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Research Article

A quantitative determination of penbutolol and its metabolite in plasma by liquid chromatography tandem mass and its application to pharmacokinetic studies

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ABSTRACT

A Simple sensitive and specific tandem mass spectrometric (LC-MS/MS) method for the determination of penbutolol (PEN) and its metabolite 4-Hydroxy penbutolol (4HPEN) in human plasma was developed and validated. The detection of the analytes was achieved in positive ion in selected reaction monitoring (SRM) mode. The deuterated compounds of the analytes were used as internal standards (IS). The extraction procedure involved solid phase extraction of PEN, 4HPEN and IS from plasma by using Strata-X cartridges. The chromatographic separation of PEN, 4HPEN and IS was carried out on a Chromatopak C_{18} column with 5mM ammonium acetate (pH 4.5) buffer and acetonitrile (15:85, v/v) as the mobile phase under isocratic conditions at a flow rate of 0.6 mL/min. The nominal retention times obtained for PEN, 4HPEN, DPEN and D4HPEN were 2.42, 1.98, 2.40 and 2.00 minutes respectively. The lower limit of quantification for PEN and 4HPEN were 0.2 and 0.1 ng/mL respectively. The standard curves were linear (r^2>0.99) over the concentration range of 0.2-302.7 ng/mL for PEN and 0.1-30.0 ng/mL for HPEN. Method validation was performed as per FDA guidelines and the obtained results met the acceptance criteria. The proposed method was found to be acceptable to a pharmacokinetic study in human volunteers.

Keywords: Penbutolol, 4-Hydroxy Penbutalol LC-MS/MS, Human plasma and Pharmacokinetics.

INTRODUCTION

Penbutolol[1-2] is a beta-blocker class drug used to treat hypertension. Penbutolol binds both beta-1 and beta-2 adrenergic receptors, rendering it a non-selective beta-blocker. Penbutolol can act as a partial agonist at beta adrenergic receptors, since it is a sympathomimetric drug. Penbutolol also demonstrates high binding affinity to the 5-hydroxytryptamine receptor 1A with antagonistic effects. This binding characteristic of penbutolol is being investigated for its implications in Antidepressant Therapy. Penbutolol is contraindicated in patients with cardiogenic shock, sinus bradycardia, second- and third-degree atrioventricular conduction block, bronchial asthma, and those with known hypersensitivity. Penbutolol acts on the β1 adrenergic receptors in both the heart and the kidney. When β1 receptors are activated by catechoPENines, they stimulate a coupled G protein that leads to the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The increase in cAMP leads to activation of protein kinase A (PKA), which alters the movement of calcium ions in heart muscle and increases the heart rate. Penbutolol blocks the catechoPENine activation of β1 adrenergic receptors and decreases heart rate, which lowers blood pressure. It metabolized in the liver by hydroxylation and glucuroconjugation forming a glucuronide metabolite and a semi-active 4-hydroxy metabolite[3]. Literature survey reveals quite a few methods have been reported for the determination of Penbutolol and 4-Hydroxy

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Penbutolol in various body fluids such as urine and plasma by using spectro photometry[4], HPLC[5, 6] and GC-MS[7]. The authors now propose a fast, sensitive, accurate and precise tandem mass spectroscopic method for the determination of Penbutolol and 4-Hydroxy Penbutolol in human plasma. The entire results obtained in the present study comply with the acceptance criteria of regulatory requirements[8, 9].

EXPERIMENTAL

Chemicals and materials: The reference standards of Penbutolol, 4-Hydroxy Penbutolol, Penbutolol D9 and 4-Hydroxy Penbutolol D9 procured from Splendid Labs; Pune, India. HPLC grade methanol and acetonitrile were obtained from J.T Baker® (Phillipsburg, USA). Analytical- grade ammonium acetate and formic acid were obtained from Merck® Ltd; Mumbai, India. The water used for the analysis was prepared by Milli-Q® water purification system (Bangalore, India). The blank (drug free) human plasmas were procured from Jeevan-dhara blood bank; Hyderabad, India. The chemical structures of analytes and the internal standards were shown in Fig.1.

Instrumentation and optimized chromatographic conditions*:* The HPLC system (Shimadzu®, Kyoto, Japan) consisting of two LC-20AD prominence pump, an auto sampler (SIL-HTc), CTO 10 ASvp column oven, a solvent degasser (DGU-20A3) and connected with a Chromatopak C_{18} column (100 mm X 4.6 mm, 5µm ID) were used for the chromatographic separation. 10µL of the sample volumes were injected into the column, which was maintained at 40ºC in the column oven. The optimized isocratic mobile phase consists a mixture of 5mM ammonium acetate and acetonitrile (15:85 v/v) with a flow rate of 0.6 mL/min into the ionization chamber of mass spectrometer. The quantitation was achieved with daughter ion detection in positive ion polarity with multiple reaction monitoring (MRM) mode for both analytes and internal standards using a MDS Sciex® API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turbo Ion Spray ™ interface at 400ºC. The ion spray voltage was set at 5000 Volts. The source parameters viz. the nebulizer gas, curtain gas, auxillary gas and collision gas were set at 40, 45, 45 and 5 pressure per square inch (psi) respectively. The compound parameters viz. the de-clustering potential (DP), entrance potential (EP), collision energy (CE), Collision Cell Entrance Potential (CEP) and collision cell exit potential (CXP) were 29.0, 10.0, 20.0, 18.35 & 5.0 Volts for Penbutolol; 40.0, 10.0, 23.0, 18.88 & 5.0 Volts for 4-Hydroxy Penbutolol. Detection of the ions were carried out in MRM mode, by monitoring the ion transition pairs of m/z 292.1 → 236.1 for Penbutolol, 308.1 → 252.1 for 4-Hydroxy Penbutolol, 301.1 → 237.1 for Penbutolol D9 and 317.1 → 253.1 for 4-Hydroxy Penbutolol D9. The Positive MRM scan mass spectra of Penbutolol and 4-Hydroxy Penbutolol for precursor and product ions were shown in Fig.2, 3. Both the quadrupoles Q1 and Q3 were set at unit resolution. The retention times obtained for the analytes PEN, 4HPEN & IS DPEN, D4HPEN were 2.42 ± 0.3, 1.98 ± 0.3 min & 2.40 ± 0.3, 2.00 ± 0.3 respectively over a total run time of 3.0 minutes. The analysis data obtained was processed by using Analyst Software™ (Version 1.4.2).

