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HPLC assay and pharmacokinetic study of atorvastatin in beagle dogs after oral administration of atorvastatin self-microemulsifying drug delivery system

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A specific and accurate reversed-phase HPLC with UV detection was developed for the assay of atorvastatin in beagle dog plasma. Indomethacin was used as the internal standard. Atorvastatin was extracted by protein precipitation, the extracts were injected into a Kromasil C_8 column (150 mm × 4.6 mm, 5 μm) with UV wavelength set at 270 nm. The mobile phase consisted of acetonitrile : 0.1 mol/L ammonium acetate buffer (pH 4.0) (65 : 35% v/v) at a flow rate of 1.0 ml/min. The column was at ambient temperature (25 °C). The injection volume was 25 μl. The blank plasma did not interfere with the determination of atorvastatin and indomethacin. A good linear relationship was obtained between the peak area ratio of atorvastatin to indomethacin and the concentration of atorvastatin over the range of 0.05 to 2.5 μg/mL. The limit of quantification was 25 ng/mL, the limit of detection was 8 ng/ml. The total chromatographic analysis time was within 9 min. The method is accurate, precise and fast for the assay of atorvastatin in plasma following oral administration of an atorvastatin SMEDDS to healthy beagle dogs.

1. Introduction

Atorvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor efficiently and widely used to lower cholesterol and triglyceride levels in patients with hypercholesterolemia (Lea et al. 1997; Malhotra et al. 2001). Atorvastatin undergoes extensive first-pass metabolism mainly by CYP3A4 in the liver and its oral bioavailability is approximately 14%. The low systemic availability has been attributed to presystemic clearance in gastrointestinal mucosa and hepatic first-pass metabolism (Lennernas 2003). Self-microemulsifying drug delivery systems (SMEDDS) have received great attention recently for their potential use as drug delivery systems for drugs with poor oral bioavailability (Pouton 2000). We developed an atorvastatin SMEDDS and an assay of atorvastatin in beagle dog plasma after oral administration of the atorvastatin SMEDDS.

2. Investigations and results

2.1. Chromatographic separation

A baseline separation of atorvastatin and indomethacin (internal standard) was obtained in beagle dog plasma. No endogenous sources of interference were observed at the retention time of the analyte. The retention time of atorvastatin and indomethacin were 6.6 min and 7.8 min, respectively (Fig. 1). The total chromatographic analysis time was within 9 min.

An aliquot of 200 μl of various concentrations (0.05, 0.1, 0.2, 0.5, 1.0, 2.5 μg/ml) of atorvastatin and 200 μl indomethacin was added to 0.5 ml blank beagle dog plasma. The sample were prepared as described in section 4.6. The standard curve for atorvastatin was $Y = 2.6108\ X + 0.0082$ ($r = 0.9995$), where Y is the peak area ratio of atorvastatin to indomethacin, and X is the concentration of

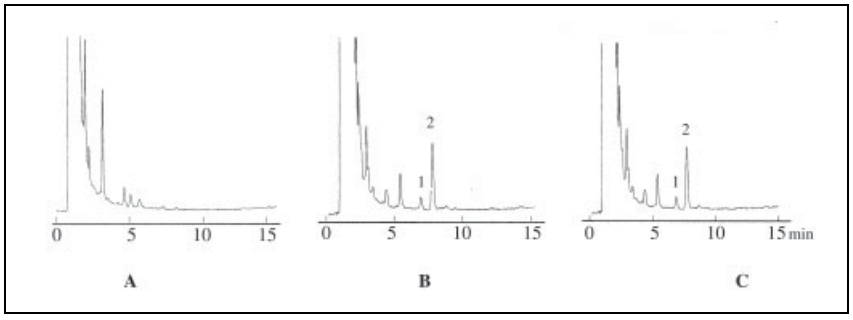

Fig. 1:
HPLC of atorvastatin and indomethacin (I.S.) in beagle dog plasma A– blank plasma, B– blank plasma spiked with atorvastatin and indomethacin, C– plasma sample 1. atorvastatin 2. indomethacin

Table: Within-day and between-day precision and recovery of atorvastatin in plasma

Added concentration (μg/ml)	Precision, RSD(%)		Accuracy (n = 6)
	Within-day (n = 6)	Between-day (n = 6)	Recovery (mean ± SD)
0.1	4.0	5.3	89.5 ± 5.1
1	2.7	3.6	91.5 ± 3.0
2	1.6	2.0	92.9 ± 2.7

atorvastatin, and good linearity was observed over the concentration range from 0.05 to 2.5 μg/mL. The limit of quantification (LOQ) was 25 ng/ml. The limit of detection (LOD) was 8 ng/ml.

