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Simultaneous determination of propranolol and 4-hydroxy propranolol in human plasma by solid phase extraction and liquid chromatography/electrospray tandem mass spectrometry

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ABSTRACT

A very sensitive, reliable, reproducible and highly selective assay for the simultaneous determination of free and total (conjugated and unconjugated) propranolol and its equipotent hydroxyl metabolite, 4-hydroxy propranolol, in human plasma was developed and validated. The analytes were simultaneously extracted from 0.300 mL of human plasma using solid phase extraction and detected in positive ion mode by tandem mass spectrometry with a turbo ionspray interface. Deuterium-labeled propranolol and 4-hydroxy propranolol, propranolol-d7 and 4-hydroxy propranolol-d7, were used as internal standards. The method has a lower limit of quantitation (LOQ) of 0.20 ng/mL for both analytes with the limits of detection (LOD) 50 and 100 pg/mL for propranolol and 4-hydroxy propranolol, respectively, based on a signal-to-noise ratio of 5. The assay was linear over a range 0.20–135.00 ng/mL for free propranolol and 0.20–25.00 ng/mL for free 4-hydroxy propranolol and linear over range 1.00–500.00 ng/mL for total propranolol and 1.00–360.00 ng/mL for total 4-hydroxy propranolol, with coefficient of determination greater than 0.99 for both analytes. The extraction recoveries were >96 and >64% on an average for propranolol and 4-hydroxy propranolol, respectively. The analytes were found stable in human plasma through five freeze (−15 °C)–thaw (room temperature) cycles and under storage on bench-top for at least 6.5 h, and also in mobile phase at 10 °C for at least 48 h. The method has shown tremendous reproducibility, with intra- and inter-day precision <11.3% (RSD), and intra- and inter-day accuracy <11% of nominal values, for both analytes, and has proved to be highly reliable for the analysis of clinical samples.

1. Introduction

Propranolol, [1-(isopropylamino)-3-(1-naphthyloxy)-2-propranolol; (Fig. 1)], a synthetic aminoalcohol, is a competitive non-selective β-adrenoreceptor antagonist possessing no other autonomous nervous system activity, has been used widely to treat various cardiovascular disorders like hypertension, angina pectoris, cardiac arrythmias and prophylaxis of secondary myocardial infarction [1]. In healthy volunteers, propranolol is completely absorbed from gastro intestinal tract after oral administration [2]. It has a plasma half-life of 4–5 h. Being highly lipid soluble and basic in nature, it is heavily bound to plasma proteins (~85 to ~95), particularly α_1-acid glycoprotein and albumin [3–5].

Propranolol gets extensively metabolized in human liver into a number of products primarily by three routes: (i) a naphthalene ring hydroxylation at -4, -5 and -7 position, catalyzed mainly by cytochrome P4502D6 (CYP2D6), (ii) N-dealkylation of the isopropylamine side chain oxidation, (iii) side chain *O*-glucuronidation [6–10]. Among different metabolites, naphthyl acetic acid and 4-hydroxy propranolol were reported to be major metabolites in humans and animals. 4-Hydroxy propranolol was first identified in urine of humans and several animal species by Bond [11]. Later, it was being identified as an equipotent to propranolol for its β-adrenoreceptor blocking activity [12,13]. 4-Hydroxy propranolol reaches similar peak plasma concentration in man after an oral administration of propranolol but has a significant shorter half-life [2,14]. As that of propranolol it gets extensively metabolized in human liver and excreted mainly as a glucuronic acid and sulfate conjugate [2,15–17].

The glucuronic acid conjugate of propranolol, propranolol glucuronide, may serve as a storage pool for propranolol which undergoes systemic and enteric deconjugation, like enterohepatic recirculation, to propranolol [18]. Hence to evaluate the major pharmacokinetic and pharmacodynamic variables of propranolol in humans must include the determination of both conjugated and unconjugated forms of propranolol and its equipotent hydroxyl metabolite, 4-hydroxy propranolol.

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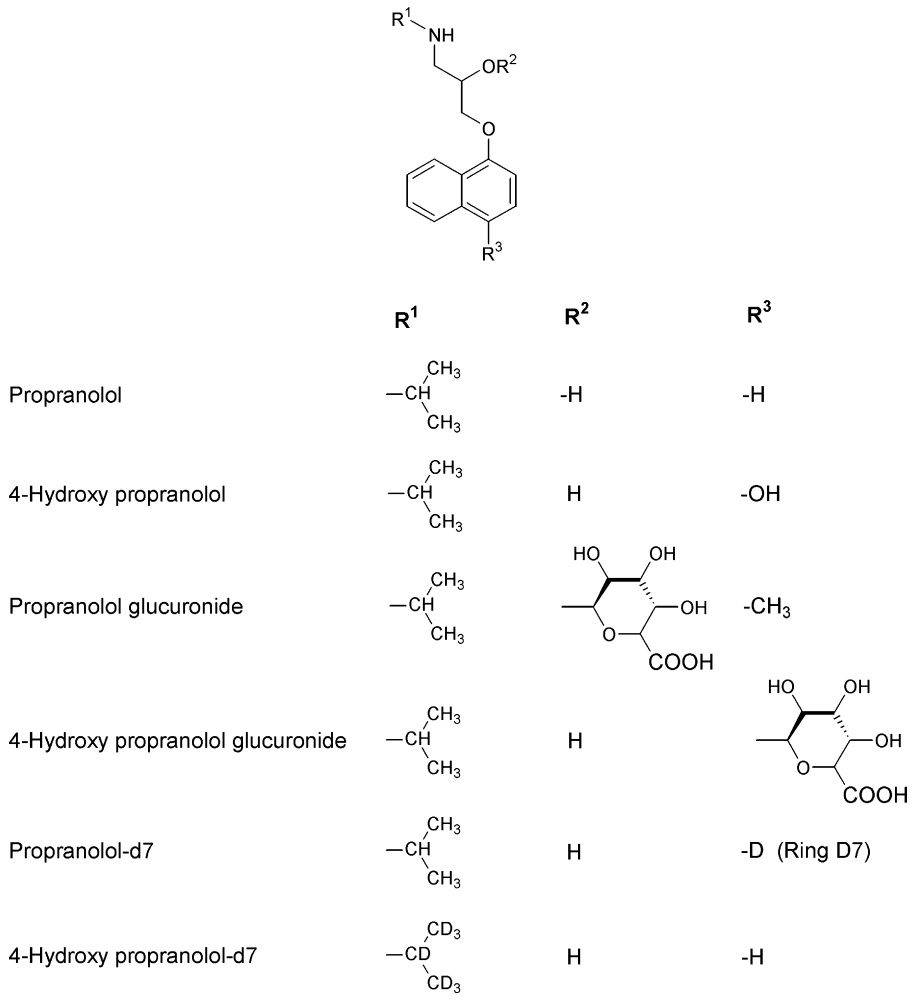

Fig. 1. Chemical structures.