Preparation of calibration curve standards and quality control samples: Primary stock solutions of PEN and 4HPEN for preparation of standards (CC) and quality control (QC) samples were prepared from separate weighing. The primary stock solutions about 1.0 mg/mL of PEN, 4HPEN were prepared in methanol and stored 1-10ºC; they were found to be stable for 16.25 days (data not shown). Appropriate dilutions for calibration curve were made from primary stock of CC using a 80:20 v/v mixture of methanol and water as a diluent to produce the CC spiking solutions containing concentrations (from Standard-8 to Standard-1) of 30273.89, 12012.68, 50082.73, 10016.55, 2504.14, 500.83, 20.01, 10.01 ng/mL for PEN and 3005.16, 1192.45, 655.85, 250.53, 50.11, 25.05, 10.02, 5.01 ng/mL for 4HPEN. Similarly QC spiking solutions were made by using primary stock of QC. These concentrations (DIQC, HQC, MQC, LQC & LLOQ QC) were 30146.14, 11515.83, 6333.71, 29.64 & 10.07 ng/mL and 2977.58, 1143.39, 628.87, 14.97 & 5.06 ng/mL for PEN and 4HPEN respectively. The primary stock solutions of Penbutolol D9 and 4-Hydroxy Penbutolol D9 about 1.0 mg/mL were prepared in methanol. Working dilution of DPEN and D4HPEN was prepared at concentration of 1000 ng/mL by using the above diluent. These ISTD primary stock solutions were stored at 1-10ºC.

The plasma calibration curve and quality control samples were prepared by spiking individually PEN & 4HPEN each 20 µL into 960µL of screened plasma. Calibration curve standard for PEN and 4HPEN were made at concentration of 0.200, 0.400, 1.000, 5.053, 25.265, 132.139, 240.254, 302.739 ng/mL and 0.100, 0.200, 0.501, 1.002, 5.011, 13.117, 23.849, 30.052 ng/mL respectively. Similarly quality control (LLOQ QC, LQC, MQC, HQC & DIQC) samples for PEN and 4HPEN were prepared at concentration of 0.201, 0.593, 126.674, 230.317 & 602.923 and 0.101, 0.299, 12.577, 22.868 & 59.552 ng/mL.

Extraction process of plasma samples: Two hundred micro liters of the spiked plasma calibration curve standards and the quality control samples were transferred into a set of pre-labeled polypropylene tubes containing 50 µL of internal standard dilution (approx.100ng/mL of PEN & 4HPEN). The tubes were added 100 µL of 0.5% formic acid solution in water and vortexed for well mixing. The Strata-X 30mg/1CC cartridges placed on the solid phase extraction (SPE) chamber were conditioned with 1 mL of methanol followed by equilibrating with 1 mL of Milli-Q water. The above samples were loaded on to the cartridges and the cartridges were washed with 1 mL of Milli-Q water followed by 1 mL of 10% methanol in water. The cartridges were dried for approximately 1 min and eluted with 1 mL of methanol. The eluents were evaporated in a stream of nitrogen at 50^0C and the residues in the dried tubes were reconstituted with 300µL of the mobile phase. These tubes were vortexed and transferred into auto-sampler vials and loaded. An aliquot of 10µL of the sample was drawn each time from the loaded vials and then analyzed by LC-MS/MS.

