

Multi-Dimensional Column Chromatographic Method with UV Detection, for the Determination of Propranolol at Therapeutic Levels in Human Plasma

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Aim

The ultimate goal of this work is to develop a sensitive, rapid and easy new method for the determination of the beta-adrenergic blocking agent propranolol in human blood, which involves on-line multidimensional chromatography and has the sensitivity required for determination of propranolol in human plasma at therapeutic dose levels and pharmaco-therapeutic studies.

Abstract

A simple and rapid HPLC assay method for the estimation of propranolol (Inderal[®]) in human plasma was developed and validated. The method totally eliminates the extraction procedure; sample clean-up was achieved by on-line solid-phase extraction. The separation was achieved with μ Bondapak 10 μ m C₁₈ column (octadecylsilane, 30 cm \times 3.9 mm). The mobile phase consisted of a mixture of water, methanol, acetonitrile, acetic acid and triethylamine in the proportion of 160 ml: 80 ml: 70 ml: 2.5 ml: 125 μ l, respectively. The pH was adjusted to 3.4 using 1 N NaOH before the addition of triethylamine. The mobile phase was filtered (0.2 μ m filter) and degasified in a ultrasonic bath. The mobile phase flow rate was 0.5 ml/min. Detection was by UV detector at 291 nm and the retention time (RT) observed at around 8 minutes. The recovery of the drug from plasma was assessed by comparing the peak height of the extracted plasma samples with the peak height of authentic (un-extracted) standards which were directly injected (i.e. without column switching) into the analytical column at these concentration levels. The recovery values showed differences lower than 4.0% between the added amount and the founded amount, and were independent of the concentration. The response was linear over a range of 20-100 ng/ml with a limit of detection of 1 ng/ml and limit of quantification at 8 ng/ml plasma. This limit of quantification is adequate for clinical analysis and pharmaco-therapeutic studies and comparable to those values obtained by other workers. The same method was used for the bioavailability study of propranolol formulation in healthy, human and male volunteers.

Keywords: Propranolol; High Performance Liquid Chromatography (HPLC); Therapeutic range; On-line column switching; Human plasma; Bioavailability

Introduction

Propranolol (Figure 1) is a beta-adrenergic blocking agent; it inhibits the cardiac accelerator responses to sympathetic stimulation, reduces myocardial blood flow, and increases myocardial resistance to flow. It also decreases heart rate, cardiac output, mean arterial pressure, and left ventricular minute work in response to exercise. The drug is used to reduce the heart's work and to lower blood pressure. It is also effective to reduce the frequency and severity of angina attacks. Other uses of the drug includes; treatment of hyperthyroidism, cirrhosis,

migraine and glaucoma. Propranolol is a highly lipophilic substance and is almost completely absorbed from the gastrointestinal tract following oral administration [1]. It is 85 to 95% protein bound in the circulation. Evidence indicates that protein-bound drug is unable to cross cell membranes. Therefore, only free drug is transported across cell membranes and possesses pharmacological activity. The therapeutic range for plasma propranolol concentrations remains unknown due to a large interindividual variation and to associated diseases [2-5]. The high interindividual variation of plasma propranolol concentration after peroral administration, approximately 20-fold, depends largely on hepatic metabolism and total body clearance [6].

Reduction of blood pressure and heart rate correlates with plasma propranolol levels [7,8]. Because the concentration of free propranolol in plasma correlates best with beta adrenergic blockade activity, its

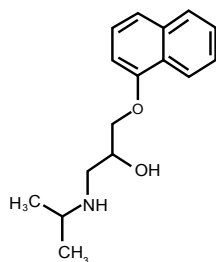


Figure 1: Basic structural formulae of propranolol. Molecular Formula: C₁₆H₂₁NO₂. Systemic name: (Isopropylamino)-3-(1-naphthoxy)-2-propanol. Average mass: 259.157227.

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determination in plasma is important [9]. A number of analytical methods have been published in the literature describing the assay of this drug in human and its pharmacokinetics [10-14]. But most of these methods off-line liquid-liquid extraction procedures may cause loss of the analyte through adsorption of the drug onto glassware during the extraction processes. Adsorptive and evaporative losses may also occur during the solvent extraction or following solvent removal 'blow down' steps, when analyte is allowed to form a dry residue on the inside surface of the container [15]. Such procedures also require elaborate sample preparation and can take up to 1 hr before injection of the sample into the chromatographic system.

