

Full Length Research Paper

Quantification of propranolol in rat plasma by LC-MS/MS using tramadol as an internal standard: Application to pharmacokinetic studies in TAA-induced liver fibrotic rats

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A simple, rapid and selective liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is developed and validated for quantification of propranolol without the sample extraction step in rat plasma using tramadol as an internal standard (IS). The analytes are separated using an isocratic mobile phase which consist of methanol and 10 mm ammonium formate (70/30, v/v) on an isocratic UK-C18 (Imtakt Unison 2.0 × 50 mm, 3 μm) column and was analyzed by MS/MS in the multiple reaction monitoring (MRM) mode using the transitions of respective (M+H)⁺ ions, m/z 260.0→116.2 and m/z 264.2→58.2 for quantification of propranolol and IS, respectively. The standard calibration curves showed good linearity within the range of 2.0 to 800.0 ng/ml ($r^2 = 0.999$, $1/x^2$ weight). The lower limit of quantification (LLOQ) was 2 ng/ml. The retention time of propranolol and IS were 1.12 and 0.939 min which means that it is the potential for the high-through put potential of the proposed method. In addition, no significant metabolic compounds were found to interfere with the analysis. Acceptable precision and accuracy were obtained for the concentrations over the standard curve range. The validated method was successfully applied for the pharmacokinetic studies after 2 mg/kg of propranolol HCl in the thioacetamide (TAA)-induced fibrotic rats.

Key words: Propranolol, liquid chromatography, mass spectrometry, pharmacokinetics, thioacetamide, liver fibrosis.

INTRODUCTION

Propranolol ([RS]-1-(1-methylethylamino)-3-(1-naphthoxy) propan-2-ol] is a competitive non-selective

β-adrenergic blockers used to treat hypertension, angina, cardiac arrhythmia, anxiety and thyrotoxicosis. It was the

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first successful β -blocker developed (Black et al., 1964) and is also one of the most widely prescribed β -blockers approved in the US (Koshakji and Wood, 1986; Shin and Johnson, 2007). Despite complete absorption, propranolol has a variable bioavailability due to extensive first-pass metabolism and is eliminated almost exclusively by hepatic metabolism by three routes called a naphthalene ring hydroxylation, N-dealkylation of the isopropylamine side chain oxidation and O-glucuronidation (Thompson et al., 1981; Walle et al., 1985; Masubuchi et al., 1993). Therefore, hepatic impairment will increase its bioavailability and high blood concentration that has more than 18 metabolites, with at least four of these having pharmacological activities (Silber et al., 1983). Among these metabolites, the main metabolite, 4-OH propranolol, has a longer half-life up to (5.2 to 7.5 h) than the parent compound (3 to 4 h) which is also pharmacologically active. The duration action of propranolol after single oral dose is longer than the half-life and may last up to 12 h, if the dose is high enough and effective serum/plasma levels encountered between 10 to 100 ng/ml with toxic levels above 2 μ g/ml in clinical situations. Many high-performance liquid chromatography (HPLC) methods for the analysis of propranolol in human plasma using both fluorescence (Rosseel and Bogaert, 1981; Rekhi et al., 1995; Kim et al., 2001) and UV (Wolf-Coporda et al., 1987; Walshe et al., 1996) detection have been described. HPLC-fluorescence detector may provide the selectivity and sensitivity necessary for routine analysis of propranolol in the blood (Hedeem et al., 1991).

Propranolol concentrations in the plasma encountered during routine propranolol therapy may range from a low 1.0 ng/ml to above 100 ng/ml, and several methods have been reported for the determination of propranolol in plasma (Rekhiet al., 1995). Probably, HPLC-UV (Zhou et al., 2002; El-Saharty, 2003; Delamoye et al., 2004) or fluorescence (Ranta et al., 2002; Satinsky et al., 2007) detection would be a more suitable choice for the routine β -blockers analysis. Due to the complex matrix of biological samples, a pretreatment procedure such as liquid-liquid extraction (Braza et al., 2000; Zhou et al., 2002; El-Saharty, 2003) pre-column clean-up (Mislanova and Hutta, 2003) and solid-phase extraction (SPE) (Satinsky et al., 2007; Hefnawy et al., 2011; Vukovic et al., 2012) were required for β -blockers separation and concentration before HPLC analysis.