Method Validation: A through validation of the method was carried out as per the USFDA guidelines[17]. The method was validated for selectivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and various stability studies. Selectivity of the method was assessed by analyzing eight blank human plasma matrixes which includes each one lot of hemolytic & lipemic plasma. The responses of the interfering substances or background noises at the retention time of the PEN and 4HPEN are acceptable if they are less than 20% of the response of the lowest standard curve point or LLOQ. The responses of the interfering substances or background noise at the retention time of the internal standard are acceptable if they are less than 5 % of the mean response of internal standard in selectivity LLOQ samples. Matrix effect was investigated to ensure that precision, selectivity and sensitivity were not compromised by the matrix. The matrix effect was checked with eight different lots of EDTA plasma which inclusive of hemolytic & lipemic plasma lots. Triplicate samples each of LQC and HQC were prepared from different lots of plasma (48 QC samples in total). Linearity was tested for PEN and 4HPEN in the concentration ranges 0.200-302.739 and 0.100-30.052 ng/mL respectively. For the determination of linearity, standard calibration curves containing at least eight points (nonzero standards) were plotted and checked. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences, but these data were not used to construct the calibration curve. The acceptance limit of accuracy for each of the back-calculated concentrations was ± 15% except for LLOQ, where it was ± 20%. For a calibration run to be accepted at least 75% of the standards, including the LLOQ and ULOQ, were required to meet the acceptance criteria and no two consecutive standards should fail, otherwise the calibration curve was rejected. Five replicate analyses were performed on each calibration standard. The samples were run in the order from low to high concentration. Intra-assay precision and accuracy were determined by analyzing six replicates at four (H, M, L, LLOQ) different QC levels on five different runs. The acceptance criteria included accuracy within ± 15% deviation from the nominal values, except the LLOQ, where it should be ± 20%, and a precision of ≤ 15% co-efficient variance or relative standard deviation (%CV or RSD), except for LLOQ, where it should be ≤ 20%. Recovery of the analytes from the extraction procedure was determined by comparing the areas of the analytes in spiked plasma (six each of low, medium and high QCs) with the those of the analytes in samples prepared by spiking the extracted drug-free plasma with the same amounts of the analytes at the step immediately prior to chromatography. Similarly, recovery of the IS was determined by comparing the mean peak areas of the extracted QC (H, M & L) samples (n=18) with those of the IS in samples prepared by spiking the extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography. The dilution integrity exercise was performed with the aim of validating the dilution test to be carried out on higher analyte concentrations above the upper limit of quantitation (ULOQ) during real-time analysis of unknown subject samples. The dilution integrity experiment was carried out at 2.0 times the ULOQ (Higher Standard) concentration for both analytes. Six replicates each of quarter concentrations were prepared and their concentrations were calculated by applying the dilution factor 4.These dilution integrity quality control (DIQC) samples were included in the precision and accuracy batch as DIQC samples. The acceptances of these samples were similar to QC samples. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (1-10^{o}C) was determined by comparing area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability for 10.33 hours, auto sampler (processed sample) stability for 48.66 hours, freeze thaw stability for six cycles and long term stability for 50.0 days were tested at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within

the acceptance limits of accuracy ± 15% for their nominal values and precision ≤15% CV.

Pharmacokinetic study design: A pharmacokinetic study was performed in healthy (n=6) male subjects. Blood samples were collected following oral administration of Penbutolol (20 mg) and 4-Hydroxy Penbutolol (300 mg) pre-dose at 0.00, 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 36.00, 48.00 and 72.00 hours in K2 EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 20 min at 4^0C and the separated plasma was collected in new labeled tubes. The collected plasma samples were stored at -70 ± 10^0C until their use. Plasma samples were spiked with IS and processed as per extraction method described above. Along with clinical samples, QC samples at low, medium and high concentrations were assayed minimum of two sets or 5% QC samples when calculated against unknown clinical samples which were distributed among the unknown samples in the analytical run; not more than 33% of the QC samples were greater than ±15 of the nominal concentration and minimum 50% at each individual QC's should pass. Time profile of Penbutolol and 4-Hydroxy Penbutolol was analyzed by non-compartmental method using WinNonlin® version 5.2 (Pharsight Corporation, Mountain View, CA, USA).

RESULTS

Method development: As both the drug and its metabolite were containing acidic as well as basic functional groups in their chemical structures, the mass spectrometric parameters were tuned in both positive and negative polarity ionization modes. Good and reproducible responses were achieved in positive ionization mode. Data from the MRM mode were considered to obtain selectivity. The protonated form of each analyte and IS, the $[M + H]^+$ ion, was the parent ion in the Q_1 spectrum and was used as precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was monitored from m/z 292.1 → 236.1 for PEN, from m/z 308.1 → 252.1 for 4HPEN, from m/z 301.1 → 237.1 for DPEN (IS) and from m/z 317.1 → 253.1 for D4HPEN (IS). We aimed to develop a simple chromatographic method with a lesser run time. Separation was tried using various combinations of methanol and buffer with varying contents of each component on a variety of columns, such as C_8 and C_{18} of different makes like Kromosil, Ace, Intersil, Hypersil, Hypurity Advance, Zorbax, Discovery and Chromatopak. The use of ammonium acetate buffer at concentration of 5mM helped to achieve a good response for MS detection in the positive ionization mode. To get a good chromatographic separation with the desired response, it was observed that mobile phase as well as selection of column was an important criterion. It was found that an isocratic mobile phase system consisting of 5mM ammonium acetate and acetonitrile (15:85, v/v) achieves good reproducible response, which was finally adopted. The retention times of PEN, 4HPEN, DPEN and D4HPEN were 2.42, 1.98, 2.40 and 2.00 min respectively. A flow rate of 0.6 mL/min produced good peak shapes and permitted a run time of 3.00 min. A good internal standard must mimic the analytes during extraction and compensate for any analyte on the column, especially with LC-MS/MS analysis, where matrix effects can lead to poor analytical results. In the initial stages of this work, several compounds were investigated to find a suitable internal standard and finally deuterated compounds of both the interested analytes were found to be best available for the present purpose. Clean samples are essential for minimizing ion suppression or enhancement in LC-MS/MS technique to reduce matrix effect. Hence, solid phase extraction was used for the sample preparation in this work. Other extraction technique like LLE and PPT were also tried, but SPE was found to be optimal; it can produce a clean chromatogram for a blank sample and yields good reproducible recovery for analytes from the plasma.

