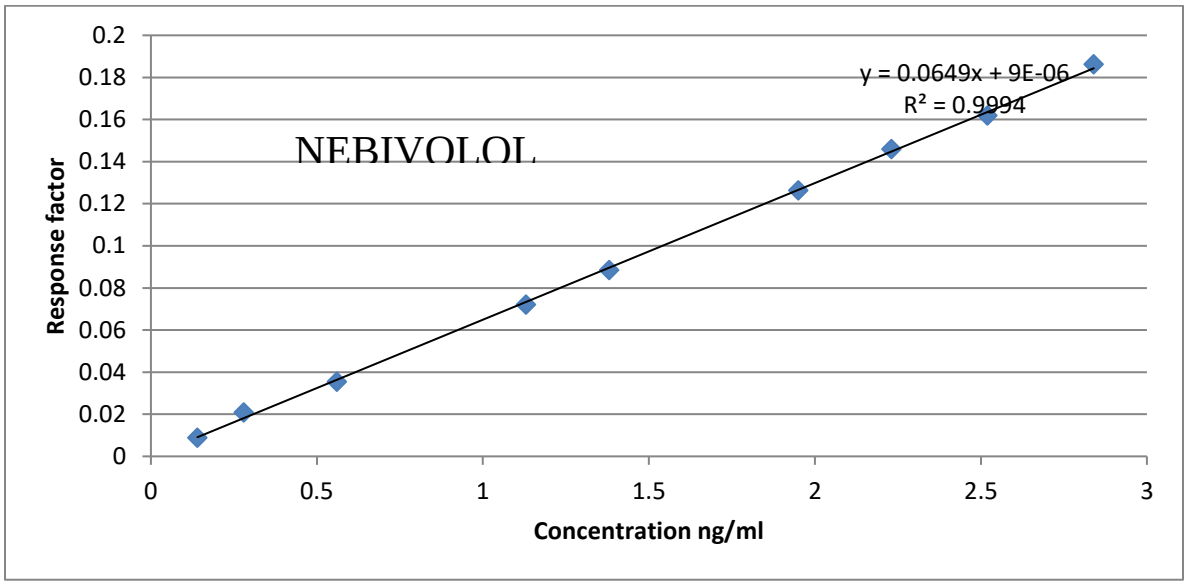


Fig17 : CALIBRATION CURVE OF HYDROCHLOROTHIAZIDE

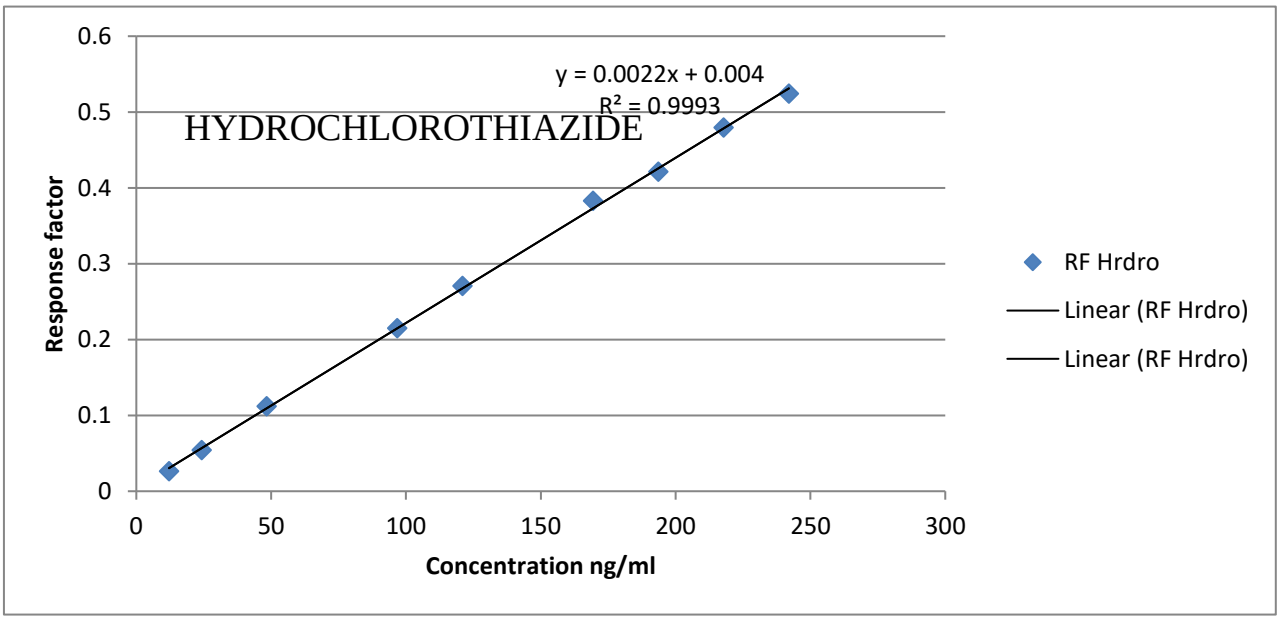
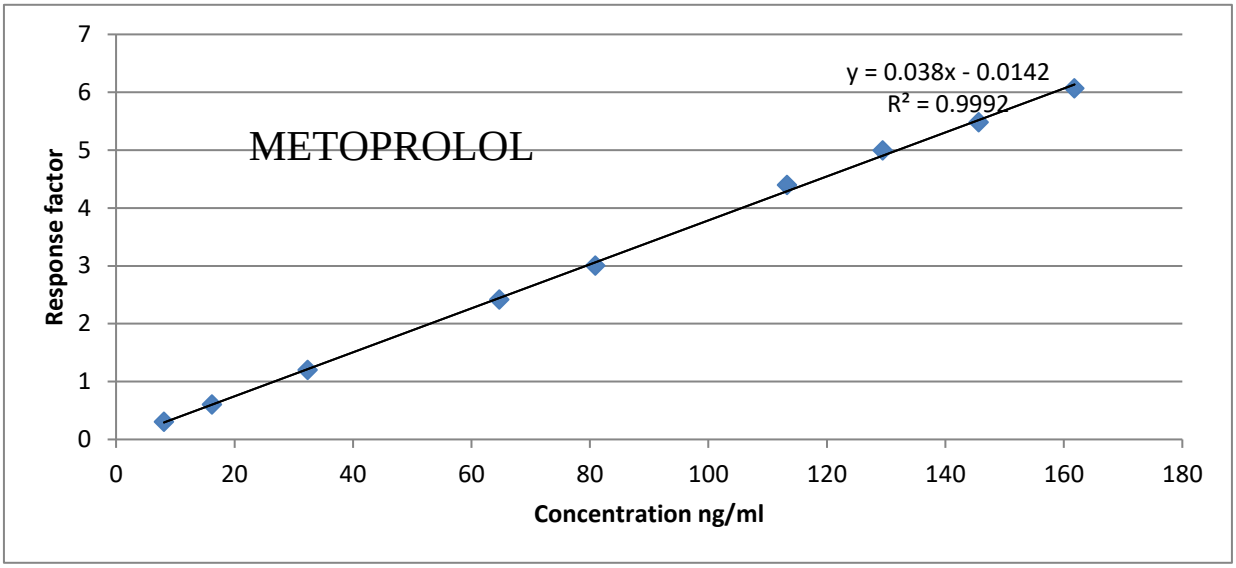


Fig18 : CALIBRATION CURVE OF METOPROLOL



Three freeze-thaw cycles were used to test the stability of drug spiked human plasma samples at three levels. The theoretical values were compared to the mean concentrations of the bench top stability samples, which were likewise short term (6 hours) (8 h). The data demonstrated that the drugs were stable in plasma after being frozen **(table 4a, 4b, 4c)**.