Recently, combined techniques such as liquid chromatography (LC) and mass spectrometry (MS) have considerably improved analytical selectivity and sensitivity. LC-MS/MS facilitates rapid data turn around and requires minimal method development. Unfortunately, due to the complexity and protein components of biological fluids, the direct injection of these samples is not compatible with most chromatographic systems (Mullett, 2007). Biological samples are problematic due to the irreversible absorption of proteins on the stationary phase, resulting

in the substantial loss of column efficiency and increase in back pressure (Souverain et al., 2004). Therefore, appropriate sample preparation is critical and is a key consideration for the development of quantitative HPLC methods for measuring drugs in biological fluids. The role of sample preparation also continues to be an important area for development, since the increase of acceptance of high-throughput instrumentation, such as LC-MS/MS, has shifted the analysis bottleneck backward towards sample preparation (Berna et al., 2002). One of the more common procedures for overcoming the difficulties associated with high complex and proteineous biological fluids is simple precipitation of proteins with an organic solvent, with or without an acid, followed by centrifugation (Gage and Stopher, 1998; Jayewardene et al., 2001).

This research paper describes a simple, rapid and sensitive LC-MS/MS for the analysis of propranolol in rat plasma obtained from male thioacetamide (TAA)-induced liver fibrotic rats after dosed intravenously with propranolol, with simple precipitation mobile phase, followed by centrifugation and its application to pharmacokinetic studies.

MATERIALS AND METHODS

Chemicals

Propranolol HCl ($C_{16}H_{21}NO_2 \cdot HCl$, MW=260.0 g/mol) and tramadol HCl (IS, internal standard, $C_{16}H_{25}NO_2 \cdot HCl$, MW=263.2 g/mol) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), respectively (Figure 1). Purity was found to be more than 99% of all the compounds. Acetonitrile, ammonium formate and methanol are all HPLC-grade purchased from Sigma Co. (St. Louis, MO, USA) while other reagents and solvents used were of analytical grade. All aqueous solutions including the buffer for the HPLC mobile phase was prepared with water purified by Milli-Q water purification system (Millipore, Milford, MA, USA).

Stock solutions and quality control standards

Primary stock solutions of propranolol hydrochloride and IS were prepared with methanol solution to a final concentration of 1 mg/ml and 10 μ g/ml, respectively and stored at $-20^\circ C$. A set of six non-zero calibration standards ranging from 2.0 to 800 ng/ml were prepared in blank rat plasma, with an appropriate amount of propranolol. The quality control (QC) samples were prepared in blank rat plasma at its concentrations of 10, 100 and 400 ng/ml. Blank rat plasma was tested before spiking to ensure that no endogenous interference was found proximal to retention times of propranolol and IS.

Sample preparation

After dilution of stock solution of propranolol to a concentration of 2.0 to 800 ng/ml with blank plasma, 50 μ l aliquot of rat plasma was pipetted into a screw cap glass tube. Briefly, 100 μ l of acetonitrile (containing IS with concentration of 100 ng/ml) were added to the 80 μ l aliquot of rat plasma for deproteinization. The mixture was briefly vortex-mixed for 10 s, followed by centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to another set of

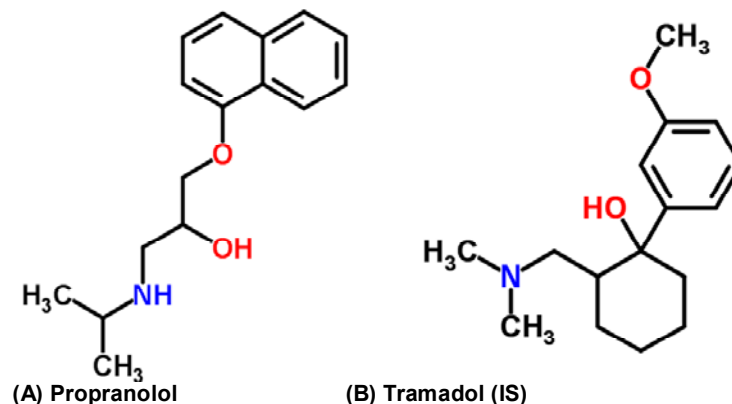


Figure 1. Chemical structures of (A) propranolol [MW = 259.34 g/mol, C₁₆H₂₁NO₂, 1-(isopropylamino)-3-(1-naphthoxy)-2-propanol and (B) tramadol [IS, MW = 263.4 g/mol, C₁₆H₂₅NO₂, 2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol.(from ChemSpider free chemical data base)

clean glass tubes and then 200 μ l of mobile phase added and vortex-mixed. After centrifugation at 13,000 rpm for 10 min, 2 μ l of the supernatant were directly injected into the LC-MS/MS.