Selectivity: The degree of interference by endogenous plasma constituents with the analyte and IS was assessed by inspection of chromatograms derived from a processed blank plasma sample (pure blank & IS added blank). As shown in Fig.4, 5. for individual analyte PEN and 4HPEN respectively, no significant interference in the processed blank plasma samples were observed at the retention times of the analyte and internal standard.

Sensitivity: The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration was found to be 5.3 CV & 103.7% for PEN and 6.9 CV & 101.7% for 4HPEN.

Extraction efficiency: A simple solid phase extraction with strata-X cartridges were found to be rugged and provided the cleanest samples. The recoveries of the analytes and internal standards were good and reproducible. The overall mean recoveries with precision range of Penbutolol and 4-Hydroxy Penbutolol was presented in Table.1.

Matrix effect: There was no significant matrix effect was observed in all the eight lots of human plasma for the analyte at low and high QC level

concentrations. The precision and accuracy for PEN & 4HPEN at LQC was 4.6, 5.4 CV & 99.5, 106.3%. Similarly at HQC was 7.1, 3.8 CV & 102.7, 100.2% respectively for PEN & 4HPEN.

Linearity: The eight-point calibration curve was found to be linear over the concentration range of 0.200-302.739 ng/mL for PEN and 0.100-30.052 for 4HPEN. After comparing the weighting factor models at none, 1/X and $1/X^2$, the regression equation with weighting factor $1/X^2$ of the analytes to the internal standards concentration was found to be the best fit in plasma samples. The mean, where n = 4; correlation coefficient (r) of the calibration curves generated in the validation was 0.999 for both PEN and 4HPEN.

Precision and accuracy: Precision and accuracy data for intra, inter day samples for all the analytes were presented in Table. 2. The results obtained in both the criteria were well within acceptance limits.

Stability studies: The different stability experiments carried out, viz. bench-top stability for 10.33 hours, auto-sampler stability for 48.66 hours, repeated freeze-thaw cycles for 6 cycles, re-injection stability for 40.17 hours, dry extract stability for 36.50 hours at 1-10ºC and long-term stability in matrix at -70ºC for 50.0 days. The mean percentage nominal values of the analytes were found to be within ± 15% of the predicted concentrations for the analyte at their LQC and HQC levels. Thus, the results shown in Table. 3 were found to be within the acceptable limits during the entire validation.

Application to a pharmacokinetic study: In order to verify the sensitivity and selectivity of this method in a real-time analysis, the present method was used to test for Penbutolol in human plasma samples collected from healthy male volunteers (n=6) in between the age 18-45 years. Institutional review board approval was obtained before study start and all subjects given written informed consent before participation. Each subject received single oral dose of penbutolol 20 mg tablets and plasma samples obtained were analysed for Penbutolol and 4-Hydroxy Penbutolol. The mean plasma concentration verses time profiles of PEN and 4HPEN are shown in Fig. 6. Following oral administration the maximum concentration (C_{max}) in plasma, 202.3ng/mL for PEN and 27.7 ng/mL for 4HPEN was attained at 1.11 and 1.83 Hours (T_{max}) for PEN and 4HPEN respectively. The plasma concentration verses time curve from zero hour to the last measurable concentration (AUC_{0-t}) & area under the plasma concentration-time curve from zero hour to infinity ($AUC_{0-\infty}$) for PEN were 647.4 & 665.2 ng.h/mL and for 4HPEN were 195.7 and 200.0 ng.h/mL. These results were given in Table. 4. which matches with the published data[10].

DISCUSSION

So far no published methods are available to determine at this lower level for the quantification of PEN and HPEN in human plasma samples with tandem mass detection. Highly sensitive methods are essential for the determination of PEN and HPEN concentrations in human plasma for bioequivalence studies. To the best of our knowledge, this is the first time determination in the plasma without compromising on the reported sensitivity for drug and the metabolite with pharmacokinetic data. The proposed method is specific, sensitive, rugged and rapid owing to the utilization of a shorter run time of 3 minutes. Here we have developed a method for the determination of PEN and HPEN in human plasma with good sensitivity (0.200-302.739 ng/mL for PEN & 0.100-30.052 for 4HPEN). The method uses deuterated internal standards with solid phase extraction technique provides best clean sample for mass spectrometry analysis.

CONCLUSION

In summary, we have developed and validated a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantify of Penbutolol and 4-Hydroxy Penbutolol. The outcome of all the validation parameters obtained with good quality, thereby we can conclude that the developed method can be useful for BA/BE studies and routine therapeutic drug monitoring with the desired precision and accuracy.