Many high-performance liquid chromatographic (HPLC) methods for the determination of propranolol [19–23] and the simultaneous determination of propranolol and 4-hydroxy propranolol have already been reported [24–28]. Most of these reverse phase liquid chromatographic methods use fluorescence detector for determination of these analytes, since both propranolol and 4-hydroxy propranolol exhibit natural fluorescence property. Due to duality in fluorescence excitation and/or emission nature of these analytes, either one has to take advantage of dual detector connected in series with appropriate setting for estimation of each analyte or one has to find the middle ground between the excitation and emission wavelength to avoid the dual detector connectivity, which undeniably leads to loss of sensitivity. The work presented here has various merits over the earlier HPLC methods like higher sensitivity, improved selectivity and simpler sample processing techniques with shorter analytical run time.

Determination of propranolol and 4-hydroxy propranolol in plasma by mass spectrometry coupled with gas chromatography (GC–MS), using single ion monitoring was first examined by Walle et al. [29]. This proposed GC method requires derivatization of analytes prior to analysis, which makes the sample preparation more laborious and time consuming. In one of the methods, multiple extraction methods needed to be employed separately for estimation of propranolol, 4-hydroxy propranolol and their respective glucuronide conjugate in human body [30]. Besides we would like to quote that the method developed by Ray et al. had actually estimated protein unbound and protein bound form of propranolol while claiming to estimate free and total form of propranolol [31]. Recently, a method to quantify propranolol using LC-electrospray ionization (ESI)/MS/MS was reported [32] that had a lower limit of quantitation (LLOQ) of 2.00 ng/mL; however, this assay was not created to simultaneously quantify 4-hydroxy propranolol other than propranolol.

To our knowledge, none of the existing liquid chromatographic methods were able to achieve adequate sensitivity <1.0 ng/mL and not proficient enough to estimate the percentage of unconjugated to conjugated, free to total, form of propranolol and 4-hydroxy propranolol. To evaluate these variables a validated highly sensitive, selective and simple analytical method is required for the simultaneous estimation of propranolol and 4-hydroxy propranolol. We report here for the first time a novel liquid chromatography–tandem mass spectrometric (LC–MS/MS) method capable of quantifying propranolol and 4-hydroxy propranolol to 0.20 ng/mL with 0.300 mL of plasma using solid phase extraction technique. We anticipate that this method will better allow us to define the drug disposition of propranolol and 4-hydroxy propranolol both in its conjugated and unconjugated forms.

2. Experimental

2.1. Chemicals and materials

Working standards of propranolol (99.9%) and 4-hydroxy propranolol (99.7%) were synthesized by Ranbaxy Research Labora-

tories, Gurgaon, India, where as standard compounds of propranolol glucuronide (99.0%) and 4-hydroxy propranolol glucuronide (99.0%) were procured from TLC Pharma Chem, Canada. Deuterium-labeled propranolol and 4-hydroxy propranolol, propranolol-d7 (99.0%) and 4-hydroxy propranolol-d7 (99.0%), were obtained from CDN Isotopes, Canada and were used as an internal standard for propranolol and 4-hydroxy propranolol, respectively. β-Glucuronidase/arylsulfatase enzyme was purchased from Roche Diagnostics, GmbH, Germany. Ammonium formate, acetic acid (glacial) and formic acid were of LC–MS grade and were obtained from Sigma–Aldrich, Steinheim, USA. Acetonitrile and methanol of HPLC grade were obtained from Spectrochem, Mumbai, India and Qualigens Fine Chemicals, Mumbai, India, respectively. Different individual lots of EDTA human plasma, used to prepare calibration standards and quality control (QC) samples, were obtained from the clinical unit of Ranbaxy Research Laboratories. Oasis HLB, 30 mg; 1 cc, solid phase cartridges were obtained from Waters Corporation, Milford, USA. All aqueous solutions and buffers were prepared using water that was purified using Milli-Q® Gradient A10® (Millipore, Moscheim Cedex, France).

2.2. Preparation of stock solution, standard and quality control samples

Stock solutions of propranolol, 4-hydroxy propranolol, propranolol-d7, 4-hydroxy propranolol-d7, propranolol glucuronide and 4-hydroxy propranolol glucuronide were prepared by dissolving accurately weighed standard compounds in methanol to yield for each compound a concentration of 1 mg/mL. All concentrations were calculated based on the free acid or neutral molecule form. The prepared stock solutions were stored at 1–10 °C protected from light.

2.2.1. For determination of free propranolol and free 4-hydroxy propranolol

Working solutions of propranolol ranging from 0.02 to 13.50 μg/mL and 4-hydroxy propranolol ranging from 0.02 to 2.50 μg/mL were prepared by serially diluting the respective stock solution in methanol–water (80:20, v/v). Aliquot of 0.200 mL from each working solution of propranolol and 4-hydroxy propranolol was added to control human plasma (19.6 mL) to yield calibration concentration at eight different concentrations ranging from 0.20 to 135.00 ng/mL for propranolol and 0.20 to 25.00 ng/mL for 4-hydroxy propranolol. Simultaneously quality control samples in human plasma at four concentration levels were prepared in the same manner as that of calibration standards for propranolol (100.00, 40.00, 0.55 and 0.20 ng/mL) and 4-hydroxy propranolol (20.00, 8.00, 0.50 and 0.20 ng/mL) representing HQC, MQC, LQC, and LOQQC, respectively.

2.2.2. For determination of total propranolol and total 4-hydroxy propranolol

As stated above, in same manner eight different concentrations of propranolol and 4-hydroxy propranolol were prepared in human plasma over a range 500.00–1.00 and 360.00–1.00 ng/mL, respectively. Quality control samples at four concentration levels were prepared by adding the working solution of propranolol glucuronide and 4-hydroxy propranolol glucuronide to human plasma to produce final concentration of 629.68, 251.87, 4.70 and 1.68 ng/mL for propranolol glucuronide and 442.71, 163.97, 4.92 and 1.64 ng/mL for 4-hydroxy propranolol glucuronide, respectively, which upon enzymatic hydrolysis yield propranolol and 4-hydroxy propranolol at concentration 375.00, 150.00, 2.80, and 1.00 ng/mL and 270.00, 100.00, 3.00, and 1.00 ng/mL, respectively.

The internal standard (IS) working solution was prepared by diluting both internal standard stock solutions with methanol:water (80:20, v/v) into a single working solution with a final concentration of 200.00 ng/mL of propranolol-d7 and 500.00 ng/mL of 4-hydroxy propranolol-d7.

Calibration curve plasma standards and quality control samples were stored at −15 °C until assayed or used for validating the analytical method.