The present paper describes a new improved reproducible and selective online multi-dimensional HPLC method using a UV detector at 291 nm. The method was optimized and successfully applied to determination of propranolol in human plasma at therapeutic dose levels. This method performs in a single step for an efficient extraction and clean-up of the drug from human plasma. The accuracy, precision, specificity, linearity, and reproducibility meet the requirements of current recommendations in bio-analytical method validation. The whole procedure takes ca. 10 min, the newly developed method compares favourably with previously reported methods for the analysis of this drug in terms of limit of detection and recovery. Since the sensitivity of this method allows satisfactory detection of propranolol signal at concentration below 10 ng/ml, it is suitable for high altitude pharmacokinetic studies and therapeutic dosage regimen in which the quantitation of the drug is required. The method can also help in monitoring propranolol levels in people with liver disease and diabetic patients where propranolol can reduce blood sugar levels and affect how the body responds to low blood sugar levels, as a result it hides the warning signs of excessively low blood sugar. The results of some pharmacokinetic parameters obtained from healthy adult subject received a single oral dose of the drug under fasting conditions are presented [16].

Experimental

Chemicals and reagents

All reagents and chemicals were ACS reagent grade, they were purchased from Sigma (Pool, Dorset, UK) and were used without purification. Water was distilled and then further purified by passing through a Milli-Q water purification system (Millipore, Milford, MA, USA). All analyses were performed utilizing an M6000 solvent delivery system, a U6K universal injector and a C18 j-Bondapak column, 300 mm×3.9 mm i.d. (Waters Assoc., Inc., Milford, Mass.). Propranolol (Inderal[®]) is Astra Zeneca product (Astra Zeneca, Bedfordshire, UK) was purchased from the Libyan market, where it is widely available.

Plasma samples were obtained from a pool of normal human plasma, collected from healthy volunteers. Heparinized plastic tubes used for whole blood collection 'LiHeparin Menovette', were supplied by Sarstedt (Numbrecht, Germany).

Apparatus

A liquid chromatograph (HPLC) equipped with a Waters Model 501 HPLC pump (pump B) at a rate of 1 ml.min⁻¹ (1800 psi). For the purpose of extraction by column switching, the injector was fitted with a 1 ml loop. A second pump (pump A), and the concentration column were collected to the analytical assembly via a Rheodyne Model 7000 six-port switching valve. The peaks were detected using a Shimadzu SPD-6A variable-wave length detector. UV absorbance was measured at 291 nm.

Chromatography

The mobile phase consisted of a mixture of water, methanol, acetonitrile, acetic acid and triethylamine in the proportion of 160 ml: 80 ml: 70 ml: 2.5 ml: 125 µl, respectively. The pH was adjusted to 3.4 using 1 N NaOH before the addition of tri-ethylamine. The mobile phase was filtered (0.2 µm filter) and degasified in an ultrasonic bath. The mobile phase flow rate was 0.5 ml/min, the injection volume of 1 ml blood samples was injected directly into the HPLC system and the peak height was recorded. To avoid tailing of the drug, the analytical column was primed and recycled overnight at a flow rate of 0.3 ml/min. The detection was done by ultraviolet detector set at 291 nm, 0.04 aufs [12]. The overall performance of the system was verified periodically by injection of standard samples. The column in use retained good efficiency and resolution despite prolonged exposure to aqueous solutions and the matrix effects of the samples.

Standard solutions and linearity

Calibration standards of propranolol (2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/ml) were prepared by spiking appropriate amount of the standard solutions in human blood. All solutions were stored in a refrigerator at 2°C and were stable at least for 3 days at this temperature. In order to prevent photo degradation, all solutions of propranolol were stored in brown volumetric flask in the dark. Under these conditions, decomposition of propranolol was less than 2% within 1 month.

Extraction recovery

The extraction efficiency and recovery of the assay was assessed at concentrations of 10, 20, 30 and 40 ng/ml. Six replicates of each concentration containing the drug in human plasma injected into the column and extracted according to the method described. The recovery of the drug from plasma was assessed by comparing the peak height of the extracted plasma samples with the peak height of authentic (un-extracted) standards which were directly injected (i.e. without column switching) into the analytical column at these concentration levels. The assay recovery was calculated using the following equation:

$$\% \text{ recovery} = (\text{mean peak height plasma extract})/(\text{mean peak height standard}) \times 100$$

Validation

The chromatographic method was validated, determining its linearity, sensitivity, precision and accuracy.