ACKNOWLEDGEMENTS

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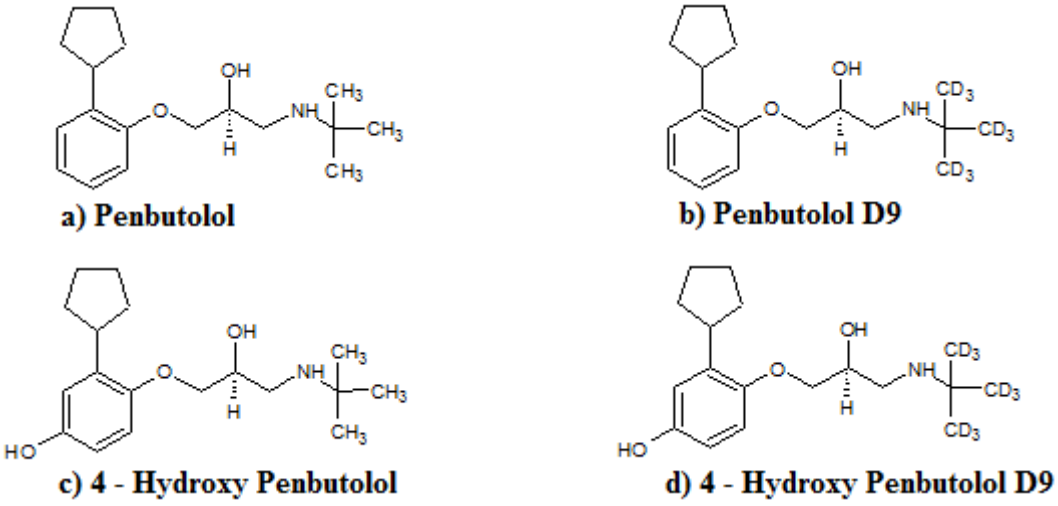

Fig.1. Penbutolol (a), Penbutolol D9 (b) and 4-Hydroxy Penbutolol (c), 4-Hydroxy Penbutolol D9 (d).

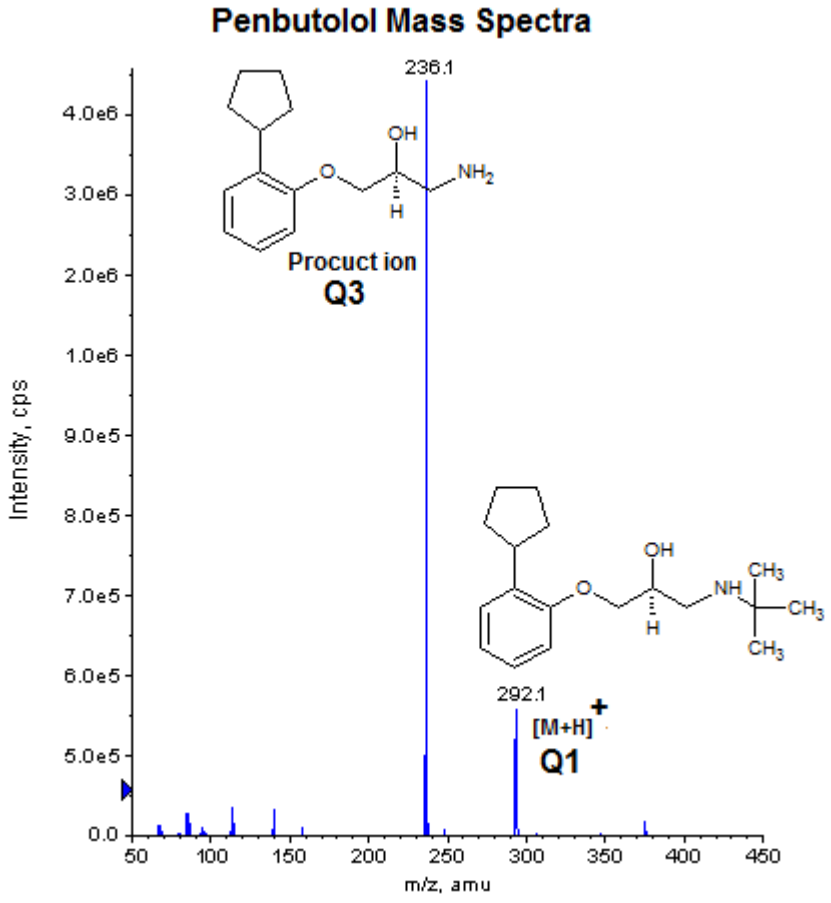

Fig.2**.** Positive MRM scans mass spectra of Penbutolol for precursor and product ions.

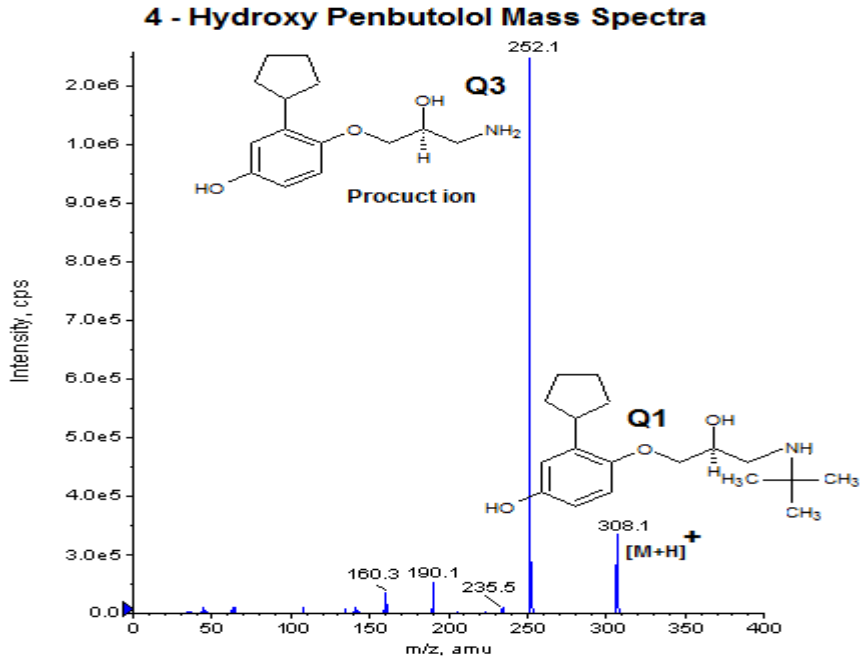

Fig.3. Positive MRM scans mass spectra of 4-Hydroxy Penbutolol for precursor and product ions.

