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Research Article

Method Development and Validation of Labetalol and Nadolol in Human Plasma by Lc-Ms/Ms

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ABSTRACT

LC-MS/MS technique is used for method development and validation of labetalol and nadolol in human plasma. Liquid chromatography separates mixture components in the basis of differences in affinity for stationary and mobile phase. It removes undesired impurities. It increases reproducibility, sensitivity, robustness, detection of low-level proteins. C18 Column (Phenomenex Luna C18, 5 µm, 50*4.6 mm ID) is used to for high resolution and peak area. Calibration curve is constructed with the help of linear regression. During the entire process no instability is found. The mass to charge ratio of labetalol and nadolol is 329.3→162 and 310.2→254.2. The average recovery of Labetalol is 91.3% and Nadolol is 100.6%.

Keywords: Labetalol, Nadolol, Human plasma, Metoprolol, LC-MS/MS, ESI

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ABBREVIATIONS

CXP: Collision Cell Exit Potential; CE: Collision Energy; EP: Entrance Potential; DP: Declustering Potential; LLOQ: Lower Limit of Quantification; ULOQ: Upper Limit of Quantification; LQC: Lower Quantity Control; MQC: Medium Quantity Control; HQC: High Quality Control; SD: Standard Deviation; CV: Coefficient of Variation; MRM: Multiple Reaction Monitoring; FDA: Food and Drug Administration; HPTLC: High Performance Thin Layer Liquid Chromatography; RP-HPLC: Reverse phase-High Performance Liquid Chromatography; %RSD: Percentage Relative Standard Deviation; LOD: Limits of Detection; LOQ: Limits of Quantification; ICH: International Conference on Harmonization

INTRODUCTION

Labetalol is used for antihypertensive which acts as a blocker for alpha and beta-adrenergic receptors. It is used alone or with the combination of other antihypertensive agents. It is used for the management of hypertension, chronic stable angina pectoris and sympathetic over activity syndrome associated with severe tetanus. In order to reduce immediate blood pressure in severe hypertension labetalol is used in emergency situation for patients with pheochromocytoma and pregnant women with preeclampsia. Also used to reduce bleeding during surgical procedures and controlled hypotension during anesthesia. Labetalol HCl combines selective and non-selective, competitive and non-competitive, alpha-1-adrenergic and beta-adrenergic blocking activity in a single substance. By oral and intravenous administration alpha and beta blockade is 1:3 and 1:7 in human. Within the myocardium it blocks the adrenergic simulation of β 1-receptors and β 2-receptors in bronchial and vascular smooth muscle and α 1-receptors in vascular smooth muscle. Without substantial reduce in resting heart rate, systemic arterial blood pressure and vascular resistance is decreased, because of combination of α and β adrenergic blocking activity.

Nadolol is used to treat angina pectoris and hypertension which is non-selective beta-adrenergic antagonist. Also used in migraine disorders and tumors. For binding at sympathetic receptor sites nadolol competes with adrenergic neurotransmitters such as catecholamines like other beta-adrenergic antagonists. In the heart and vascular smooth muscle, nadolol binds at beta-1-adrenergic receptors inhibiting the effects of the catecholamines epinephrine and norepinephrine and reduces the heart rate, systolic and diastolic blood pressure. In bronchiole smooth muscle causing vasoconstriction beta-2 adrenergic receptors are blocked. Nadolol is structurally similar to propranolol. Nadolol has no intrinsic sympathomimetic activity unlike some other beta-adrenergic blocking agents. It has little myocardial depressant activity and does not have an anesthetic-like membrane-stabilizing action *(Figure 1a, 1b)*.

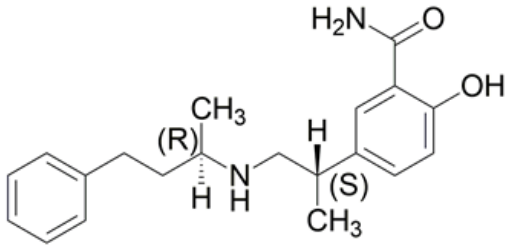

Figure 1a: Structure of labetalol

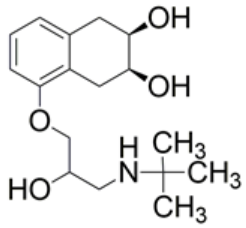

Figure 1b: Structure of nadolol

According to the literature survey several techniques were used for the analysis of Labetalol and Nadolol. Labetalol is analysed by LC-MS (Ganesan M, et al., 2010), HPTLC (Bhalerao MR, 2014), UV-spectrophotometry (Kadam S, et al., 2020; Suryawanshi AD, et al., 2019; Manasa O, et al., 2014; Karuppasamy C, et al., 2014), RP-HPLC (Kulkarni VC, et al., 2015; Chakravarthy AV, et