2.3. LC/ESI–MS/MS instrumentation and analytical conditions

The liquid chromatography separation was performed using a Shimadzu scientific instruments (Shimadzu Corporation; Kyoto, Japan) consisted of two LC-20AD delivery pumps, an on-line DGU-20A3 prominence solvent degasser, a SIL-HTc Shimadzu autosampler and a CBM-20A prominence column oven. Liquid chromatographic separations were achieved using LiChrospher® 60 RP Select B column (125 mm × 4 mm i.d., 5 μm particle size) (Merck Scientific, USA). An injection volume of 15 μL was used for each analysis. Mobile phase consisted of a mixture of 1 mM ammonium formate in water titrated to pH 3.1 ± 0.1 with formic acid and acetonitrile in the ratio of 20:80 (v/v). The flow rate of the mobile phase was 1.3 mL/min and splitting ratio was set at 4:10. The column and autosampler were maintained at 35 ± 1 and 10 ± 1 °C, respectively.

Samples were analyzed with API-4000 triple quadrupole mass spectrometer (MDS Sciex®; Toronto, Canada) equipped with an electrospray ionization source operating in positive ion mode. Nitrogen was used as the nebulizer, auxillary, collision and curtain gases. Analytes were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) of precursor–product ion transitions with 150 ms dwell time, at *m*/*z* 260.1/116.1 for propranolol, *m*/*z* 267.0/116.2 for propranolol-d7, *m*/*z* 276.1/173.0 for 4-hydroxy propranolol and *m*/*z* 283.2/123.2 for 4-hydroxy propranolol-d7. Nitrogen was used as the nebulizer, auxillary, collision and curtain gases. The main working source/gas parameters of the mass spectrometer were optimized and maintained as follows: collision activated dissociation (CAD) gas, 6; curtain gas, 40; gas 1 (nebulizer gas), 50; gas 2 (heater gas), 50; turbo ionspray (IS) voltage, 2500 V; source temperature, 500 °C. The compound parameters like, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were optimized and set at 55, 10, 26 and 10 V, respectively, for propranolol and propranolol-d7 and 55, 10, 24 and 10 V, respectively, for 4-hydroxy propranolol and its deuterated analogue.

Data acquisition and processing were performed using Analyst version 1.4.1 software (MDS Sciex; Toronto, Canada).

2.4. Solid phase extraction (SPE) procedure

Plasma samples frozen at −15 °C were thawed on the day of extraction at room temperature followed by vortexing to ensure homogeneity.

For the determination of free propranolol and free 4-hydroxy propranolol, 0.300 mL of spiked plasma was transferred to polypropylene tube followed by 0.050 mL of IS working solution (containing 200 and 500 ng/mL of propranolol-d7 and 4-hydroxy propranolol-d7, respectively), and vortexed for 30 s.

For the determination of total propranolol (propranolol plus propranolol glucuronide) and total 4-hydroxy propranolol (4-hydroxy propranolol plus 4-hydroxy propranolol glucuronide), 0.300 mL of spiked plasma was transferred to glass tube followed by 0.050 mL of IS working solution, vortexed for 30 s. Then 0.250 mL of sodium acetate buffer (0.1 M, pH 5.0) and 0.050 mL of β-glucuronidase/arylsulfatase (≥100,000 Units/mL) were added to each tube. After vortexing for 30 s, the tubes were incubated at 35 ± 2 °C for ∼1.5 h; the samples were then assayed for propranolol and 4-hydroxy propranolol.

From this step on, the samples for free and total propranolol and 4-hydroxy propranolol analyses were processed under the same conditions. To each tube 0.300 mL of 5% formic acid solution was then added, and vortexed again for 30 s. The tubes were centrifuged at 4000 rpm for 5 min and the supernatant was loaded on SPE cartridge and centrifuged at 1500 rpm for a minute. Before that the Oasis HLB cartridge was conditioned with 1 mL of methanol followed by 1 mL of water. After loading of the samples, the cartridge was washed with 25% methanol in water and analytes were eluted with 100% methanol. The extracted samples were evaporated to dryness using a Zymark TurboVap LV evaporator (Caliper, Hopkinton, MA, USA) and reconstituted with 0.400 mL of mobile phase [1 mM ammonium formate in water titrated to pH 3.1 ± 0.1 with formic acid–acetonitrile (20: 80, v/v)]. 15 μL was injected into the LC–MS/MS system for analysis.

2.5. Method validation

A full method validation was performed according to guidelines set by the US FDA [33]. The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity of response, accuracy, precision, recovery, matrix effect, and stability of analytes during both short-term sample processing and long-term storage.

2.5.1. Linearity and LLOQ

The calibration curves of propranolol and 4-hydroxy propranolol were both constructed using standard plasma samples at eight concentrations. Curves were best fitted using a least square linear regression model $y = mx + b$, weighted by $1/x^2$, in which y is the peak area ratio, m is slope of the calibration curve, b is the y-axis intercept of the calibration curve and x is the analyte concentration. The LLOQ is defined as the lowest concentration on the calibration curve at which an acceptable accuracy within ±20% and a precision below 20% can be obtained.

2.5.2. Accuracy and precision

Intra- and inter-day accuracy expressed as a percentage of deviation from the respective nominal value and the precision of the assay was measured by the percent coefficient of variation (%CV) at concentrations. Intra-day precision and accuracy were determined by analyzing six replicates of the quality control samples at four levels for both analytes during a single analytical run. The inter-day precision and accuracy were determined by analyzing 18 replicates of the quality control samples at each level for both analytes through three analytical runs made on different days.

2.5.3. Recovery

The extraction recoveries for each analyte and IS at three QC concentration levels were determined by measuring the mean peak area response of six replicates of extracted quality control samples against the mean peak area response of six replicates of aqueous (unextracted) quality control samples and IS at concentrations representing approximately 100% extraction of quality control samples at low, middle and high concentration.

2.5.4. Selectivity

Selectivity was assayed by processing six different lots of blank plasma samples. Among the analyzed batch, plasma batch showing no or minimal interference at the retention time of analytes and internal standards was selected. To 5 mL of the plasma 50 μL each of working solution containing propranolol, 4-hydroxy propranolol was added to achieve concentration near to the limit of quantitation. Processed 6 aliquots of spiked LLOQ as per the set extraction protocol and analyzed.

2.5.5. Matrix effect and matrix factor

Matrix effect was assayed by two different ways. First, the regions of ion suppression or enhancement were confirmed using a post-column (T-joint) infusion of analytes and IS mixture made of higher strength from an infusion pump at the rate of 10 μL/min, while injecting a blank extracted plasma. Secondly, matrix effect was assayed at two concentration levels (low and high quality control concentrations) for both analytes. Six different blank plasma lots, free of any significant interference at the RT of analytes and IS, were selected and spiked with the working solutions of low and high quality control sample. Spiked samples were processed in duplicates and quantitated against freshly spiked calibration curve containing propranolol and 4-hydroxy propranolol. The matrix effect is nullified if the accuracy is within ±15% and precision is ≤15% at the low and high QC concentrations.