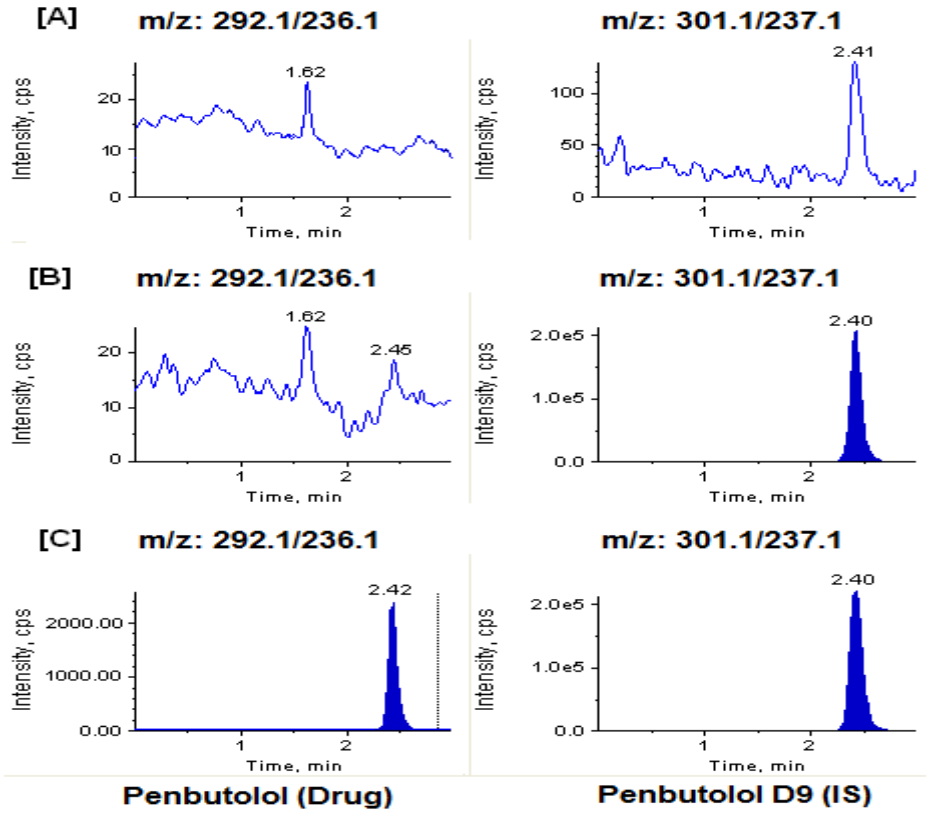

Fig.4. Typical multiple reaction monitoring mode chromatograms of Penbutolol (left panel) and internal standard (right panel) in (A) human blank plasma; (B) human plasma spiked with internal standard; (C) a lower limit of quantitation sample along with internal standard.