Table 4a: STABILITY OF NEBIVOLOL, HYDROCHLOROTHIAZIDE, METOPROLOL AND AMLODIPINE IN PLASMA DURING STORAGE AND SAMPLE HANDLING

Freeze and Thaw Stability Data

Nebivolol				Hydrochlorothiazide				Metoprolol		
SI.NO	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**	**SI.NO**	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**
1	131.98	1382.98	2806.2	**1**	10.21	110.21	233.41	7.01	76.01	157.56

2	122.02	1372.02	2815.1	**2**	10.48	114.48	232.48	7.02	74.02	155.98
3	124.78	1374.78	2809.5	**3**	9.58	113.08	233.08	6.53	75.33	157.33
Mean	126.60	1376.5	2810.3	**Mean**	10.09	112.59	232.99	6.85	75.12	156.95
S.D (+/-)	5.14	5.70	4.48	**S.D (+/-)**	0.46	2.17	0.47	0.28	1.01	0.85
C.V	4.07	0.41	0.15	**C.V**	4.57	1.93	0.20	4.08	1.34	0.54

Table 4b: STABILITY OF NEBIVOLOL, HYDROCHLOROTHIAZIDE, METOPROLOL AND AMLODIPINE IN PLASMA DURING STORAGE AND SAMPLE HANDLING

Short Term Stability Data (at ambient temperature)

Nebivolol				**Hydrochlorothiazide**				**Metoprolol**		
SI.NO	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**	**SI.NO**	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**
1	131.96	1382.9	2806.1	**1**	10.20	110.20	233.39	6.99	76.00	157.55
2	122.01	1372	2815	**2**	10.47	114.47	232.47	7.00	74.00	155.96
3	124.77	1374.7	2809.4	**3**	9.57	113.07	233.06	6.52	75.32	157.32
Mean	126.24	1376.5	2810.1	**Mean**	10.08	112.58	232.87	6.83	75.10	156.94

S.D (+/-)	5.13	5.69	4.5	**S.D (+/-)**	0.46	2.17	0.46	0.27	1.01	0.85
C.V	4.06	0.41	0.16	**C.V**	4.56	1.93	0.20	4.01	1.35	0.54

Table 4c: STABILITY OF NEBIVOLOL, HYDROCHLOROTHIAZIDE, METOPROLOL AND AMLODIPINE IN PLASMA DURING STORAGE AND SAMPLE HANDLING

Bench Top Stability Data

Nebivolol				**Hydrochlorothiazide**				**Metoprolol**		
SI.NO	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**	**SI.NO**	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**	**LQC (ng/ml)**	**MQC (ng/ml)**	**HQC (ng/ml)**
1	131.94	1382.8	2806	**1**	10.20	110.20	233.35	6.98	76	157.34
2	121.98	1373	2814.9	**2**	10.47	114.41	232.46	7.00	74.01	156.58
3	124.75	1374.6	2809.6	**3**	9.59	113.05	233.05	6.52	75.32	155.69
Mean	126.22	1376.8	2810.1	**Mean**	10.08	112.55	232.95	6.83	75.11	156.53
S.D (+/-)	5.12	5.25	4.47	**S.D (+/-)**	0.45	2.14	0.45	0.27	1.0	0.82
C.V	4.05	0.38	0.15	**C.V**	4.46	1.90	0.19	3.97	1.34	0.52

The detection limits (LOD) for Nebivolol, Hydrochlorothiazide, and Amlodipine were 46.86ng/ml, 3.4ng/ml, and 2.67ng/ml, respectively, while the quantitation limits (LOQ) were 142ng/ml, 12.1ng/ml, and 8.09ng/ml. Separation conditions (e.g., column, reagents, instruments, and data systems), instrumental changes (e.g., pumping systems and detectors), and the use of non-HPLC grade solvents can all affect these values, resulting in signal-to-noise ratio variances **(table 5).**