Results and Discussion

The main objective of method validation was to demonstrate the reliability of a particular method for the quantitative determination of an analyte concentration in a specific biological matrix. The characteristic of a bio-analytical method essential to ensure the acceptability of the performance and reliability of analytical results are: separation and specificity, recovery, linearity, accuracy and precision (inter-day and intraday variability must be low), limit of quantification (LOQ) and minimum quantified concentration (MQC), and finally analyte stability.

Separation of the drug in human blood

Reversed-phase chromatography appears to be most useful in the analysis of propranolol in biological mixtures which possess both acidic and basic organic functional groups. The first step in the set-up of the switching/separation system (Figure 2) involved the selection of a suitable pre- (or concentration) column, which would retain

the analyte while other endogenous components are eluted to waste. Among the different columns used for this purpose was an octyl-bonded reversed phase column (125 cm×4.0 mm I.D. 5 µm-particle size, Lichrosorb RP-8 Hibar), this column showed the most favourable retention characteristics for propranolol. The next stage was to find two compatible eluents of different elutropic strengths; one is a washing eluent which should provide a good clean-up of the blood components without causing the analyte to elute from the pre-concentration column (i.e. having a poor elution capability). The washing eluent should also be miscible with the mobile phase, as even slight incompatibility could result in a slug of solvent travelling down the analytical column, partially carrying sample components which may cause band broadening. The use of a 0.5 M solution of perchloric acid allowed the purification of the injected blood samples by washing out any endogenous substances while retaining and concentrating the analyte in the pre-column. Finally a strongly eluting solvent (mobile phase) was chosen to elute the drug off the pre-column and onto the analytical column. The use of the two eluents and the two columns allowed the co-elution of the peaks of interest from the first column “pre-concentration column” into the second column “analytical column”, where separated from the excess reagents and other side products in the sample. This particular type of column switching is often referred to as ‘heart-cutting’ [16]. A wash time (defined as the length of time between injection and switching of the valve) of 1 min was found to be satisfactory to provide a good clean-up of the blood components without causing the analyte to elute. Six replicate injections of blood samples containing the analyte yielded a relative standard deviation (RSD) >5%, using the above conditions. The mobile phase was optimised by careful examination of all major variables, including pH and ionic strength, to determine their influence on enhancing peak separation without prolonging the analysis time for each sample. The pH selected for the analysis did not significantly alter the peak characteristics, even after 6 months of continuous use. Under the experimental conditions, the drug was well resolved from endogenous plasma peaks flanking it (Figure 3).

Linearity and detection limits

Linear calibration graphs of peak heights of standards versus the concentration of the standards were constructed by analysing spiked blank blood samples over four days in the concentration range (2-100 ng/ml). This concentration range is within the therapeutic range for propranolol. Plasma propranolol levels higher than 20 ng/ml, defined as minimum effective concentration were sufficient to obtain a clinical response by beta-adrenoceptor blockage, but better results for the antihypertensive effect can be achieved at plasma levels ranging from 50 to 100 ng/ml [8,17,18]. The plotted points represented the means of at least five replicate injections per standard. For each of the four regression lines (Table 1), the correlation coefficients (r^2) were all greater than 0.9993. A useful way to describe the sensitivity of the method is often expressed as the limit of detection (LOD) and quantisation (LOQ). The use of the washing proved cycle resulted in reduction of the back ground noise and improved LOD and LOQ values. In our case the LOD for propranolol was found to be 1 ng/ml and limit of quantification at 8 ng/ml. This limit of quantification is

	Equation of the regression line	Correlation coefficient, r
Day 1	$y=0.027+0.022x$	0.9998
Day 2	$y=0.016+0.024x$	0.9980
Day 3	$y=0.040+0.021x$	0.9990
Day 4	$y=0.033+0.034x$	0.9964

Table 1: P Linear regression data ($y=mx+b$) and correlation coefficients (r) for plots of propranolol.