The matrix factor (MF) is defined as the peak response in the presence of matrix ions versus the peak response in the absence of matrix ions. Since this method involves terminal drying step, biological matrix samples were prepared by reconstituting the post-extracted blank plasma samples with reference solution containing propranolol, 4-hydroxy propranolol at concentration representing medium QC concentration and their respective deuterated analogue. The control sample was the same reference solution prepared in mobile phase. Matrix factor was evaluated using six different blank plasma lots and determined by measuring the peak area response and analyte/IS peak area ratio of biological matrix sample against the peak area response and analyte/IS peak area ratio of reference solution.

2.5.6. Stability

The stability of propranolol, 4-hydroxy propranolol, propranolol glucuronide and 4-hydroxy propranolol glucuronide in human plasma was determined under a variety of storage and processing conditions. The freeze–thaw stability was evaluated by analyzing QC samples spiked collectively with propranolol and 4-hydroxy propranolol as well as QC samples spiked collectively with propranolol glucuronide and 4-hydroxy propranolol glucuronide at two concentrations separately after undergoing five freeze (−15 °C)–thaw (room temperature) cycles against respective freshly spiked calibration standards spiked with propranolol and 4-hydroxy propranolol. The bench-top storage stability was assessed for both QC samples (free as well as glucuronide) at two concentrations by placing at room temperature for a ∼6.5 h before being extracted and analyzed against a freshly spiked calibration standard spiked with propranolol and 4-hydroxy propranolol. The autosampler storage stability was determined by storing the reconstituted QC samples at two concentrations for 48 h under autosampler condition (maintained at 10 °C) before being analyzed. The −15 °C freezer storage stability of the analytes was determined by extracting and analyzing QC samples at two concentrations after being stored at −15 °C for 158 days.

2.6. Pharmacokinetic and bioequivalence studies in healthy volunteers

An open label, balanced, randomized, two-treatment, two-period, two-sequence, single-dose, crossover design was used for the assessment of pharmacokinetics and bioequivalence. Hundred healthy adult male volunteers who gave written informed consent took part in this study. After an intake of high fat meal diet, all subjects were given a single oral dose of propranolol hydrochloride 160 mg extended release (ER) capsules of Ranbaxy Laboratories Limited and INDERAL® long acting (LA) capsule (containing propranolol hydrochloride 160 mg) of Ayerst Laboratories was administered during each period of the study. Blood samples were collected before and at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,

11, 12, 13, 14, 16, 18, 22, 26, 30, 36, 48, 60 and 72 h post-dose in each period. The plasma was immediately separated by centrifugation and stored frozen below −15 °C until analysis. The pharmacokinetics parameters were calculated by a non-compartmental analysis using WinNonlin Professional software (Version 5.0, Pharsight Corp., Mountain View, CA, USA). The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were read directly from the experimental data. The total area under the plasma concentration–time curve from time zero to infinity ($AUC_{0\to\infty}$) and from time zero to the last measurable concentration ($AUC_{0\to t}$) was calculated using the trapezoidal rule-extrapolation method.

An incurred sample reanalysis (ISR) was performed on 40 sample points from 10 different subjects selected randomly from the study population. Two time points from each period of these identified 10 subjects were taken up for ISR of which one time point was T_{max} and second time point was the last time point wherein the concentration obtained is at least three times of LOQ. The basic objective of ISR was to reconfirm the initial values and to demonstrate that the assay is reproducible. The conformity of the original result with the ISR sample is calculated as a % difference. The % difference should be within 20% for at least 67% or 2/3rd of the total reanalyzed incurred samples [34].

$$\%\text{difference} = \frac{\text{absolute (reanalyzed value} - \text{original value)}}{\text{average of reanalyzed and original value}} \times 100\%$$

3. Results and discussion

3.1. LC–MS/MS condition optimization

Fig. 2 shows the MS/MS spectra of propranolol, 4-hydroxy propranolol and internal standards. Under turbo ionspray ionization condition, all analytes formed protonated molecules $[M+H]^+$ as major ion peaks due to the addition of proton to the amine group, which easily get protonated under acidic condition. Adduct ions of sodium $[M+Na]^+$ were also observed for both analyte and IS (data not shown). Several fragment ions were observed in the product ion spectra of both compounds and the respective internal standard. Fragment ion at m/z 116 was chosen as product ion, for propranolol, and propranolol-d7, where as fragment ion at m/z 173, 123 was selected for 4-hydroxy propranolol and 4-hydroxy propranolol-d7, respectively as these ions presented a higher abundance, stability and no cross-talk effect.

The strength and pH of ammonium formate buffer, 1.00 ± 0.05 mM and 3.1 ± 0.1, respectively, in the mobile phase was optimized to avoid potential charge competition at higher concentration in calibration curve; as an increase in buffer strength >5 mM elutes both analytes and IS at retention time (RT) ~2.8 min, leads to a charge competition and saturation over the stated calibration range. The retention time for 4-hydroxy propranolol was ~3.0 and ~4.0 min for propranolol. A representative chromatogram of double blank, blank, lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) sample are shown in Fig. 3.