LC-MS/MS analyses

The LC system used was an Agilent (Agilent Technologies, Inc., Palo Alto, CA, USA) chromatograph equipped with an isocratic pump (1200 series) and interfaced with an autosampler (Reliance, Spark, Holland). The analytical column was an isocratic UK-C18 (Imtakt Unison 2.0 \times 50 mm, 3 μ m) column (SIS Inc., Co. Ringoes, NJ, USA) which was placed in an oven at 40°C. The mobile phase consisted of methanol [10 mm ammonium formate with 0.1% formic acid (70:30, v/v)] and the flow rate was 250 μ l/min. MS analysis was performed using an AB Sciex 400 QTRAP™ mass spectrometer system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization (ESI) interface and Ionics EP 10+ system, and operated in the positive ionization mode. The ion source parameters were set as follows: curtain gas = 40 psi, GS1 = 50 psi and GS2 = 50 psi, ion spray voltage = 5500 V, ion source temperature = 350°C, collision-activated dissociation (CAD) = medium. This system was set up in multiple-reaction monitoring (MRM) mode, monitoring the transitions m/z 260.13 \rightarrow m/z 116.1 and m/z 264.20 \rightarrow m/z 58 for quantification of propranolol and IS, respectively. Data acquisition and analysis were performed using the analyst software peak simple chromatography data system version 1.4.1 (Applied Biosystems, Foster City, CA, USA). Total chromatographic run time per sample was about 3 min.

Assay validation

Assay validation was performed according to the Food and Drug Administration (FDA) guidance on bioanalytical methods validation (FDA, 2001). Linearity was determined using a linear least-square regression with $1/x^2$ weighting, which was performed on the peak area ratios of propranolol/IS versus propranolol concentrations of the six rat plasma standards. The sensitivity of the method was expressed as the lowest limit of quantification (LLOQ) that could be quantitatively determined with acceptable accuracy and precision. The accuracy and precision were assessed by analyzing four concentrations of QC samples with 2.0 to 800 ng/ml from five

different validation batches and was calculated using one-way analysis of variance (ANOVA). Specificity was performed and six randomly selected blank rat samples collected under controlled conditions were passed through the similar extraction procedures. The samples were analyzed to determine the extent to which endogenous plasma components could interfere with the analytes or IS at the retention time. To evaluate the inter-day precision and accuracy, validation control samples with drug concentration of 2.0, 10, 100 and 400 ng/ml propranolol and IS (100 ng/ml) solutions were analyzed together with one independent calibration curve. The accuracy and precision of the inter-day assay were evaluated at the same concentration and was calculated for five different days. Inter- and intra-day precision were expressed as relative standard deviation (RSD). The accuracy was expressed as the percentage ratio between the experimental and nominal concentrations for each sample. The lowest limit of quantification (LLOQ) was defined as the lowest plasma concentration of each propranolol analyzed with an error of 20% or lower, that corresponds to a signal five times greater than the analytical background noise in our experiment (FDA, 2001).

Pharmacokinetic study

Animals were handled and housed in a protected environment in conventional plastic cage with free access to food and water. The animals were maintained at a temperature of 22 to 25°C with a light cycle of 12 h of light and 12 h of darkness (12L:12D), with the lights on at 0700 h and off at 1900 h (Wersinger and Martin, 2009). Hepatic fibrosis was induced by intraperitoneal injection of thioacetamide (TAA, 200 mg/kg), 2 times per week, during 12 weeks and then 3 times per week during 7 and 11 weeks in minimal and severe liver fibrotic groups, respectively. The fibrotic change of each animal were confirmed by light microscopic observation after connective tissue-specific Masson's trichrome staining after propranolol pharmacokinetic studies. To evaluate the applicability of this method, the pharmacokinetic characteristics of propranolol were examined in the TAA-induced liver fibrotic rat model (male SD rat, 200 to 250 g, SLC, Inc, Japan). Experimental animals were divided into two groups with seven rats; minimal and severe liver fibrotic rats. A 2.0 mg/kg bolus dose of propranolol was administered through tail vein. After restraining the rat by rat restraint cuff, 0.5 ml of venous blood sample was drawn via lateral vein by

heparinized syringe just prior to and at 0.5, 1, 2 and 4 h after propranolol administration and the samples were centrifuged at 2,500 rpm for 10 min and the plasma obtained was frozen at -70°C until analysis. The total plasma propranolol concentration at various time points was analyzed by LC-MS/MS. The initial plasma concentration (C_0) is anticipated to be intercept on the plasma concentration axis when the line is extrapolated back to time zero in the semi-logarithmic concentration-time plot. The area under the plasma concentration-time curve (AUC) was calculated for the total propranolol concentration (C_t) using the linear trapezoidal rule extrapolated to infinity according to a PK Solutions 2.0 (SUMMIT Research Services, Montrose, USA) (Summit Research Services, 2005).