al., 2016) and Nadolol is analysed by RP-HPLC (Arafah RS, et al., 2020), UV-spectrophotometry (Veeramanikandan V, et al., 2020; Vijayalakshmi R, et al., 2015; Adegoke OA, et al., 2006).

MATERIALS AND METHODS

Experiment

Chemicals and reagents: From the Akshaya labs, Hyderabad, India, working standard of Labetalol and Nadolol were obtained. From Merck, Acetonitrile HPLC grade and from Qualigens fine chemicals, Ammonium Acetate AR grade and from Milli-Q RO system, water HPLC grade was used for analysis.

LC-MS/MS conditions and parameters: In positive ion mode, the LC-MS/MS instrument was functioned and equipped with ESI using nebulizer, curtain and collision gas which is embraced with AB Sciex API-4000 mass spectrometer. The HPLC 95 setup contains online DGU-20A3 soluble degasser, segment boiler CTO20A, LC20AD drives-2, SIL-HTc sampler. C18-XBridge segment was split into 60:40 proportion with a stream rate of 1.0 mL/min kept at 40°C. Changeable phase contains 0.1% methanoic acid (A) and ethanenitrile (B). In the primary phase up to 0.8min it is arranged as 95% A/5% B then it gets changed to 5% A/95% B for 2.2 min. Later it slowly stimulated to 95% A/5% B by 2.4 minutes and remained the same at 3.5 min. The MS condition is shown in *Table 1*.

Table 1: Positive ion mode (for labetalol and nadolol)

Parameter	Value
Ionization type and polarity	ESI, positive ion mode
Ion source	Turbo spray
Scan type	MRM
Ion spray voltage	5500 V
Q1 resolution	Unit(0.7)
Q3 resolution	Unit(0.7)
Temperature	5500C
Gas 1	50
Gas 2	50
CUR gas	30
CAD gas	8
ihe	On

Selection of HPLC column: In HPLC column inverted C-18 columns were regularly used for the objective analyte. Primarily Sio2 based C-8 columns are verified and these results in poor separation. Several aspects were taken, such as the composition of the mobile phase and PH stages were examined, yet no enhancement in chromatographic condition. C18 results in fine resolution but presents the uneven peaks. Finally, Phenomenex Luna C18 showed substantially good resolution, symmetrical peaks and less co-elution between target compounds due to its effectiveness in polarity and aromatic selectivity.

Internal standard: For positive mode compounds metoprolol was used as an internal standard. Labetalol and nadolol requires neutral PH but metoprolol requires acidic PH for elution, and after extraction supernatant was obtained when diluted with water. Hence it is operated in positive mode which gives good resolution.

Sample preparation: Accurately transferred 100 mg of labetalol and nadolol working standard into a 100 ml volumetric flask and dissolved in acetonitrile and made the final volume with water and acetonitrile (1:1) to give 1.0 mg/ml solution of labetalol and nadolol labeled and stored the solution in a refrigerator below 8°C. Preparation of labetalol and nadolol standard solution Standard solution for Calibration curve Prepared, 10 ml each of 16, 20, 40, 80, 120, 240, 260 ng/ml of labetalol and nadolol standard solutions using the labetalol and nadolol standard stock solution and mobile phase and labeled and stored at -2 ± 2°C until analysis.

RESULTS AND DISCUSSION

Validation

It is the second state in analysis. By using validation method precise and accurate results were obtained. FDA guidelines should have to be satisfied in method validation, if not it is considered as unsuitable and inappropriate.

For concentration determination in human plasma, it should be appropriate, and then the process begins with the construction of calibration curve which should be in the range of 1-1000 ng/mL. Labetalol and nadolol standards were extracted in the presence of internal standard and analyzed using MRM transitions *(Table 2)*. From the chromatograms peak area ratio of analyte to internal standard was calculated. Human plasma without analytes, and analytes without human plasma were analyzed. Therefore, it helps to find the stability at room temperature in plasma for 24 hrs.