Table 5: SYSTEM SUITABILITY PARAMETERS

Parameters	Nebivolol	Hydrochlorothiazide	Metoprolol
Linearity and Range	142-2840ng/ml	12.1-22ng/ml	8.09-161.8ng/ml
Regression Equation	0.0649x= 0.0203	0.0022x+ 0.004	0.038-0.0142
Correlation Coefficient	0.9994	0.9993	0.9992
Theoretical Plates	35552	21664	3561
Asymmetric factor	1.0	1.05	1.1
Limit of Detection(LOD)	46.86ng/ml	3.4ng/ml	2.67ng/ml
Limit of Quantification(LOQ)	142ng/ml	12.1ng/ml	8.09ng/ml

Changing the experimental conditions was used to investigate the approaches' ruggedness and resilience. When the experimental settings (operators and columns of same type) and optimum there were no notable changes in the chromatographic parameters when the circumstances were modified (pH, mobile phase ratio, and flow rate).

The optimised methods' system suitability parameters, such as column efficiency (theoretical plates) and resolution factor, were determined to be satisfactory.Finally, the established method for estimating Nebivolol, Hydrochlorothiazide, and Metoprolol in plasma is quick, sensitive,

exact, selective, and linear, and thus may be used in a bioequivalence research to further test its applicability.

4.3 Estimation of Nebivolol, Hydrochlorothiazide, Metoprolol and Amlodipine by LC-MS/MS methods

Estimation of the supernatant obtained from plasma samples was carried out using the optimized chromatographic conditions. The standards were injected and chromatograms, spectra's were recorded. The typical spectra's and chromatograms of the Nebivolol, Hydrochlorothiazide and Metoprolol **(figure 1 – 15)**

During the pre-study and in-study validation processes, calibration curves for spiking plasma containing Nebivolol, Hydrochlorothiazide, and Metoprolol, as well as an internal standard, were created frequently.**(figure 16- 18).**

The medicines, internal standard, and endogenous components were all separated well in the mobile phase employed for the estimate. The blank plasma showed no interference at the retention time of both the Nebivolol, Hydrochlorothiazide and Metoprolol and internal standard.

The response factor of Nebivolol, Hydrochlorothiazide and Metoprolol was calculated. The concentration of Nebivolol, Hydrochlorothiazide present in plasma samples were calculated and presented.

5. CONCLUSION

The developed method is rapid, sensitive, and rugged and reproduction with high recovery LC-MS/MS method for determination of Nebivolol, Hydrochlorothiazide, amlodipine and Metoprolol in human plasma was developed and validated as per FDA guidelines. Bio-analytical method validation and regulated bio-analysis are an integral part of a drug development program. Need of performing the study is to develop a,simultaneous estimate of Nebivolol, Hydrochlorothiazide, Metoprolol, and Amlodipine in human plasma by LC-MS/MS was bio-analytically developed and validated.The method's validation parameters satisfied the acceptance criteria. The stability of LQC, MQC, and HQC was shown to be sufficient to allow sample analysis to be completed. Based on the findings, I can say that the created bio-analytical method is simple, quick, accurate, and exact in accordance with the requirements.LOD for Nebivolol,

Hydrochlorothiazide and Metoprolol were found to be 46.68, 3.4 and 2.67ng/ml and LOQ for Nebivolol, Hydrochlorothiazide and Metoprolol were found to be 142, 12.1 and 8.09ng/ml.

In this thesis provided a new bio-analytical methodology LC-MS/MS that can be improves the analysis throughput and cycle times.

Guidance documents from regulatory bodies across the globe are revised to reflect the current technologies but are not yet fully harmonized. At the onset of method development, bio-analysts should carefully evaluate the properties of the analyte of interest, its metabolites, and assay requirements such as LQC, MQC, HQC and LLOQ.

Once a desired method is developed, the bio-analysts can then proceed with method validation and bio-analysis in accordance with the different regulatory guidelines and laboratory-specific SOPs.

Precision and Accuracy are within the acceptable criteria as per USFDA guidelines and method was proved to be rugged by different analysts and different laboratories.

Considering that, above conclusions on the method and stability of the analyte. Method can be taken as quantification for Nebivolol, Hydrochlorothiazide, Metoprolol and Amlodipine.

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