The total area under the first moment-time curve (AUMC) was calculated by integration of time (t) of first moment ($C_t \cdot t$) ($\text{AUMC} = C_t \cdot t \cdot dt$). Dose/AUC calculated the plasma clearance (CL) ($\text{CL} = \text{dose}/\text{AUC}$) and the mean residence time (MRT) of the drug in the body was calculated by AUMC/AUC ($\text{MRT} = \text{AUMC}/\text{AUC}$). The apparent volume of distribution at steady-state (V_{dss}) was calculated by $\text{CL} \cdot \text{MRT}$ ($V_{\text{dss}} = \text{CL} \cdot \text{MRT}$) and terminal half-life ($t_{\beta 1/2}$) was calculated by $\ln 2/\beta$ (β = slope of the terminal phase). All values presented are expressed as the mean \pm SD of seven rats. The pharmacokinetic parameters of the two groups were compared for the statistical differences by independent samples t-test using statistical package for social sciences (SPSS) 20.0 ver. and a probability of $p < 0.05$ was considered significant.

RESULTS

Separation

The molecular structures of propranolol hydrochloride and IS are shown in Figure 1. The simple preparation procedure including the simple liquid extraction of propranolol with acetonitrile and mobile phase and centrifugation of extracted sample and supernatant were directly injected into the isocratic HPLC separation. The chromatograms of (A) double blank plasma, (B) blank plasma with 100 ng/ml of IS, (C) blank plasma with 2.0 ng/ml (LLOQ) of propranolol and 100 ng/ml of IS and (D) rat plasma was taken 2 h after a bolus dose of 2 mg/kg propranolol hydrochloride spiked with 100 ng/ml of IS shown in Figure 2. The retention times of propranolol and IS was about 1.12 and 0.94 min, respectively, that means the potential for the high throughput potential of the proposed method. Blank rat plasma had no significant endogenous peaks at the retention time of propranolol or IS (Figure 2A). To avoid the interference from exogenous/endogenous compounds co-eluted with the target compound, MS/MS (termed tandem MS) detection was performed. Ionization of analytes was carried out using the electrospray ionization (ESI) technique with positive polarity and multiple reactions monitoring (MRM) mode. From full-scan mass spectra via the Q1 mass filter, the protonated molecular ions $[\text{M}+\text{H}]^+$, at m/z 260.13 for propranolol and m/z 264.20 for IS were chosen for the precursor ion (Figure 3A and B). The MS/MS fragmentation was achieved by introducing the $[\text{M}+\text{H}]^+$ ions into the second quadrupole (Q2) cell with the best collision energy set of 25.0 eV for propranolol and 35.0 eV for IS.

After collision-induced dissociation, the MS/MS transition m/z 260.13 \rightarrow m/z 116.10 for propranolol and m/z 264.20 \rightarrow m/z 58.00 for IS was selected. The most abundant ions in the production mass spectrum at m/z 116.10 for propranolol and m/z 58.00 for IS were monitored for quantification (Figure 3A and B).

Method validation

The standard calibration curves showed good linearity within the range of 2.0 to 800 ng/ml using least-squares regression analysis ($y = 0.00101x + 0.000142$, $r^2 \geq 0.999$, $1/x^2$ weighting). Intra- and inter-day precisions and accuracies were determined by analyzing QC samples against a calibration curve on the same day ($n = 5$) and on different days ($n = 5$). As shown in the Table 1, this method allowed good precision and accuracy. The relative standard deviation values of both intra- and inter-day were 0.45 to 8.61% and 0.69 to 5.82%, respectively. Intra- and inter-day accuracies were 97.8 to 106.8% and 100.9 to 108.8%, respectively. Under the described analytical conditions, the LLOQ which is defined as the lowest concentration of propranolol at which both the precision and accuracy were less than or equal to 20% (FDA, 2001), was 2.0 ng/ml.

DISCUSSION

Application in pharmacokinetic study

The mean plasma concentration-time profiles of propranolol after a single bolus dose (2 mg/kg) are illustrated in Figure 4A and the main estimated pharmacokinetic parameters are listed in Table 2. Plasma concentrations of propranolol were in the standard curve range and remained above the LLOQ (2 ng/ml) for the entire sampling period. Because the single bolus dose 4 mg propranolol hydrochloride resulted in a mean plasma concentration of 238.09 ± 96.47 and 439.52 ± 39.74 ng/ml at 0.5 h and 22.42 ± 3.53 and 25.07 ± 8.00 ng/ml at 4 h for the minimal and severe liver fibrosis rat models, respectively, the LLOQ of this method appeared to have enough sensitivity. The sampling schedule should also cover the plasma concentration-time curve long enough to provide a reliable estimate of the extent of exposure which is achieved if area under the curve (AUC_t) covers at least 90% of AUC_∞ (Europe, the Middle East and Africa (EMEA), 2010).