The method was evaluated in terms of within-day and between-day precision and recovery (Table). The mean recoveries were 89.69%, 91.53% and 92.89% at concentration of 0.1, 1,0, and 2.0 μg/ml, respectively (n = 6). The mean recovery was 91.4%. The within-day RSD was less than 5% and the between-day RSD was less than 10%.

The HPLC method presented has good reproducibility, is easy to deal with, and needs little time for assay of atorvastatin in biological samples after isolation by protein precipitation with acetonitrile.

2.2. Pharmacokinetic study in beagle dogs

Analysis of atorvastatin in beagle dog plasma samples was performed by HPLC with UV detection following oral administration of 6 mg/kg atorvastatin. Figure 2 shows the atorvastatin concentration-time curve within 24 h in beagle dog plasma after oral administration of atorvastatin SMEDDS (n = 6). Pharmacokinetic parameters and statistical analysis were derived by non-compartmental methods. The mean peak plasma concentration (C_{max}) was 512.98 ± 52.60 ng/ml, and the time of C_{max} (t_{max}) was 1.25 ± 0.38 h. The area under the plasma concentration-time curve ($AUC_{0\rightarrow 24\,h}$) was 2612.96 ± 367.64 ng h/ml estimated by the linear trapezoidal method.

3. Discussion

Several HPLC procedures have been presented for the analysis of atorvastatin and its metabolites in biological fluids based on MS/MS detection (Black et al. 1998; Jemal et al. 1999; Bullen et al. 1999; Jacobson et al. 2000; Fukazawa et al. 2003), while GC/MS assay and enzyme inhibition assay with liquid scintillation counting have also have been reported (Cilla et al. 1996; Shum et al. 1998; Black et al. 1999; Asberg et al. 2001). LC/MS/MS methods are sensitive, with low quantitation limits, but are not applied widely because the equipment is sometimes unavailable. The enzyme inhibition assay is nonspecific, measuring atorvastatin and its active metabolites on the basis of the inhibition of HMG-CoA reductase activity.

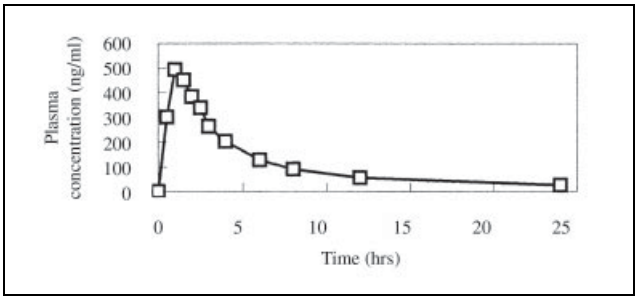

Fig. 2: Plasma concentration profiles of atorvastatin after a single oral administration of atorvastatin SMEDDS in six beagle dogs

Atorvastatin is insoluble in aqueous solution at pH 4 and below, very slightly soluble in water, soluble in acetonitrile and methanol, and at least 98% bound to plasma proteins. Atorvastatin has been reported as being isolated from plasma by liquid-liquid extraction, liquid-solid extraction or protein precipitation. During the extraction of atorvastatin from plasma matrix, these procedures were examined. Based on recovery data, the protein precipitation method was chosen to provide optimal recovery. The precipitation solvents were also compared. Acetonitrile provided good separation of atorvastatin from the plasma matrix. Acetonitrile also minimizes possible ester formation with the atorvastatin molecule compared with methanol. Indomethacin used as the internal standard achieved good baseline separation from atorvastatin, no interference with endogenous sources in plasma matrix, and little chromatography time.

In this paper, we improved a specific HPLC method with UV detection (Prueksaritanont et al. 1999) to determine atorvastatin in beagle plasma after protein precipitation with acetonitrile, and the assay procedure was validated and successfully applied to the pharmacokinetic evaluation of six male healthy beagle dogs following oral administration of atorvastatin SMEDDS.