Construction of calibration curve

To determine the linearity, five sets of quantitation standards were prepared on alternate day using the same plasma and prepare the dilute stock solution each having 9 different concentration levels in human plasma. It is referred as inter and intra-day date which is reproducible on alternate days. The main aim is to assess the lowest level which is quantified by LC-MS/MS. As per the FDA simple form of regression is chosen to obtain the linearity *(Figure 2a, 2b)*.

Table 2: Summary of MRM transition conditions

Compound	Mode of ionization	Q1 mass (m/z)	Q3	DP	EP	CE	CXP
Labetalol	Positive	329.3	162	100	10	33	10
Nadolol	Positive	310.2	254.2	100	10	24	10

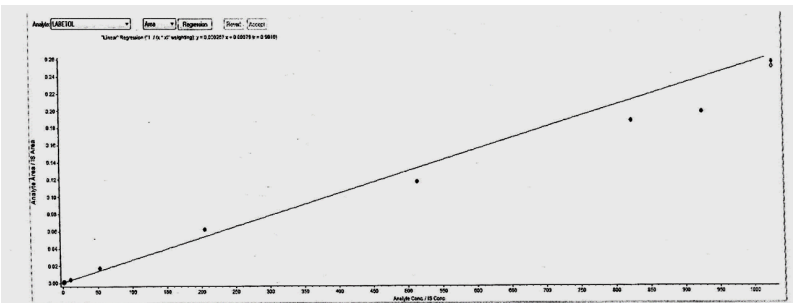

Figure 2a: Calibration curve of labetalol

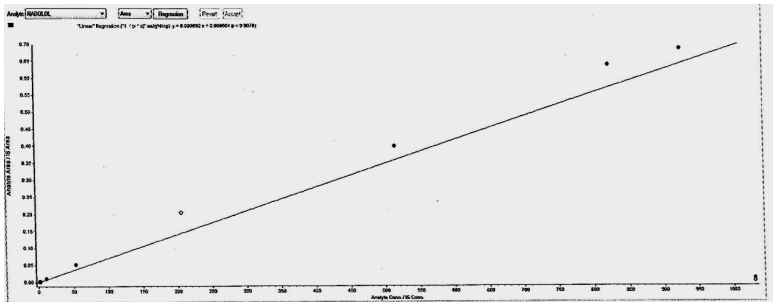

Figure 2b: Calibration curve of nadolol

Carryover

After the highest standard, sample without internal standard is injected immediately to the verify the carryover is less than 20% of the LLOQ on a peak area basis.

MRM transitions

To provide the quantifiable spectra multi-reaction monitoring tracks precursor and product ions through mass spectrometer. Different collision energies are present when transition from precursor to product ion *(Figure 3a, 3b, 3c, 3d)*.

Linearity

The calibration curve was constructed using linear regression by the analyst 1.5 software. The back calculated standard curve data for labetalol and nadolol is shown in *Table 3 and 4*. Standards with a back-calculated accuracy outside the range of 85-115% of the nominal concentration were excluded from the regression statistics. The correlation coefficients for all calibration curves were more than 0.99 *(Figure 4a,4b,4c,4d,4e)*.

Accuracy and precision

Table 5 and 6 shows calculated concentrations and inter and intra batch precision and accuracy values of labetalol. *Table 7 and 8* shows calculated concentrations and inter and intra batch precision and accuracy values of nadolol. In multi-step preparation procedure, the values obtained are acceptable, which are nominal concentrations between 90-110%. Coefficient of variation is below the nominal criteria i.e., less than 15%.

Recovery

The recovery of analytes from human plasma in the form of LQC, MQC, HQC was determined by comparing with their aqueous quality control samples. The average recovery of labetalol is 91.3% and nadolol is 100.6% *(Table 9) (Figure 5a,5b,5c,5d,5e,5f)*.