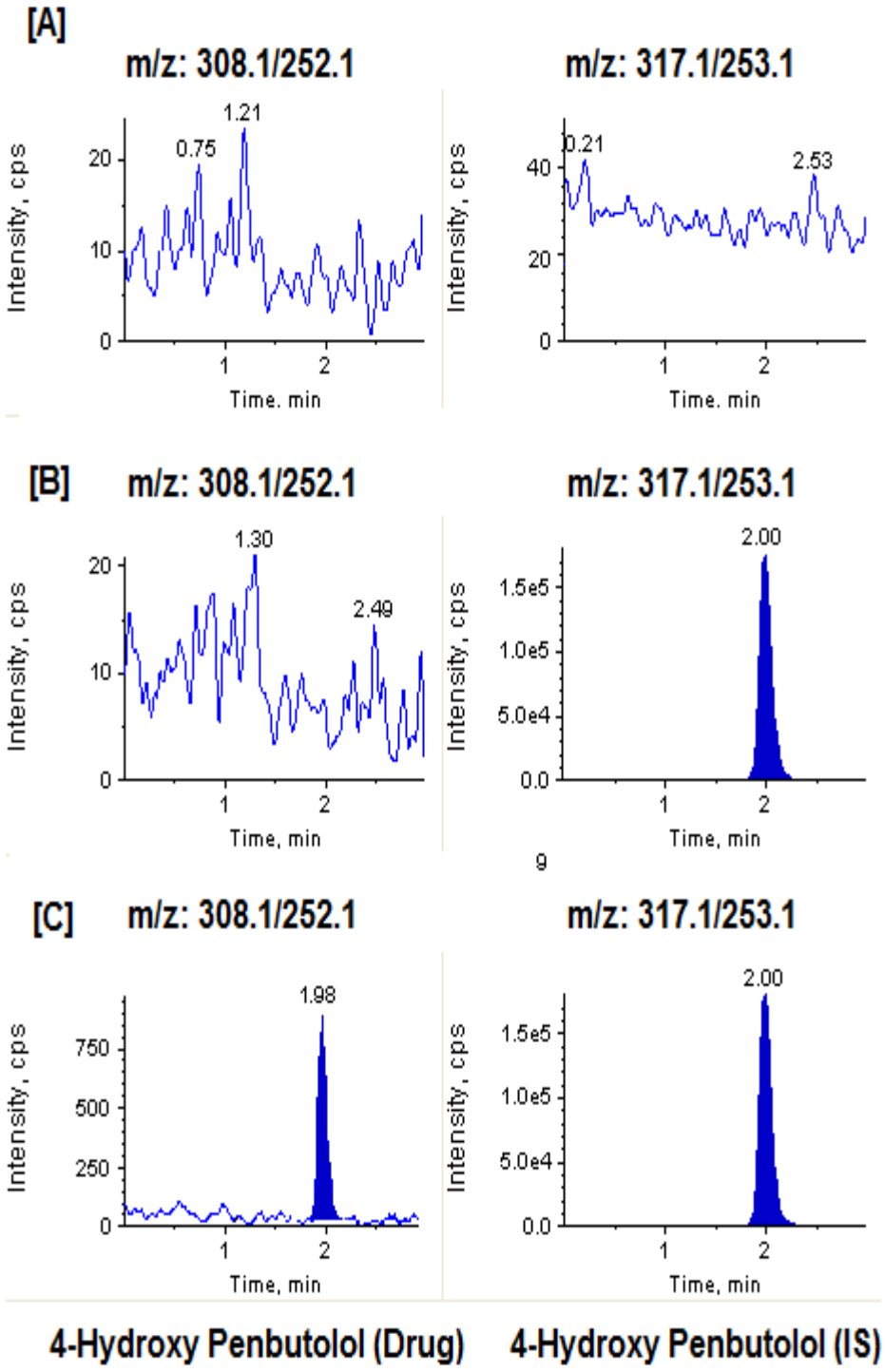

Fig.5**.** Typical multiple reaction monitoring mode chromatograms of 4-Hydroxy Penbutolol (left panel) and internal standard (right panel) in (A) human blank plasma; (B) human plasma spiked with internal standard; (C) a lower limit of quantitation sample along with internal standard.

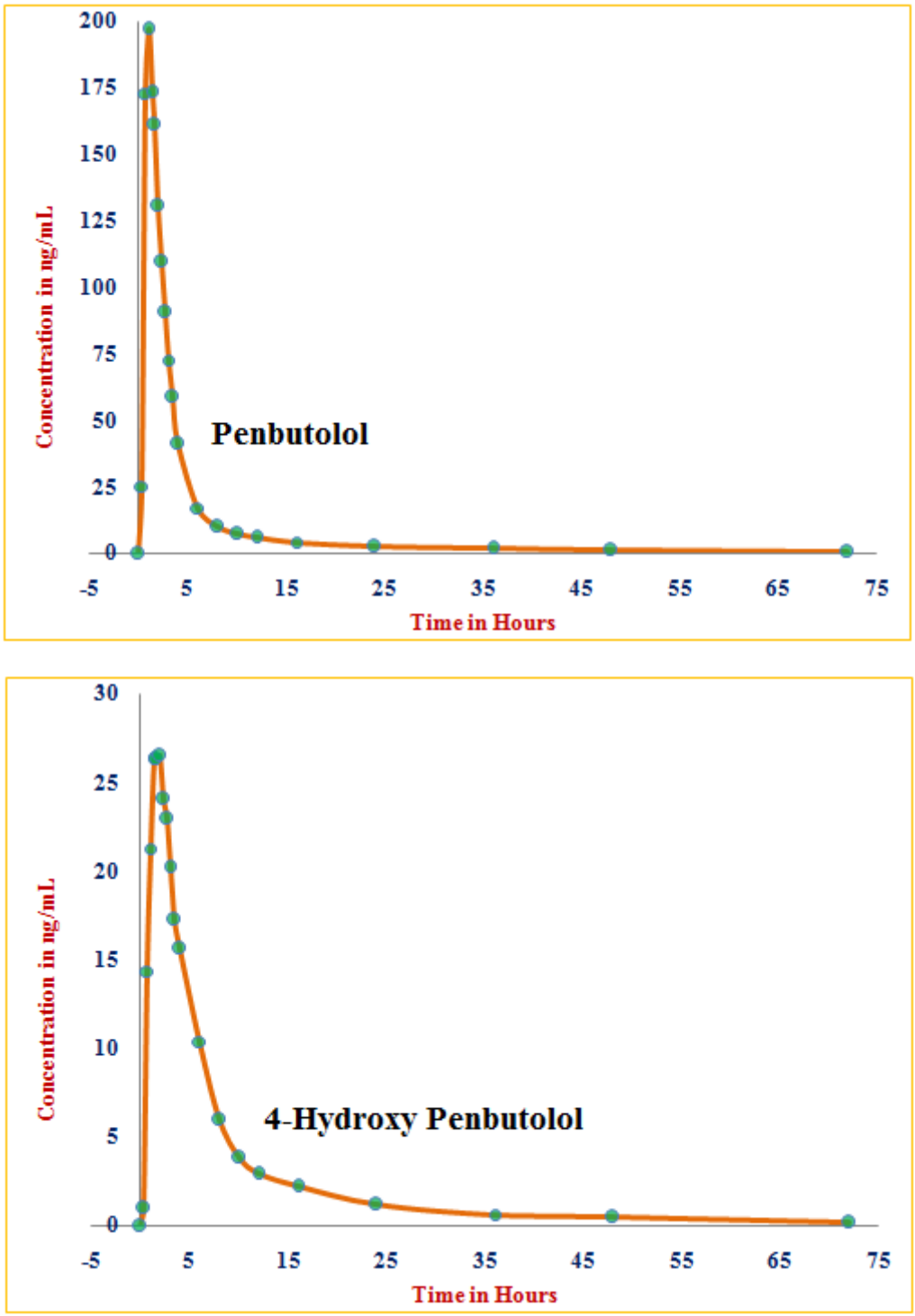

Fig.6**.** Mean plasma concentration Vs Time curve for 6 volunteers for Penbutolol and 4-Hydroxy Penbutolol