Pharmacokinetic characteristics in normal and liver fibrosis groups

In our study, because AUC_t is about 91.3 and 95.5% in the minimal and severe liver fibrosis groups, sampling

Propranolol

Tramadol (IS)

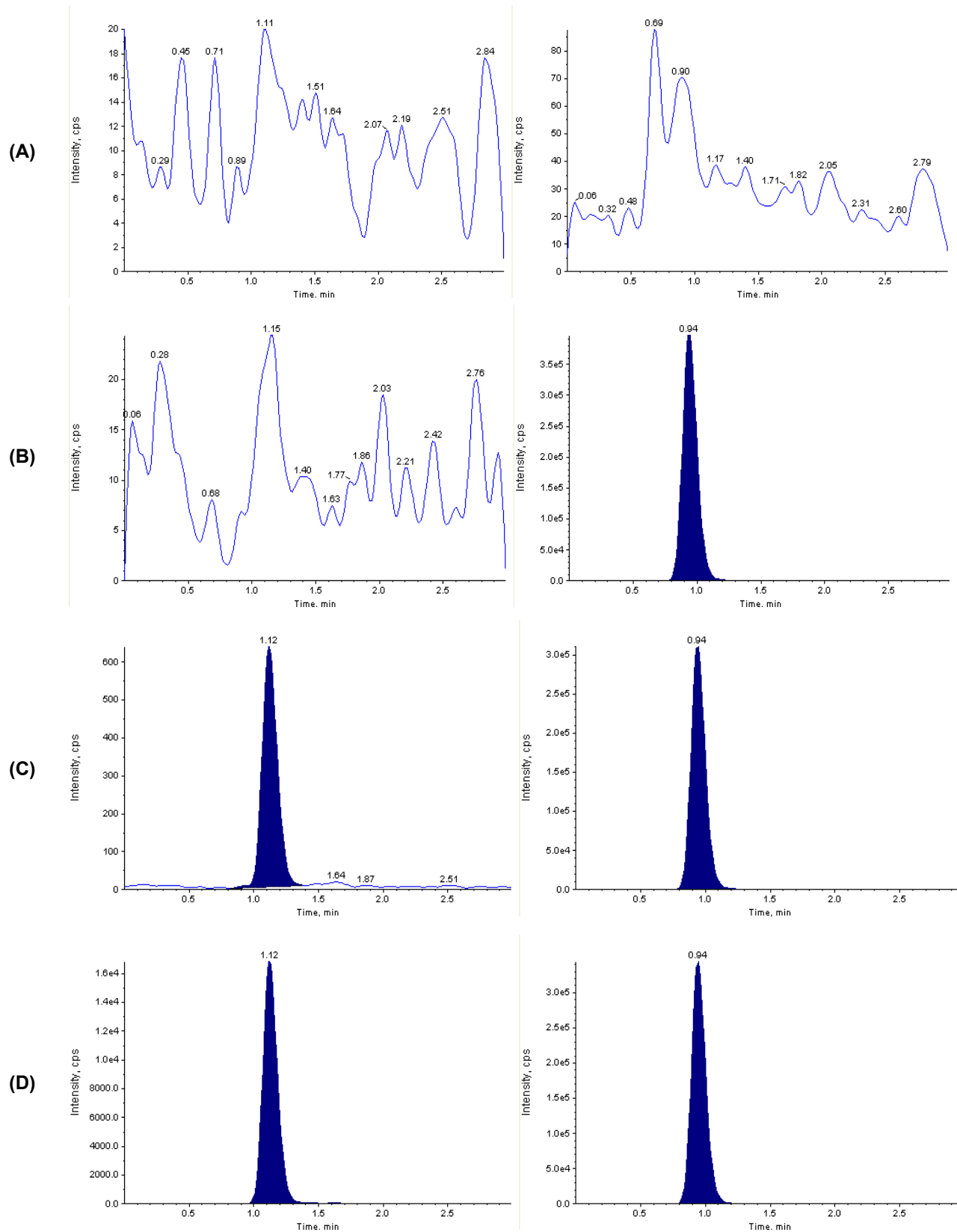


Figure 2. Chromatograms of (A) double blank plasma, (B) with IS (100 ng/ml), (C) with propranolol (LLOQ, 2.0 ng/ml) and IS (100 ng/ml), and (D) rat plasma taken 2 h after a bolus dose (2 mg/kg) of propranolol hydrochloride spiked with IS (100 ng/ml).