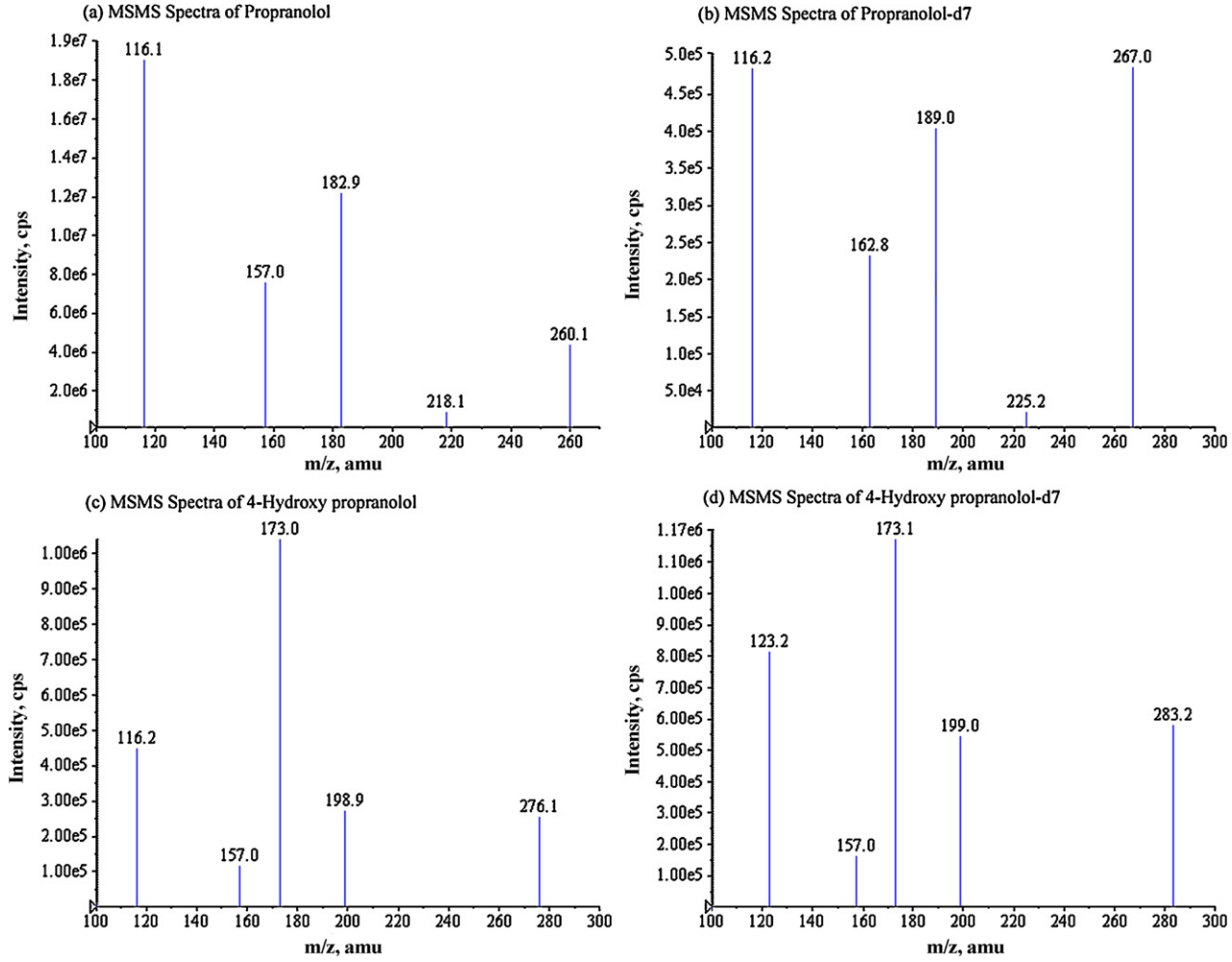

Fig. 2. The product ion spectra of: propranolol, propranolol-d7, 4-hydroxy propranolol and 4-hydroxy propranolol-d7.

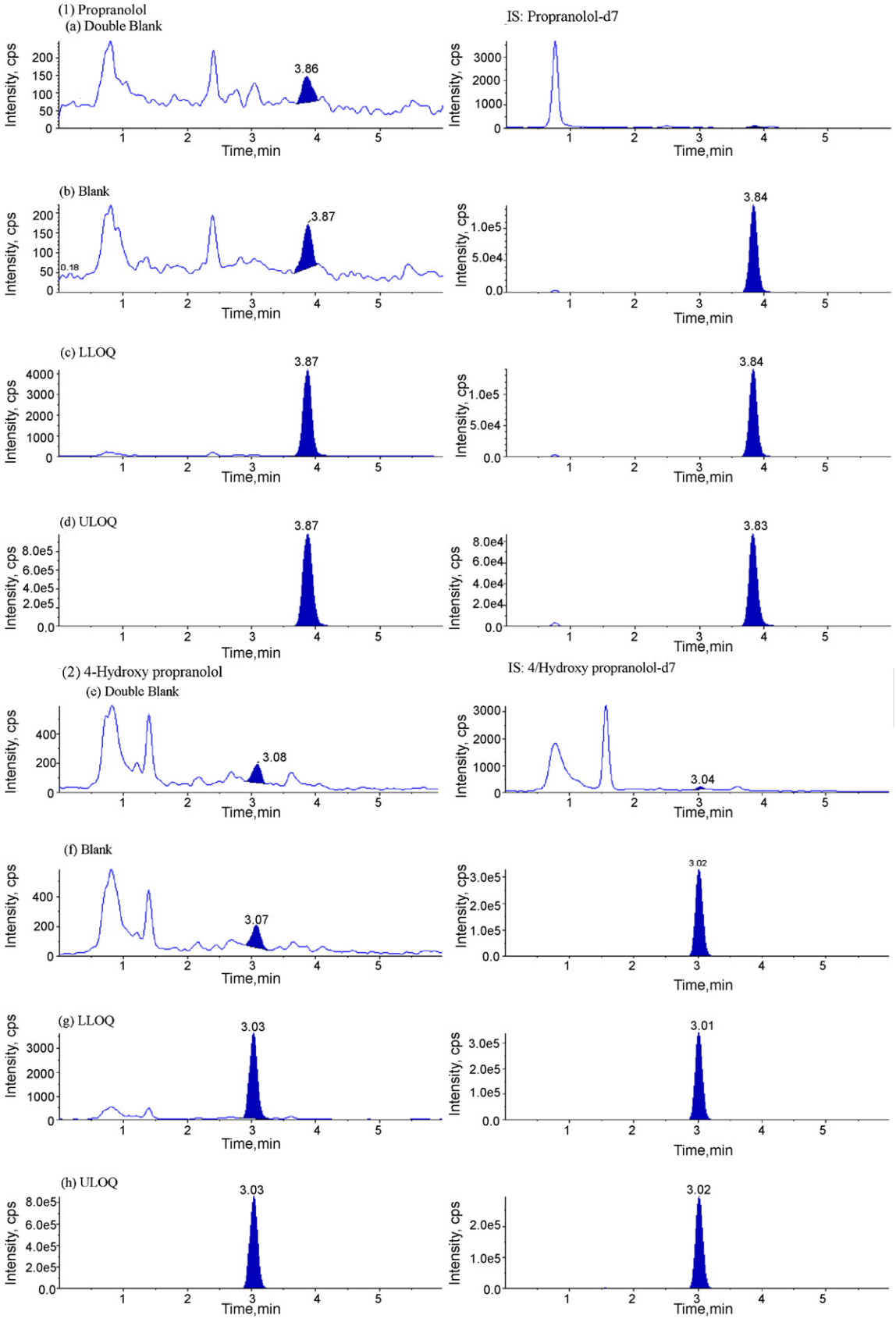

Fig. 3. Representative chromatograms of (1) propranolol and (2) 4-hydroxy propranolol in human plasma: (a and e) double plasma blank; (b and f) plasma blank; (c and g) LLOQ, 1.00 ng/mL; and (d and h) ULOQ. Propranolol (left panels, a–d) and its IS (right panels); 4-hydroxy propranolol (left panels, e–h) and its IS (right panels).

Table 1
Precision and accuracy of quality control samples for free and total propranolol and 4-hydroxy propranolol.