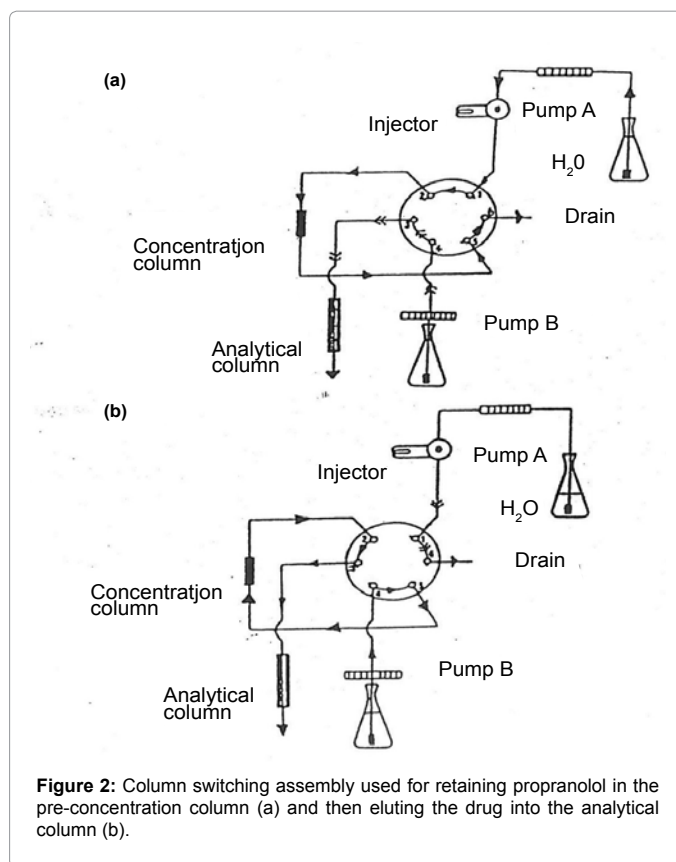


Figure 2: Column switching assembly used for retaining propranolol in the pre-concentration column (a) and then eluting the drug into the analytical column (b).

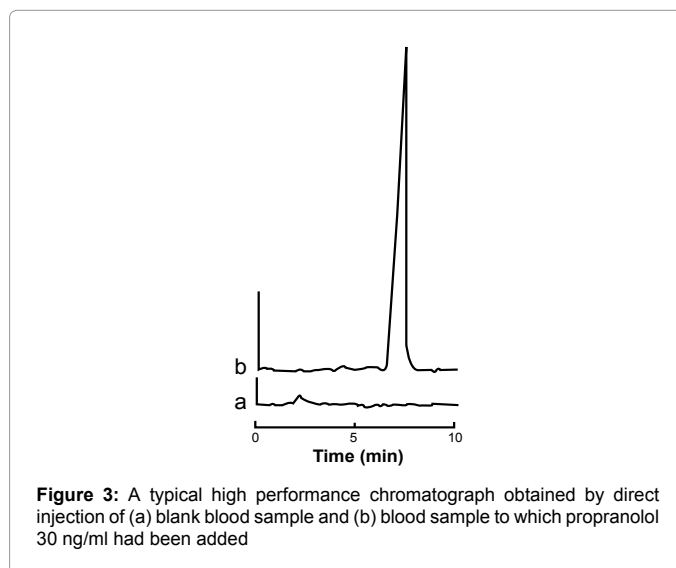


Figure 3: A typical high performance chromatograph obtained by direct injection of (a) blank blood sample and (b) blood sample to which propranolol 30 ng/ml had been added

adequate for clinical analysis and pharmaco-therapeutic studies and comparable to those values obtained by other workers.

Selectivity and stability studies

The possible potentials of various compounds to interfere with the analysis of the drug in the plasma were studied (Table 2). These compounds were selected according to (a) their common use by most of patients and (b) might be administered with propranolol. The selected drugs were: Acetaminophen, aspirin, codeine, salicylamide,

Compound	Analyte : Interferent (w:w)	Relative Sensitivity (%)*
Acetaminophen	1:1	95.86
Caffeine	1:1	96.43
Salicylamide	1:1	96.57
Codeine	1:1	97.28
Aspirin	1:1	97.13
Digoxin	1:1	96.25

*Expressed as the ratio of the signal of the analyte in the presence of interferent to that of the analyte alone

Table 2: Interference by various compounds on the analysis of propranolol.

digoxin and caffeine, interference from these substances was tested in two different studies using the proposed technique. The separation peak height, retention times and the recovery of the analyte were not affected by the presence of these substances. Errors less than 2% were calculated by comparing the results with the corresponding results obtained with a solution containing the drug alone. There was no interference from the heparin and sodium meta-bisulphite, which added to the blood as anticoagulant and antioxidant, respectively.