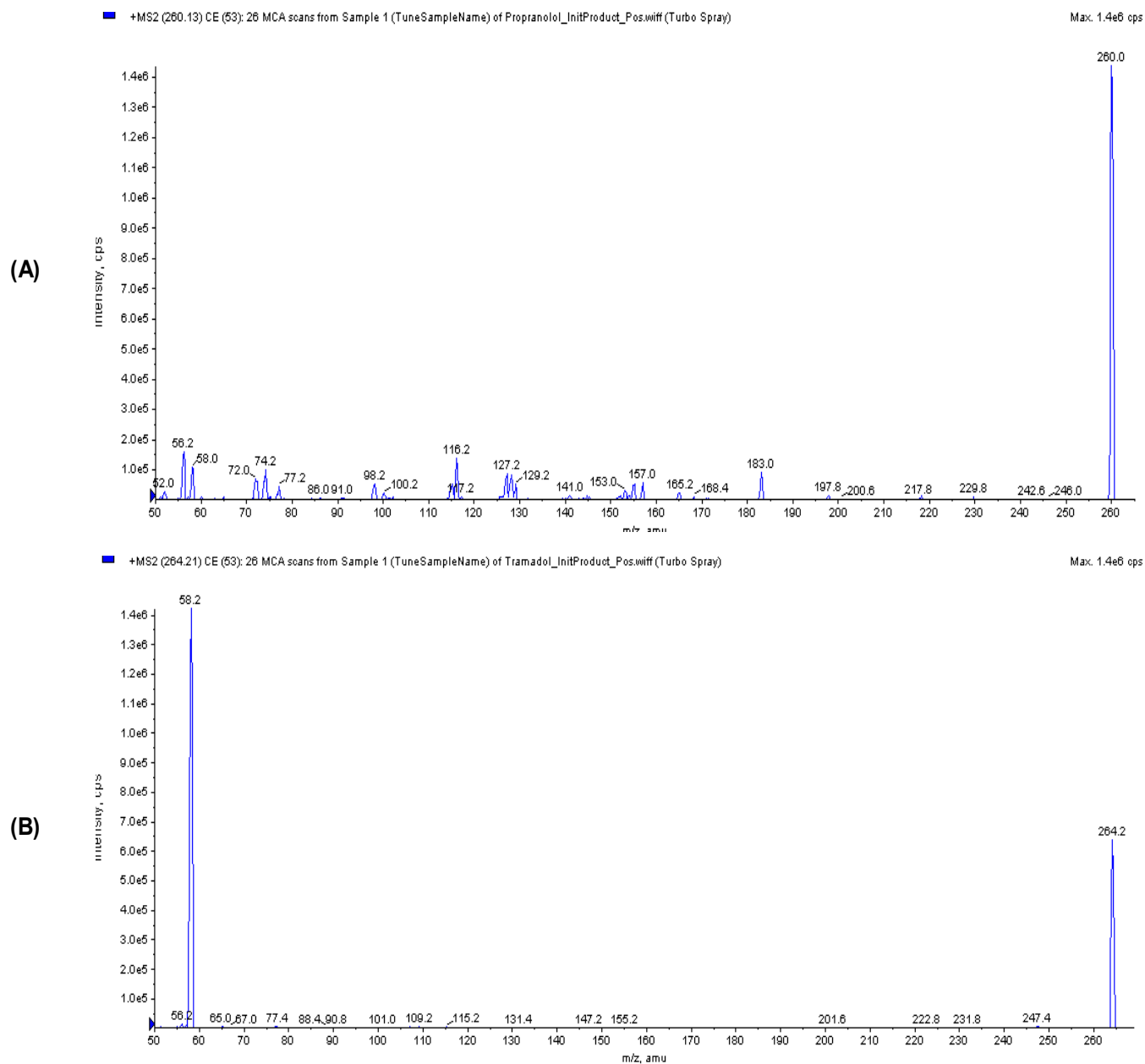


Figure 3. Full-scan mass spectra of precursor and product ions of propranolol; (A) m/z 260.0 \rightarrow 116.2 and tramadol (B) m/z 264.2 \rightarrow 58.2.

schedule may be enough for pharmacokinetic study. The mean initial plasma concentrations (C_0) and AUC_t of the propranolol of severe liver fibrotic rats, which was dependent on a period of TAA treatment, are significantly increased to 201 and 167.8% of those of minimal liver fibrotic rats shown in Table 2. The mean plasma propranolol concentration-time curves after single bolus dose (2 mg/kg) in the minimal and severe fibrotic rats showed an open two-compartment pharmacokinetic model along with a rapid initial distribution and slower terminal elimination phase that are described in the Figure 4B (Fagan et al., 1982). The plasma profiles of the

mean propranolol concentration versus time after a single bolus dose in two groups exhibited marked different patterns and other pharmacokinetic parameters such as AUC , V_d , MRT , elimination rate constant and CL are different between two groups (Table 2). We successfully applied the proposed method to determine propranolol plasma concentration in a preclinical pharmacokinetic study in the TAA-induced hepatic fibrosis rat model as shown in Figure 4C. Therefore, we conclude that this method is proven to be good enough for the determination of propranolol for pharmacokinetic studies using micro-volume of plasma.