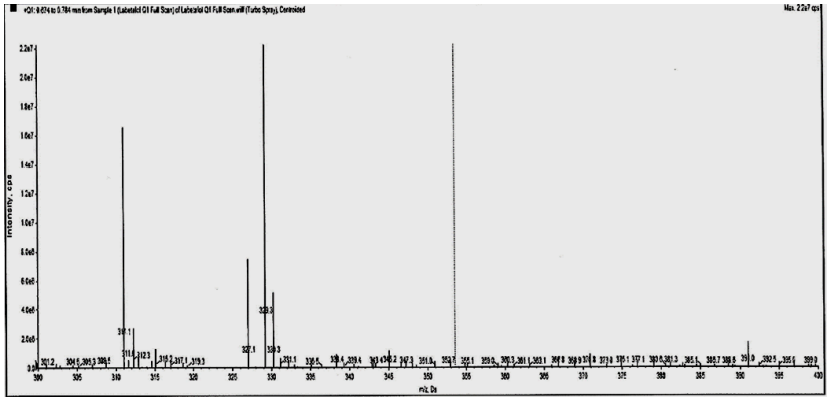

Figure 3a: Parent ion at m/z of labetalol

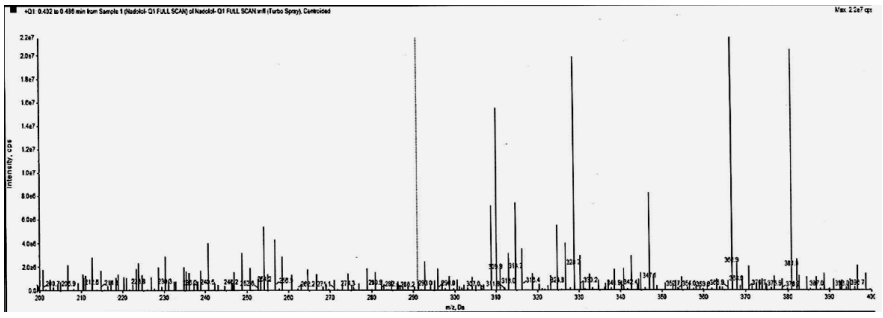

Figure 3b: Parent ion at m/z of nadolol

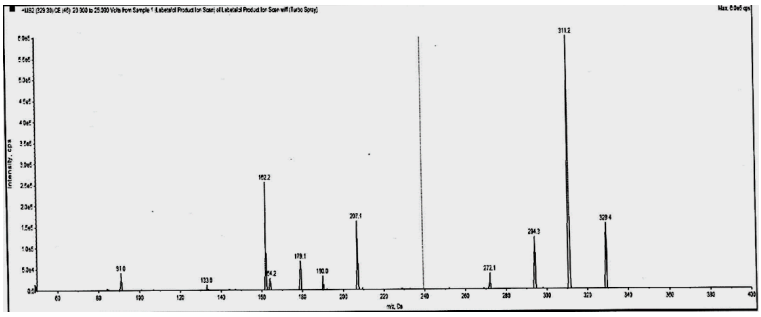

Figure 3c: Product ion mass spectra of labetalol in positive ionization mode

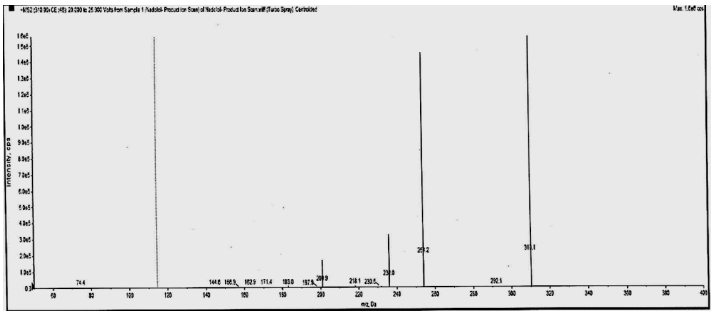

Figure 3d: Product ion mass spectra of nadolol in positive ionization mode

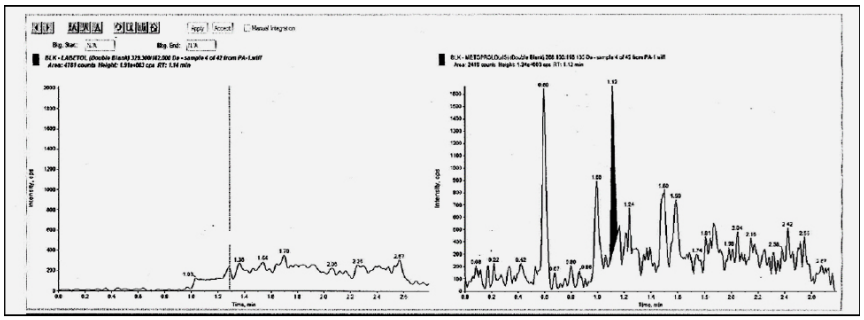

Figure 4a: Representative example of blank chromatogram for labetalol

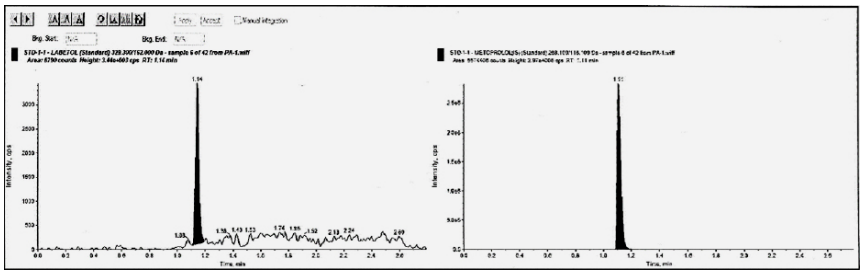

Figure 4b: Representative example of LLOQ chromatogram of labetalol

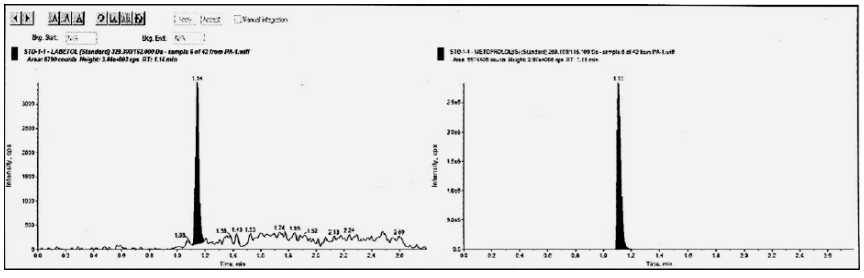

Figure 4c: Representative example of LQC chromatogram of labetalol

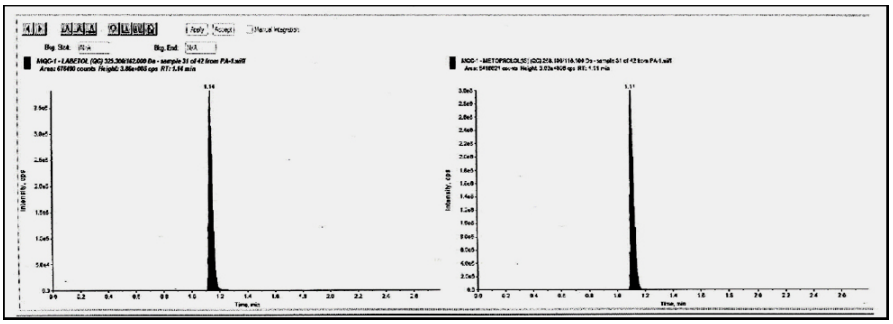

Figure 4d: Representative example of MQC chromatogram for labetalol

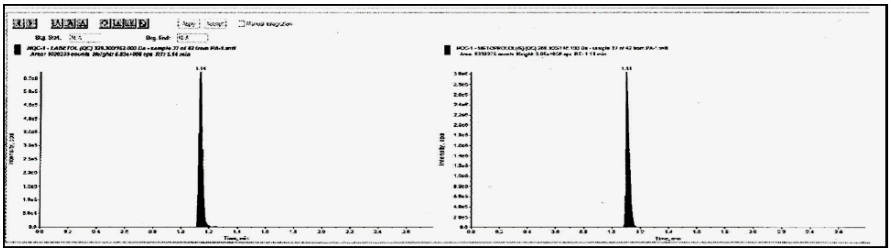

Figure 4e: HQC chromatogram for labetalol

Table 3: Back-calculated standard curve data for labetalol in human plasma

Concentration (ng/mL)							