Analyte	Nominal concentration (ng/mL)	Intra-day ($n=6$)			Inter-day ($n=18$)		
		Mean	CV (%)	Accuracy (%)	Mean	CV (%)	Accuracy (%)
Free propranolol	0.20	0.204	4.3	101.9	0.209	11.3	104.6
	0.55	0.528	2.3	95.9	0.533	5.4	96.9
	40.00	39.103	1.5	97.8	39.047	1.4	97.6
	100.00	95.442	1.8	95.4	94.711	1.9	94.7
Free 4-hydroxy propranolol	0.20	0.206	9.3	103.2	0.221	7.8	110.4
	0.50	0.473	5.9	94.5	0.481	6.3	96.2
	8.00	7.938	0.8	99.2	7.968	2.1	99.6
	20.00	19.108	1.4	95.5	19.261	2.7	96.3
Total propranolol	1.00	1.007	4.0	100.7	1.021	4.7	102.1
	2.80	2.717	1.6	97.0	2.779	3.7	99.2
	150.00	157.230	2.7	104.8	154.778	3.6	103.2
	375.00	381.235	1.2	101.7	377.673	1.7	100.7
Total 4-hydroxy propranolol	1.00	1.025	6.0	102.5	1.105	9.1	110.5
	3.00	2.887	5.0	96.2	3.117	10.0	103.9
	100.00	104.043	1.6	104.0	108.014	7.2	108.0
	270.00	259.070	2.3	96.0	270.871	9.3	100.3

As we have used isotopic-labeled internal standards, interference from internal standards to analytes due to the 'cross-talk' effect and/or impurities in working standards were examined by injecting a neat solution containing working concentration of propranolol-d7 and 4-hydroxy propranolol-d7 and monitoring the other compounds i.e. analytes on the extracted ion chromatograms (data not shown). No 'cross-talk' effect was observed from both labeled internal standard.

3.2. Sample preparation optimization

Solid phase extraction (SPE) and liquid–liquid extraction (LLE) techniques are often used in the preparation of biological samples due to their ability to improve the sensitivity and robustness of assay. Most of the methods reported till date employed LLE for the extraction of active moiety(s) from biological matrix. When we employed LLE for determination of propranolol and 4-hydroxy propranolol by tandem mass spectrometry, we encountered certain limitation factors which are firstly, desired sensitivity could not be achieved to estimate free form of 4-hydroxy propranolol. Secondly, basic mode condition is must for extraction which eventually can lead to oxidative degradation of 4-hydroxy propranolol. Thirdly and finally high percentage of ion suppression was observed at the (RT) of 4-hydroxy propranolol. In the present work, a SPE method was adopted that provides higher recovery, sensitivity and stability for both analyte and IS than that reported in the literature. An added advantage over the earlier methods was the proposed solid phase extraction procedure was simple and easy to automate.

3.3. Method validation

3.3.1. Linearity and sensitivity

The method was validated using the above criteria and found to be linear from the concentration 0.20 to 135.00 ng/mL for estimation of free propranolol and 0.20–25.00 ng/mL for estimation of free 4-hydroxy propranolol. Where as for total propranolol and total 4-hydroxy propranolol determination, found linear over the concentration range 1.00–500.00 and 1.00–360.00 ng/mL, respectively. Typical equation for calibration curves were: $y = 0.0278x + 0.00316$, $r = 0.9995$ for propranolol and $y = 0.00864x + 0.00143$, $r = 0.9993$ for 4-hydroxy propranolol. The described assay has the LOQ of 0.20 ng/mL in human plasma for both free propranolol and free 4-hydroxy propranolol, where as 1.00 ng/mL for total propranolol and total 4-hydroxy propranolol. The limits of detection (LOD) for the method was 50 and 100 pg/mL for propranolol and 4-hydroxy propranolol, respectively, based on a signal-to-noise ratio of 5. No carry over peaks were observed at the retention times and ion channels of both propranolol and 4-hydroxy propranolol or their respective IS.

3.3.2. Precision and accuracy

At the eight calibration standards, the inter-day precision ranged from 0.3 to 3.6% and 1.2 to 10.7% and accuracy ranged from 95 to 108% and 96 to 106% for free propranolol and free 4-hydroxy propranolol, respectively, where as inter-day precision ranged from 1.4 to 5.1% and 1.1 to 3.8% and accuracy ranged from 96 to 105% and 97 to 105% for total propranolol and total 4-hydroxy propranolol, respectively. The intra-day and inter-day precision and accuracy of QC samples for free and total propranolol as well as 4-hydroxy propranolol is summarized in Table 1. The intra-day precision ranged from 1.5 to 11.3% and 0.8 to 9.3% with accuracy ranging from 94 to 105% and 94 to 110% for free propranolol and free 4-hydroxy propranolol, respectively. The inter-day precision ranged from 1.2 to 4.7% and 1.6 to 10.0% with accuracy ranging from 97 to 105% and 96 to 111% for total propranolol and total 4-hydroxy propranolol, respectively. These data confirm that the described method has a satisfactory accuracy and precision for the quantification of propranolol and 4-hydroxy propranolol both in their free as well as total form throughout a wide dynamic range.

3.3.3. Recovery

At three QC concentration levels the percent extraction recoveries (mean ± % standard deviation) after six replicates for free as well as total propranolol and 4-hydroxy propranolol were summarized in Table 2. Mean extraction recovery for the IS ($n=6$) was 95.2 ± 3.8% for propranolol-d7 (200 ng/mL) and 62.5 ± 5.3% for 4-hydroxy propranolol-d7 (500 ng/mL). Data indicated that the extraction efficiency for propranolol and 4-hydroxy propra-

Table 2
Extraction recovery of free as well as total propranolol and 4-hydroxy propranolol with solid phase extraction procedure.

Analyte	QC			% Recovery	
	LQC	MQC	HQC	Mean ± SD (%)	CV (%)
Free propranolol	98.7	94.2	96.3	96.4 ± 2.3	2.3
Total propranolol	96.5	102.0	97.1	98.5 ± 3.0	3.1
Free 4-hydroxy propranolol	70.8	66.9	60.9	66.2 ± 5.0	7.5
Total 4-hydroxy propranolol	62.5	70.2	61.5	64.7 ± 4.8	7.4

Table 3
Variability in analytes concentration in different lots of human plasma at two concentration levels.

Analyte	Propranolol		4-Hydroxy propranolol	
Matrix lots	LQC	HQC	LQC	HQC
(WTO67701)	0.53	92.17	0.50	18.98
	0.54	92.61	0.47	19.03
(WTO67702)	0.52	97.95	0.49	19.81
	0.53	91.76	0.50	18.37
(WTO67704)	0.54	90.95	0.50	18.46
	0.53	92.10	0.49	18.71
(WTO67709)	0.54	93.44	0.50	18.76
	0.51	91.97	0.47	18.52
(WTO67713)	0.54	90.63	0.50	18.73
	0.53	92.50	0.48	18.62
(WTO67716)	0.54	89.95	0.49	18.47
	0.54	92.09	0.50	18.49
Nominal concentration (ng/mL)	0.55	100.00	0.50	20.00
Mean	0.531	92.344	0.490	18.745
CV (%)	1.9	2.2	2.3	2.1
Accuracy (%), $n = 12$	96.6	92.3	98.0	93.7

nolol using solid phase extraction was satisfactory and was not concentration-dependent.

3.3.4. Assay selectivity

Selectivity was defined as non-interference from the endogenous plasma constituents at retention time of both analytes and internal standards. Selectivity of the method was determined by comparing the chromatograms of six different blank plasma lots which were extracted using the proposed extraction procedure with the corresponding spiked LLOQ sample. As can be seen in Fig. 3, no significant interference from the endogenous plasma components at the retention time corresponding to propranolol, 4-hydroxy propranolol and their respective internal standard observed.