The stability of the column is of importance in order to define the period of time over which the column can be used without a significant decrease in the sensitivity. After 6 months it was observed that a significant decrease in the sensitivity and selectivity has occurred. The column separation was at 45% of its initial value, with relative standard deviations greater than 13%. The continuous washing procedure extended the life of the column and minimised the start-up time. Stability of the a biological sample spiked with propranolol and kept in an ice bath was tested by making 5 consecutive injections of the same sample over a period of approximately 3 h. There was no significant change in the peak heights between the first injection and the last one. In addition blood samples were stable for at least 3 days when stored at -2°C. Freeze-thaw effect on propranolol recovery was investigated by freeze-thaw analysis of 3 cycles which did not show any major degradation of propranolol. The mean (n=3) recovery at drug concentration of 100 ng/ml was 97.60 ng/ml (%CV=3.20).

Inter- and intra assay variability

The reproducibility of the overall method was determined by extracting and injecting seven replicates blood standard samples at each four different concentrations. The values of amount drug found was used in the calculation of the mean, standard deviation (SD) and coefficient of variations (CV%). Both 'between-day' and 'within-day' reproducibility were assessed for the drug samples. The intra-assay precision was determined with 7 replicates of each concentration. The intermediate precision was determined by analyzing each solution 7 times (intra-day precision), analyzing the same samples on 2 different days (inter-day precision), and by two different analysts during two days (between analysts precision). The precision was expressed as variation coefficients (VC.). As shown by the results in table 3, the method had an overall mean coefficient of variation of 4.5 and 5.9% for 'within-day' and 'between-day' assays, respectively. The recovery values showed differences lower than 4.0% between the added amount and the founded amount, and were independent of the concentration.

Comparison with conventional method

The performance of the method was compared to conventional HPLC method using UV detection [11]. There were no significant differences in the recovery obtained by both methods at four different concentrations ranged between 20-100 ng/ml (Table 4). The LOD and LOQ of the multi-dimensional chromatography method were better

than that obtained by the conventional method. In addition the multi-dimensional chromatography method performs in a single step for an efficient extraction and clean-up of the drug from human blood. It also offers the advantages of being simple, less time and labour consuming, does not require additional solvents for extraction, inexpensive and suitable for routine analysis and kinetic purpose.

Clinical and pharmacokinetic application

The study was conducted in a healthy male volunteer for a single dose of 2x40 mg propranolol tablets. Blood samples were collected before and within 24 hours after drug administration. Blood propranolol concentrations were measured using the multi-dimensional HPLC method. The area under the curve (AUC_T) was calculated by the linear trapezoidal rule. Parameters of AUC_{0-24hr}=620.4 ± 275.0 ng.hr/ml, AUC_{0-∞}=660.2+285.2 ng.hr/ml, t_{max}=1.2 ± 0.3 hr and C_{p max}=100.3 ± 45.4 ng/ml were obtained and are in a good agreement with the pharmacokinetic and bioavailability parameters reported by other workers [19,20].

Conclusion

In conclusion, this work had illustrated the use of a single on-line extraction and enrichment step followed by UV detection which effectively resolved and quantitated propranolol in biological samples. The method eliminates interferences from other endogenous components present in the blood, it is not so subject to sample loss during transfer stages as off-line methods. The developed and validated method shows satisfactory linearity, precision and accuracy. The sensitivity of the method expressed as LOQ (8 ng/mL) compares favourably with previously reported methods for the analysis of this drug [11,13]. The practical extraction procedure, based on column switching extraction provides a selective method for clean-up of the drug from plasma with high and precise recovery. Consequently, the method is particularly useful for pharmacokinetic and clinical applications in essential arterial hypertensive patients, ischemic heart disease, cardiac arrhythmias and other cardiovascular diseases. A work involving the use of this method in studying the different pharmacokinetic parameters of the drug and its bioavailability in patients receiving different propranolol formulations will be conducted in our laboratory in the near future.

Compound	Analyte : Interferent (w:w)	Relative Sensitivity (%)*
Acetaminophen	1:1	95.86
Caffeine	1:1	96.43
Salicylamide	1:1	96.57
Codeine	1:1	97.28
Aspirin	1:1	97.13

*Expressed as the ratio of the signal of the analyte in the presence of interferent to that of the analyte alone

Table 3: Interference by various compounds on the analysis of propranolol.

Amount added (ng/ml)	Mean amount found* (ng/ml)	
	Multi-dimensional method	Conventional method
20	17.80 ± 0.80	18.54 ± 0.85
50	46.95 ± 1.52	46.70 ± 1.29
70	66.39 ± 2.95	65.69 ± 3.14
100	96.82 ± 4.30	95.85 ± 3.27

*The assay recovery was determined at four different concentrations, five samples from each concentration (n=5). A minimum recovery of 95% was used as criteria

Table 4: Comparison of the newly developed multi-dimensional method with conventional chromatographic method.