4. Experimental

4.1. Instrumentation

The HPLC system (Shimadzu Inc., Japan) consisted of an LC-10AD pump, SIL-10A autoinjector and SPD-10A UV detector. Data were collected and analysed by Class LC-10 software (version 1.63, Shimadzu, Japan). The chromatographic column was a Kromasil^{TM} C_8 (150 mm × 4.6 mm, 5 μm) column preceded by a 0.5 μm precolumn filter (Waters, MA, USA). The centrifuge (Sigma, 3K15) was from Sigma Corp. (USA).

4.2. Chemicals and reagents

Atorvastatin was from Honghui Biopharmceutical Inc. Indomethacin was provided by the National Institute for the Control of Pharmaceuticals and Biological Products of China, was used as the internal standard. Ammonium acetate was from Shanghai Reagent Inc. Acetonitrile and methanol were HPLC grade from Burdick & Jackson Co. (Muskegon, MA, USA). The purified water was filtered through a Milli-Q UV-Plus purification system (18MV-cm) from Millipore Inc, Milford, MA, USA. All other chemicals used were of analytical grade. All other solvents used were of HPLC grade. The mobile phase consisted of acetonitrile: 0.1 mol/L ammonium acetate buffer (pH 4.0 adjusted with glacial acetic acid) (65 : 35% v/v) which was filtered through a 0.45 μm membrane filter.

4.3. Animals

Six healthy male beagle dogs (obtained from the Laboratory Animal Center of Fudan University), weighing between 12 and 14 kg were used in the study.

4.4. Study design

The study was conducted in random order with a 7 day washout interval between the doses. Dogs were fasted for 24 h prior to the experiment. Blood samples (3 ml) were collected from limb veins into heparinized tubes at the following times: immediately before administration, and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 h after medication. Blood samples were immediately centrifuged at 3000 rpm for 10 min (4 °C). Plasma samples were collected in cap tubes and stored at −20 °C until assay. Frozen plasma samples were thawed at room temperature just prior to assay.

4.5. Sample preparation

A stock solution of atorvastatin at a concentration of 10 μg/ml was prepared and diluted to the desired concentrations for the working solutions, and stored at 4 °C. A stock solution of indomethacin at a concentration of 0.2 μg/ml was prepared as the internal standard solution, and stored at 4 °C, protected from light.

4.6. Assay

An aliquot of 200 µl indomethacin (0.2 µg/ml in acetonitrile) was added to 0.5 ml plasma samples, then 2 ml ice-cold acetonitrile was added and vigorously vibrated for 2 min to precipitate proteins. After centrifuging at 5000 rpm for 10 min, the supernatant acetonitrile was transferred to a clean tube, and the separated organic mixture was evaporated to near dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 200 µl of 50% acetonitrile (v/v) and a 25 µl fraction was injected for HPLC analysis. The column was at ambient temperature (25 °C). The flow rate was 1.0 mL/min and the detector was set at a wavelength of 270 nm.

4.7. Validation

A good linear relationship was obtained between the peak area ratio of atorvastatin to indomethacin and the concentration of atorvastatin over the range of 0.05 to 2.5 µg/ml. The linear regression of the curve for peak area ratio (Y) versus concentration (X) was $Y = 2.6108\ X + 0.0082$ ($r = 0.9995$). The limit of quantification was 25 ng/ml, and the limit of detection was 8 ng/mL. The within-day and between-day precisions were assayed using six replicates at low, medium, and high concentration, respectively. Precision was characterized by RSD%; the within-day RSD was less than 5% and the between-day RSD was less than 10%. Accuracy was evaluated as recovery and expressed by mean ± SD.

4.8. Pharmacokinetic study

The HPLC method was developed to investigate the plasma profile after oral administration of a single dose of 6 mg/kg atorvastatin SMEDDS in six beagle dogs. A pharmacokinetic study was performed using plasma concentration-time curve data over 24 h. Pharmacokinetic parameters and statistical analysis, derived by non-compartmental analysis, were performed with the WinNonlin Program (Version 4.1, Pharsight Corp. CA, USA). Data from plasma concentration-time curves after drug intake were used to obtain the mean peak plasma concentrations (C_{max}, ng/ml) and time of C_{max} (t_{max}, h). The area under the plasma concentration-time curve ($AUC_{0\rightarrow 24\,h}$) was estimated by use of the linear trapezoidal method. The pharmacokinetic parameters are expressed as mean ± SD.

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