Table 4
Matrix factor for analyzing propranolol and 4-hydroxy propranolol in human plasma.

Matrix factor				
Matrix lots	Using peak area response		Using analyte/IS peak area ratio	
	Propranolol	4-Hydroxy propranolol	Propranolol	4-Hydroxy propranolol
(WTO67701)	0.98	0.97	0.99	0.98
(WTO67702)	0.98	0.87	0.98	1.02
(WTO67704)	1.04	0.99	1.02	0.98
(WTO67709)	0.99	0.88	1.02	1.03
(WTO67713)	0.96	0.89	1.00	1.04
(WTO67716)	0.98	0.88	0.99	0.98
Mean	0.988	0.915	1.000	1.005
S.D (±)	0.0271	0.0540	0.0174	0.0280
CV (%)	2.7	5.9	1.7	2.8

3.3.5. Matrix effect

Matrix effect, ion suppression or enhancement, due to the co-eluting endogenous component of sample matrix along with analyte or internal standard may affect the chromatography and accuracy of quantitation method when developing LC–MS/MS assay. So, to ensure the selectivity of method, matrix effect on the presented method was evaluated by using post-column infusion of mixture of propranolol (100 ng/mL), propranolol-d7 (100 ng/mL), 4-hydroxy propranolol (200 ng/mL) and 4-hydroxy propranolol-

Table 5
Stability data for propranolol and 4-hydroxy propranolol under various conditions ($n = 6$).

Storage period and storage condition	Analyte	Nominal concentration (ng/mL)	Mean	CV (%)	Accuracy (%)
Five freeze/thaw cycles (−15 °C)	Free propranolol	0.55	0.506	2.8	92.1
		100.00	93.513	2.9	93.5
	Free 4-hydroxy propranolol	0.50	0.492	3.5	98.3
		20.00	18.751	2.3	93.8
	Total propranolol	2.80	2.733	2.4	97.6
		375.00	355.865	1.7	94.9
	Total 4-hydroxy propranolol	3.00	3.130	3.1	104.3
		270.00	253.357	1.9	93.8
Bench-top stability ~6.5 h	Free propranolol	0.55	0.575	7.2	104.5
		100.00	92.202	1.7	92.2
	Free 4-hydroxy propranolol	0.50	0.516	4.6	103.2
		20.00	19.342	1.2	96.7
	Total propranolol	2.80	2.698	3.4	96.4
		375.00	384.665	1.7	102.6
	Total 4-hydroxy propranolol	3.00	3.200	4.4	106.7
		270.00	259.187	1.9	96.0
In-injector stability ~48 h	Free propranolol	0.55	0.547	2.0	99.4
		100.00	93.285	1.6	93.3
	Free 4-hydroxy propranolol	0.50	0.477	3.1	95.4
		20.00	19.197	2.0	96.0
	Total propranolol	2.80	2.842	2.1	101.5
		375.00	374.807	2.1	99.9
	Total 4-hydroxy propranolol	3.00	3.033	1.5	101.1
		270.00	254.971	2.9	94.4
Long-term stability 158 days	Free propranolol	0.55	0.599	4.5	108.9
		100.00	104.749	2.5	104.7
	Free 4-Hydroxy propranolol	0.50	0.490	4.6	98.0
		20.00	20.443	1.5	102.2
	Total Propranolol	2.80	2.880	2.1	102.9
		375.00	389.126	2.6	103.8
	Total 4-hydroxy propranolol	3.00	2.936	6.3	97.9
		270.00	266.689	4.2	98.8

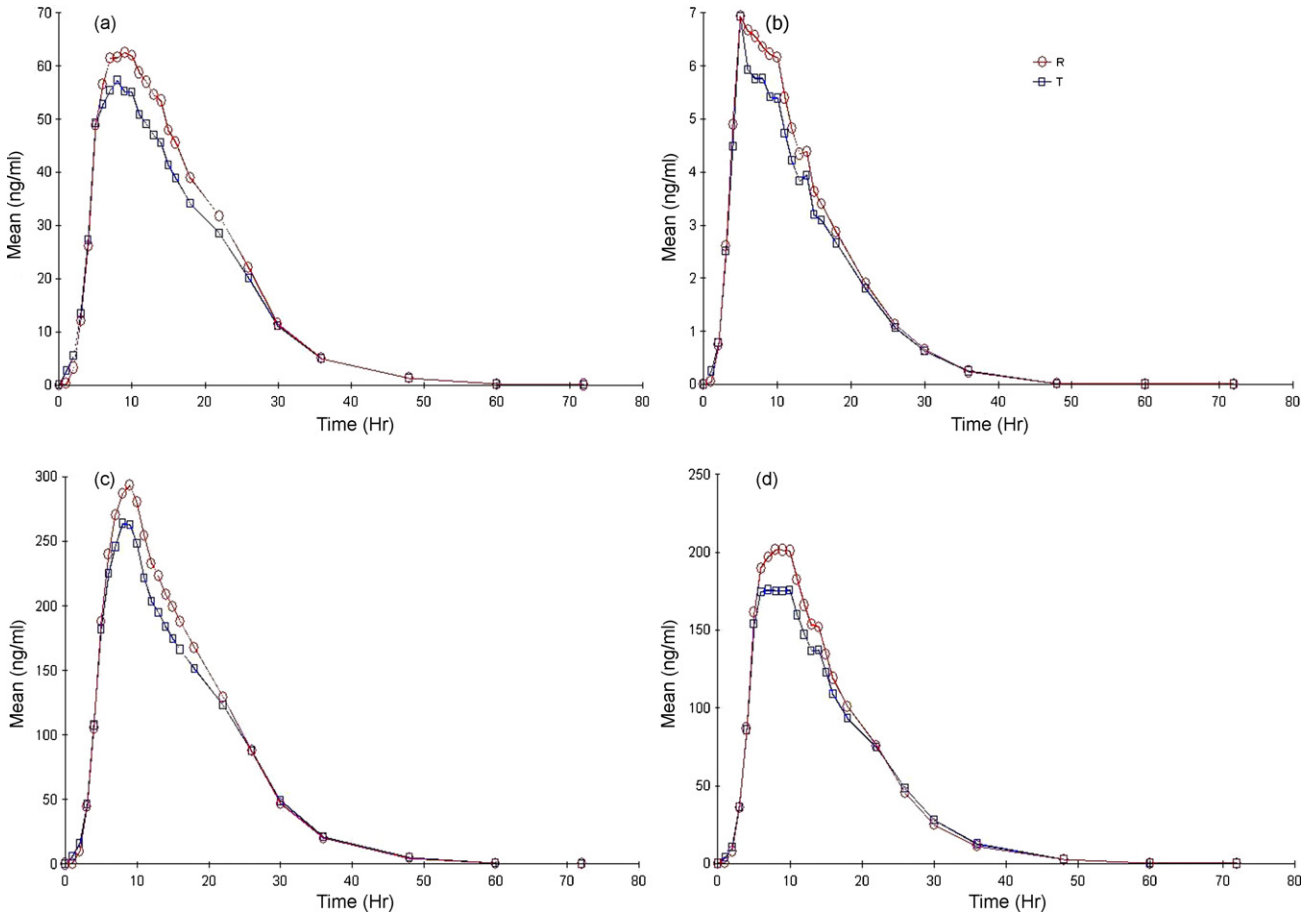

Fig. 4. The linear plasma mean concentration versus time profile of (a) free propranolol, (b) free 4-hydroxy propranolol, (c) total propranolol, (d) total 4-hydroxy propranolol.