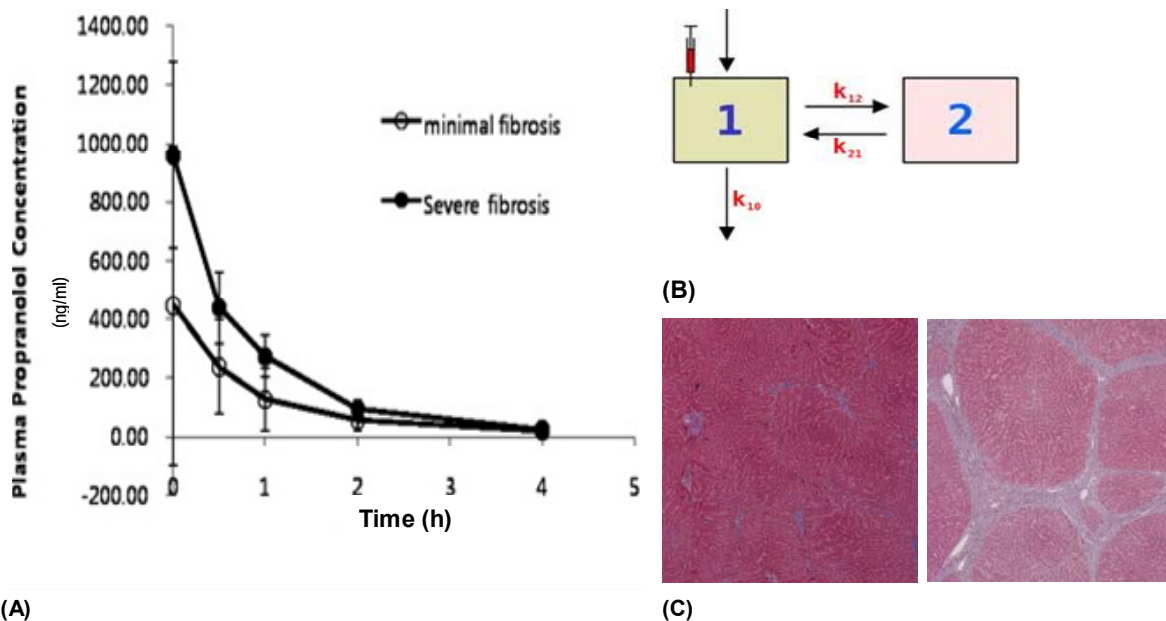


Figure 4. (A) Mean \pm SD ($n = 7$) plasma concentrations vs. time plots after single bolus dose of 4 mg/kg propranolol hydrochloride to the TAA-induced minimal and severe hepatic fibrotic rats, (B) 2-compartment open model (1 = central compartment, 2 = peripheral compartment), k = distribution rate constant and (C) liver light microscopic findings in minimal (left) and severe (right) fibrotic rat, blue = collagen septa, $\times 40$.

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Conflict of interest

Authors reported none.

REFERENCES

- Berna M, Murphy AT, Wilken B, Ackermann B (2002). Collection, storage, and filtration of in vivo study samples using 96-well filter plates to facilitate automated sample preparation and LC/MS/MS analysis. *Anal. Chem.* 74:1197-1201.
- Black JW, Crowther AF, Shanks RG, Smith LH, Dornhorst AC (1964). A new adrenergic beta-receptor antagonist. *Lancet.* 1:1080-1081.
- Braza AJ, Modamio P, Marino EL (2000). Two reproducible and sensitive liquid chromatographic methods to quantify atenolol and propranolol in human plasma and determination of their associated analytical error functions. *J. Chromatogr. B Biomed. Sci. Appl.* 738:225-231.
- Delamoye M, Duverneuil C, Paraire F, de Mazancourt P, Alvarez JC (2004). Simultaneous determination of thirteen beta-blockers and one metabolite by gradient high-performance liquid chromatography with photodiode-array UV detection. *Forensic Sci. Int.* 141:23-31.
- El-Saharty YS (2003). Simultaneous high-performance liquid chromatographic assay of furosemide and propranolol HCL and its application in a pharmacokinetic study. *J. Pharm. Biomed. Anal.* 33:699-709.
- EMA (2010). Guideline on the investigation of bioequivalence, Committee for medicinal products for human use (CHMP). London: European Medicines Agency. [online]; http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/01/WC500070039.pdf
- Fagan TC, Walle T, Walle UK, Conradi EC, Harmon G, Gaffney TE (1982). Early kinetics of intravenous propranolol. *Br. J. Clin. Pharmacol.* 13:571-574.
- FDA (2001). FDA Guidance for industry: Bioanalytical Method Validation. In: US Department of Health and Human Services, Center for Drug Evaluation and Research and center for Veterinary Medicine. Food and Drug Administration. Rockville, MD, USA. [online]; <http://www.fda.gov/CDER/GUIDANCE>
- Gage R, Stopher DA (1998). A rapid HPLC assay for voriconazole in human plasma. *J. Pharm. Biomed. Anal.* 17:1449-1453.
- Hedeem KM, Tyczkowska K, Aucoin DP, Norton RM (1991). Rapid high-performance liquid chromatographic method for the determination of propranolol levels in canine and feline plasma. *J. Chromatogr.* 572:239-245.
- Hefnawy MM, Asiri YA, Al-Zoman NZ, Mostafa GA, Aboul-Enein HY (2011). Stereoselective HPLC analysis of tertatolol in rat plasma using macrocyclic antibiotic chiral stationary phase. *Chirality* 23:333-338.
- Jayewardene AL, Kearney B, Stone JA, Gambertoglio JG, Aweeka FT (2001). An LC-MS-MS method for the determination of indinavir, an HIV-1 protease inhibitor, in human plasma. *J. Pharm. Biomed. Anal.* 25:309-317.
- Kim HK, Hong JH, Park MS, Kang JS, Lee MH (2001). Determination of propranolol concentration in small volume of rat plasma by HPLC with fluorometric detection. *Biomed. Chromatogr.* 15:539-545.
- Koshakji RP, Wood AJ (1986). A modified, sensitive liquid chromatographic method for measurement of propranolol with fluorescence detection. *J. Pharm. Sci.* 75:87-89.
- Masubuchi Y, Kagimoto N, Narimatsu S, Fujita S, Suzuki T (1993). Regioselective contribution of the cytochrome P-450 2D subfamily to propranolol metabolism in rat liver microsomes. *Drug Metab. Dispos.* 21:1012-1016.
- Mislanova C, Hutta M (2003). Role of biological matrices during the analysis of chiral drugs by liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 797:91-109.