Table 1. Overall mean recoveries of analytes and their IS

Analyte name	Sample concentration ng/mL	Response post extracted (mean ± SD)	Response Extracted (mean ± SD)	Recovery (%)	Mean ±SD recovery
PEN	0.593 (LQC)	13134 ± 377	11820 ± 503	90.0	90.92 ± 0.82 % CV 0.9
	126.674 (MQC)	2870559 ± 51266	2628804 ± 11287	91.6	
	230.317 (HQC)	5278919 ± 52440	4813327 ± 73343	91.2	
HPEN	0.299 (LQC)	11442 ± 391	9384 ± 97	82.0	82.93 ± 1.14 % CV 1.4
	12.577 (MQC)	462252 ± 53106	381660 ± 9439	82.6	
	22.868 (HQC)	861660 ± 16084	725586 ± 18269	84.2	
DPEN (IS)	101.260	3183283 ± 55309	2712871 ± 296189	85.2	
DHPEN (IS)	102.307	349950 ± 17966.	296204 ± 2713	84.6	

Table 2. Precision and accuracy of the method including dilution integrity QC sample

		Intra-day precision and accuracy (n = 6) six from each batch			Inter-day precision and accuracy (n = 24) six from each batch		
Analyte	Concentration ng/mL	Concentration found (Mean; ng/mL)	Precision (% CV)	Accuracy (%)	Concentration found (Mean; ng/mL)	Precision (% CV)	Accuracy (%)
PEN	0.201 (LLOQ)	0.202	5.0	96.4	0.194	9.5	100.3
	0.593 (LQC)	0.615	5.2	107.1	0.635	4.3	103.7
	126.674 (MQC)	124.180	0.9	98.0	125.149	1.0	98.8
	230.317 (HQC)	232.926	1.4	101.1	233.753	1.0	101.5
	602.923(DIQC)	600.327	1.0	99.6	608.080	1.5	100.9
4HPEN	0.101 (LLOQ)	0.108	3.9	106.6	0.106	5.0	104.7
	0.299 (LQC)	0.308	1.8	103.1	0.308	2.4	102.9
	12.577 (MQC)	12.791	1.3	101.7	12.673	2.9	100.8
	22.868 (HQC)	22.292	3.7	97.5	23.295	3.9	101.9
	59.552(DIQC)	60.400	4.6	101.4	62.926	4.8	105.7

Table 3. Stability data of the Penbutolol and 4-Hydroxy Penbutolol

Analyte	Stabilities	QC Conc. (ng/mL)	Mean ± SD	Precision (% CV)	Stability (%)
PEN	Auto-sampler (48.66Hr)	0.593 (LQC)	0.602 ± 0.02 221.735 ± 16.2	2.5 7.3	101.5 96.3
	Dry extract (36.50Hr)		0.582 ± 0.03 229.453 ± 14.8	4.6 4.4	98.1 99.6
	Bench top (10.33Hr)		0.594 ± 0.02 229.958 ± 14.8	3.4 6.5	100.1 99.6
	Freeze-thaw (6Cycle)		0.571 ± 0.02 223.649 ± 12.6	4.2 5.6	99.3 97.2
	Re injection (40.17Hr)	230.317 (HQC)	0.589 ± 0.02 223.649 ± 12.6	3.5 5.6	99.3 97.1
	Long term (50.00Days)		0.592 ± 0.02 230.668 ± 9.3	4.1 4.0	99.8 100.2
HPEN	Auto-sampler (48.66Hr)	0.299 (LQC)	0.291 ± 0.02 22.209 ± 1.5	6.0 6.8	97.2 97.1
	Dry extract (36.50Hr)		0.301 ± 0.02 22.487 ± 1.3	6.2 5.7	100.7 98.3
	Bench top (10.33Hr)		0.297 ± 0.02 22.410 ± 2.1	6.8 9.4	99.2 98.0
	Freeze-thaw (6Cycle)		0.303 ± 0.0.2 23.687 ± 1.5	7.8 6.2	101.4 103.6
	Re injection (40.17Hr)	22.868 (HQC)	0.302 ± 0.02 22.128 ± 0.9	6.3 4.1	100.9 96.8
	Long term (50.00Days)		0.295 ± 0.02 22.477 ± 1.5	7.0 6.8	98.6 98.3

Table 4. Pharmacokinetic data

Parameter	Penbutolol	4-Hydroxy Penbutolol
C_{max}(ng/mL)	202.3 ± 38.7	27.7 ± 0.9
T_{max}(Hrs)	1.11 ± 0.3	1.83 ± 0.4
AUC $_{(0\text{-}T)}$	647.4 ± 94.8	195.7 ± 45.2
AUC $_{(0\text{-}\infty)}$	665.2 ± 96.9	200.0 ± 46.4
$T_{1/2}$ in Hours	20.5 ± 4.5	16.4 ± 1.8

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