Standard concentration	**Batch-1**	**Batch-2**	**Batch-3**	**Mean**	**SD**	**% CV**	**% Accuracy**
1	1.01	1.03	1.07	1.04	0.03	2.95	103.67
2	2.34	2.22	2.2	2.25	0.08	3.36	112.67
10	11.73	12.31	12.08	12.04	0.29	2.43	120.4
50	62.31	61.87	61.9	62.03	0.25	0.4	124.03
200	235.94	234.88	235.35	235.39	0.53	0.23	117.68
500	452.68	451.31	451.43	451.81	0.76	0.17	90.35
800	729.38	728.87	728.94	729.06	0.28	0.04	91.12
900	870.03	867.38	867.75	868.39	1.44	0.17	96.49
1000	999.08	998.03	998.15	998.42	0.57	0.06	99.84

Table 4: Back-calculated standard curve data for nadolol in human plasma

Concentration (ng/mL)							
Standard concentration	**Batch-1**	**Batch-2**	**Batch-3**	**Mean**	**SD**	**% CV**	**% Accuracy**
1	0.95	0.96	1.05	0.98	0.021	2.13	97.7
2	2.49	2.45	2.64	2	0.09	4.47	100.2
10	13.62	13.72	12.87	11.69	0.119	1.02	116.9
50	52.43	53.61	51.95	56.69	0.956	1.69	113.4
200	206.18	224.16	203.06	221.84	4.055	1.83	110.9
500	573.34	573.08	543.21	466.18	8.635	1.85	93.2
800	919.47	919.42	904.15	788.7	9.012	1.14	98.6
900	989.23	990.76	965.06	872.25	12.553	1.44	96.9
1000	1032.15	1097.05	1023.43	955.68	17.528	1.83	95.6