- Mullett WM (2007). Determination of drugs in biological fluids by direct injection of samples for liquid-chromatographic analysis. *J. Biochem. Biophys. Methods.* 70:263-273.
- Ranta VP, Toropainen E, Talvitie A, Auriola S, Urtti A (2002). Simultaneous determination of eight beta-blockers by gradient high-performance liquid chromatography with combined ultraviolet and fluorescence detection in corneal permeability studies in vitro. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 772:81-87.
- Rekhi GS, Jambhekar SS, Souney PF, Williams DA (1995). A fluorimetric liquid chromatographic method for the determination of propranolol in human serum/plasma. *J. Pharm. Biomed. Anal.* 13:1499-1505.
- Rossee MT, Bogaert MG (1981). High-performance liquid chromatographic determination of propranolol and 4-hydroxypropranolol in plasma. *J. Pharm. Sci.* 70:688-689.
- Satinsky D, Serralheiro HS, Solich P, Araujo AN, Montenegro MC (2007). On-line coupling of sequential injection extraction with restricted-access materials and post-column derivatization for sample clean-up and determination of propranolol in human plasma. *Anal. Chim. Acta.* 600:122-128.
- Shin J, Johnson JA (2007). Pharmacogenetics of β -blockers. *Pharmacotherapy* 27:874-887.
- Silber BM, Holford NH, Riegelman S (1983). Dose-dependent elimination of propranolol and its major metabolites in humans. *J. Pharm. Sci.* 72:725-732.
- Souverain S, Rudaz S, Veuthey JL (2004). Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 801:141-156.
- Summit Research Services (2005). PK Solutions version 2.0, non-compartmental pharmacokinetic analysis program, Colorado 81401: Montrose, USA.
- Thompson JA, Hull JE, Norris KJ (1981). Glucuronidation of propranolol and 4'-hydroxypropranolol. Substrate specificity and stereoselectivity of rat liver microsomal glucuronyltransferases. *Drug Metab. Dispos.* 9:466-471.
- Vukovic G, Stereva D, Bursić V, Mladenova R, Lazić S (2012). Application of GC-MSD and LC-MS/MS for the determination of priority pesticides in baby foods in Serbian market, *LWT – Food Sci. Technol.* 49:312-319.
- Walle T, Walle UK, Olanoff LS (1985). Quantitative account of propranolol metabolism in urine of normal man. *Drug Metab. Dispos.* 13:204-209.
- 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.
- Wersinger SR, Martin LB (2009). Optimization of laboratory conditions for the study of social behavior. *ILAR J.* 50:64-80.
- Winek CL (1976). Tabulation of therapeutic, toxic, and lethal concentrations of drugs and chemicals in blood. *Clin. Chem.* 22:832-836.
- Wolf-Coporda A, Plavsic F, Vrhovac B (1987). Determination of biological equivalence of two atenolol preparations. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 25:567-571.
- Zhou Q, Yao TW, Zeng S (2002). Chiral reversed phase high-performance liquid chromatography for determining propranolol enantiomers in transgenic Chinese hamster CHL cell lines expressing human cytochrome P450. *J. Biochem. Biophys. Methods.* 54:369-376.