References

1. Paterson JW, Conolly ME, Dollery C, Hayes A, Cooper RG (1970) The pharmacodynamics and metabolism of propranolol in man. *Pharmacologia Clinica* 2: 127-133.
2. Holford NH, Sheiner LB (1981) Understanding the dose-effect relationship: clinical application of pharmacokinetic-pharmacodynamic models. *Clin Pharmacokinet* 6: 429-453.
3. Frishman WH, Furberg CD, Friedewald WT (1984) β -Adrenergic blockage for survivors of acute myocardial infarction. *N Engl J Med* 310: 830-837.
4. McDevitt DG (1979) Adrenoceptor blocking drugs: Clinical pharmacology and therapeutic use. *Drugs* 17: 267-288.
5. Walle T, Webb JG, Bagwell EE, Walle UK, Daniell HB, et al. (1988) Stereoselective delivery and actions of beta receptor antagonists. *Biochem Pharmacol* 37: 115-124.
6. Chidsey CA, Morselli P, Bianchetti G, Morganti A, Leonetti G, et al. (1975) Studies of the absorption and removal of propranolol in hypertensive patients during therapy. *Circulation* 52: 313-318.
7. Coltart DJ, Shand DG (1970) Plasma propranolol levels in the quantitative assessment of beta-adrenergic blockage in man. *Br Med J* 3: 731-734.
8. Coltart DJ, Gibson DG, Shand DG (1971) Plasma propranolol levels associated with suppression of ventricular ectopic beats. *Br Med J* 1: 490-491.
9. McDevitt DG, Frisk-Holmberg M, Hollifield JW, Shand DG (1976) Plasma binding and the affinity of propranolol for a betareceptor in man. *Clin Pharmacol Ther* 20: 152-157.
10. Ismail Z, Wahab MSA, Rahman ARA (2004) Bioequivalence study of propranolol tablets. *Eur J Gen Med* 1: 42-47.
11. Srikanth MV, Ram BJ, Sunil SA, Rao NS, Murthy KVR, et al. (2012) Development and validation of HPLC method for estimation of propranolol HCl in human plasma. *Journal of Scientific and Industrial Research* 71: 120-123.
12. Salman SA, Sulaiman SA, Ismail Z, Gan SH (2010) Quantitative determination of propranolol by ultraviolet HPLC in human plasma. *Toxicol Mech Methods* 20: 137-142.
13. Umezawa H, Lee XP, Arima Y, Hasegawa C, Izawa H, et al. (2008) Simultaneous determination of β -blockers in human plasma using liquid chromatography-tandem mass spectroscopy. *Biomed Chromatogr* 22: 702-711.
14. Walshe M, Kelly MT, Smyth MR (1996) Comparison of two extraction methods for determination of propranolol and furosemide in human plasma by mixed mode chromatography. *J Pharm Biomed Anal* 14: 475-481.
15. Henion J, Brewer E, Rule G (1998) Sample preparation for LC/MS/MS: analyzing biological & environmental samples. *Anal Chem* 70: 650A-656A.
16. Mifune M, Krehbiel DK, Stobaugh JF, Riley CM (1989) Multidimensional high-performance liquid chromatography of opioid peptides following pre-column derivatization with naphthalene-2,3-dicarboxaldehyde in the presence of cyanide ion. Preliminary results on the determination of leucine- and methionine-enkephalin-like fluorescence in the striatum region of the rat brain. *J Chromatogr* 496: 55-70.
17. Johnsson G, Regardh CG (1976) Clinical pharmacokinetics of beta-adrenoceptor blocking drugs. *Clin Pharmacokinet* 1: 233-263.
18. Frishman WH (1982) The beta-adrenoceptor blocking drugs. *Int J Cardiol* 2: 165-178.
19. Roscoe R, Cooper J, Wilson TW, Joshi NN, Midha KK (1982) The relative bioavailability of a commercial propranolol hydrochloride tablet in man. *Biopharm Drug Dispos* 3: 105-114.
20. Salman SA, Amrah S, Wahab MS, Ismail Z, Ismail R, et al. (2010) Modification of propranolol's bioavailability by *Eurycoma longifolia* water-based extract. *J Clin Pharm Ther* 35: 691-696.