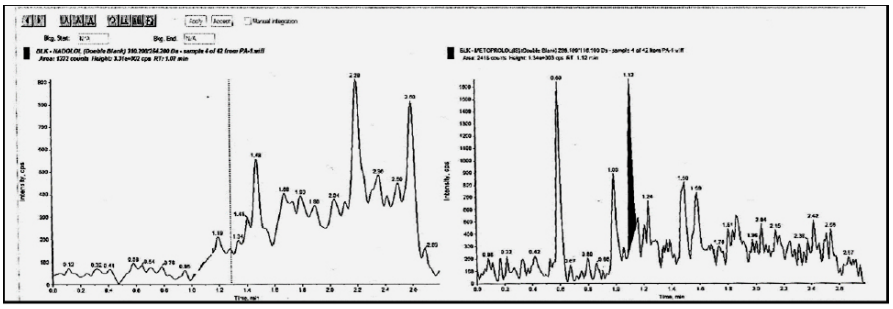

Figure 5a: blank chromatogram for nadolol

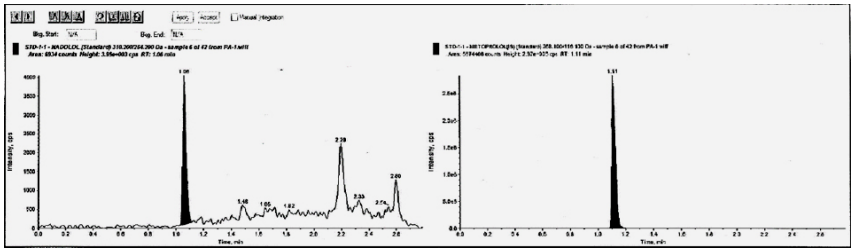

Figure 5b:LLOQ chromatogram for nadolol

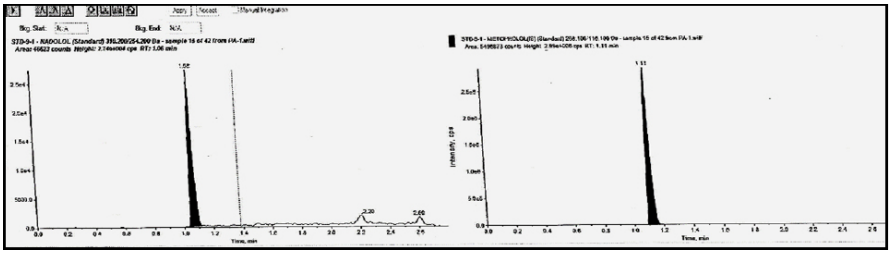

Figure 5c: ULOQ chromatogram for nadolol

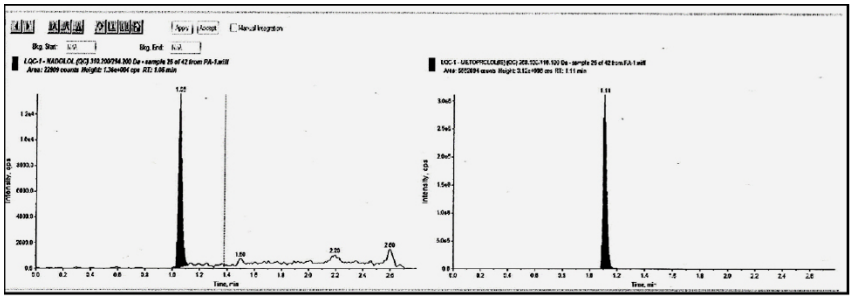

Figure 5d: LQC chromatogram for nadolol

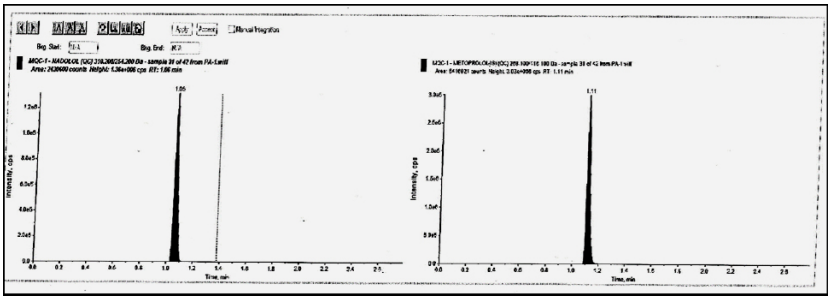

Figure 5e: MQC chromatogram for nadolol

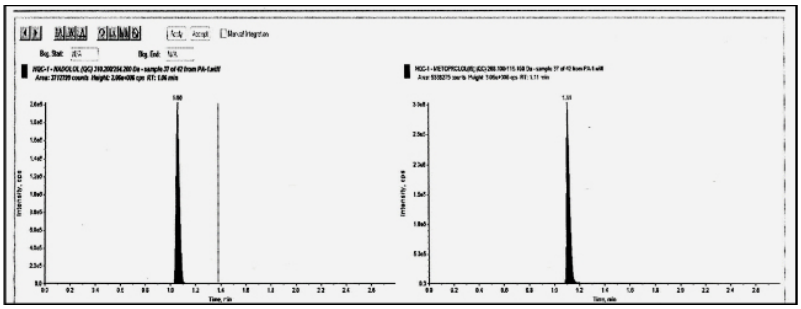

Figure 5f: HQC chromatogram for nadolol

Stability

In freeze-thaw stability LQC, HQC, MQC values are obtained by three cycles of freezing and thawing and the obtained values are within the range of precision and accuracy determined by the inter-day QC samples.

In auto-sampler stability the samples are kept in auto-sampler for 24 hrs and compared with fresh samples. The obtained values of LQC, HQC, MQC were within the range of accuracy and precision determined by inter-day QC samples.

No instability was found during freezing and thawing, in auto-sampler and bench-top process.

In freeze-thaw stability LQC, HQC, MQC values are obtained by three cycles of freezing and thawing and the obtained values are within the range of precision and accuracy determined by the inter-day QC samples.

In auto-sampler stability the samples are kept in auto-sampler for 24 hrs and compared with fresh samples. The obtained values of LQC, HQC, MQC were within the range of accuracy and precision determined by inter-day QC samples.

In bench-top stability the samples are kept in room temperature for 4hrs and then compared with the fresh samples. The obtained values of LQC, HQC, MQC were within the range of accuracy and precision which is determined by inter-day QC samples.

No instability was found during freezing and thawing, in auto-sampler and bench-top process.

CONCLUSION

This method describes the development and validation of labetalol and nadolol in human plasma by liquid chromatography and mass spectrometry. It should be operated in positive ion mode and metoprolol is used as an internal standard. The average recovery of labetalol is 91.3% and for nadolol is 100.6%. Linearity ranges from 85-115%. No instability was found in the process.

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