d7 (200 ng/mL) from an infusion pump at the rate of 10 μL/min while maintaining the chromatographic and mass spectrometric conditions as used for validation. Six different lots of blank plasma were extracted and evaluated. Matrix effect at the elution region of propranolol and propranolol-d7 was negligible from the plasma matrix, where as slight ion suppression was observed at the elution region of 4-hydroxy propranolol and 4-hydroxy propranolol-d7.

Results of the second exercise performed to check matrix effect due to the use of different plasma lots were summarized in Table 3. Results obtained therein indicate that no additional variations in plasma concentration due to the use of different plasma lots were observed. Hence the concentration of the analytes obtained from clinical study samples should therefore be considered as reliable.

3.3.6. Matrix factor

The matrix ionization suppression or enhancement of these drugs and IS was assessed by measuring the matrix factor. The mean absolute matrix factor at the medium concentration from six lots of plasma samples was 0.99 and 0.92 for propranolol and 4-hydroxy propranolol, respectively (Table 4). The CVs of absolute MF and IS-normalized MF from six lots of plasma samples were <6%. These results indicate that the assay has no significant matrix ionization suppression or enhancement.

3.3.7. Stability studies

The stabilities of propranolol and 4-hydroxy propranolol (free as well as total) were investigated at two concentrations of QC samples (low and high concentration) to cover expected conditions during analysis, storage and processing of all samples, which include the stability data from various stability exercise like in-injector, bench-top, freeze/thaw and long-term stability tests. These data were summarized in Table 5. Result in table indicates that the analytes were found to be stable in mobile phase under autosampler storage condition for at least 48 h at 10 °C, in addition both free and total propranolol as well as 4-hydroxy propranolol were found to be stable in plasma for five freeze/thaw cycles, when stored at

Table 6
Pharmacokinetic parameters (mean ± SD) of 2 types of 160 mg propranolol capsules based on its plasma concentrations.

Parameters	Free propranolol		Free 4-hydroxy propranolol		Total propranolol		Total 4-hydroxy propranolol	
	Reference	Test	Reference	Test	Reference	Test	Reference	Test
T_{max} (h)	8.897 ± 2.412	8.230 ± 2.719	7.115 ± 2.170	6.379 ± 2.206	8.805 ± 1.691	8.449 ± 1.947	8.150 ± 1.814	7.920 ± 2.114
C_{max} (ng/mL)	73.235 ± 37.697	66.710 ± 41.083	8.482 ± 3.667	7.930 ± 3.294	321.483 ± 117.307	291.163 ± 127.748	233.262 ± 107.536	205.755 ± 85.215
$AUC_{0\to t}$ (μg h/mL)	1.179 ± 0.498	1.072 ± 0.497	0.099 ± 0.034	0.091 ± 0.030	4.950 ± 1.652	4.612 ± 1.617	3.283 ± 1.249	3.078 ± 1.182
$AUC_{0\to\infty}$ (μg h/mL)	1.190 ± 0.500	1.081 ± 0.500	0.102 ± 0.034	0.094 ± 0.030	4.971 ± 1.651	4.633 ± 1.616	3.304 ± 1.249	3.100 ± 1.181
$T_{1/2}$ (h)	5.5 ± 1.1	5.4 ± 1.3	5.3 ± 1.8	5.0 ± 1.4	5.1 ± 0.9	5.2 ± 1.0	5.2 ± 1.0	5.3 ± 1.3

−15 °C and thawed to room temperature. The accuracy for bench-top stability ranged from 92.2 to 104.5% and 96.0 to 106.7% and the precision ranged from 1.7 to 7.2% and 1.2 to 4.6% for propranolol and 4-hydroxy propranolol (free as well as total), respectively. Whereas the precision for the long-term stability ranged from 2.1 to 4.5% and 1.5 to 6.3% and the accuracy ranged from 102 to 109% and 97 to 103% for free as well as total propranolol and 4-hydroxy propranolol, respectively. The results of the long-term stability data indicate that propranolol as well as its metabolite 4-hydroxy propranolol and their respective glucuronide conjugates were stable in plasma at −15 °C over 5 months period. Further long-term stability analysis is in process.

3.3.8. Hydrolysis efficiency

The glucuronide hydrolysis efficiency was investigated by the analysis of six replicates of plasma samples containing known concentration of propranolol glucuronide and 4-hydroxy propranolol glucuronide at low and high quality control concentration. The % CVs of samples in hydrolysis step was 2.1 and 3.5% for the low and high concentration, respectively. The obtained results indicate that the glucuronide hydrolysis with β-glucuronidase/arylsulfatase was repeatable and consistent under the presented experimental conditions. Thus we conclude that the difference between total and free propranolol and 4-hydroxy propranolol represents the concentration of their glucuronide.

3.4. Clinical pharmacokinetics and bioequivalence study in healthy subjects

This proposed method was successfully applied to a pharmacokinetic study of propranolol in 100 healthy male volunteers following oral administration of 160 mg of ER/LA propranolol capsule. The mean concentration–time profile of propranolol and 4-hydroxy propranolol in these volunteers is shown in Fig. 4 and the mean estimated pharmacokinetic parameters derived from the plasma concentration profiles are summarized in Table 6. The bioequivalence parameters almost overlapped between the test and reference samples.

Finally, based on the results obtained from the ISR, it was observed that 87.5 and 95.0% of sample points for propranolol and 4-hydroxy propranolol, respectively, were within ±20% of initial concentration value, further proving the proposed method is reproducible and suitable for pharmacokinetic evaluation of propranolol.

4. Conclusion

A sensitive, specific, accurate and reproducible LC–MS/MS method has been developed and validated for the estimation of propranolol and 4-hydroxy propranolol in human plasma. The method has a lower limit of quantitation of 0.20 ng/mL for propranolol and 4-hydroxy propranolol, which is more sensitive than previously reported techniques, using a 0.300 mL of plasma sample. The proposed range is suitable for the estimation of propranolol and 4-hydroxy propranolol in human plasma in the free as well as total form after an oral administration of 160, 120, 80, 60 or 40 mg of propranolol in a pharmacokinetic or bioequivalence study. An added advantage over the earlier methods was the proposed solid phase extraction procedure was simple, efficient and easy to automate. The method was used successfully to determine plasma drug concentrations in human plasma samples.

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