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Development and validation of a liquid chromatography–tandem mass spectrometry method for simultaneous determination of amlodipine, atorvastatin and its metabolites ortho-hydroxy atorvastatin and para-hydroxy atorvastatin in human plasma and its application in a bioequivalence study

Ying Zhou[a], Jie Li[a], Xiaomeng He[a], Mengmeng Jia[a], Mingzhou Liu[b], Huqun Li[a], Zhengjiang Xiong[c], Yue Fan[d], Weiyong Li[a,*]

[a] Institute of Clinical Pharmacy, Union Hospital Affiliated to Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430022, PR China
[b] Wuhan Polytechnic University, Wuhan 430023, PR China
[c] Lunan Pharmaceutical Group Corporation, Linyi 276005, Shandong, PR China
[d] Hubei College of Traditional Chinese Medicine, Wuhan 430065, PR China

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ABSTRACT

A sensitive, simple and rapid high-performance liquid chromatography coupled with positive ion electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS/MS) method was developed for the simultaneous determination of amlodipine, atorvastatin and its metabolites ortho-hydroxy atorvastatin and para-hydroxy atorvastatin in human plasma. The analytes were extracted from human plasma through liquid–liquid extraction method. A mixture of methyl *tert*-butyl ether and ethyl acetate (50:50, v/v) was used as the extractant. The chromatographic separation was achieved on a CAPCELLPAK CR 1:4 (5 μm, 150 mm × 2.0 mm i.d.) column within 6.0 min with the mobile phase consisted of acetonitrile and ammonium acetate buffer (20 mM) containing 0.3% formic acid (50:50, v/v). Data acquisition was carried out in multiple reaction monitoring (MRM) mode. The method was validated and was successfully applied to the bioequivalence study of combination tablets containing AM and AT with coadministered individual drugs in 50 healthy Chinese male volunteers.

1. Introduction

Cardiovascular disease is a multi-factorial disease and cardiovascular risk factors rarely occurred in isolation [1,2], so the combination of risk factors such as hypertension and dyslipidemia may act synergistically to increase the risk of cardiovascular disease events [3]. Clinical guidelines now recognize the importance of a multi-factorial approach to manage cardiovascular risk, which drives the development of the combination of the antihypertensive drug amlodipine besylate and the lipid-lowering medication atorvastatin calcium into a single-pill used currently in many parts of the world [4].

Amlodipine (AM) is a dihydropyridine calcium channel blocker that primarily inhibits calcium ion influx into cardiac and vessel smooth muscle cells, resulting in peripheral arterial vasodilation and a reduction in BP [5]. Atorvastatin (AT) is a selective inhibitor of HMG-CoA reductase, the enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of cholesterol and lipoproteins, thereby reduces the formation of lipids [6]. Both AM and AT have been used in routine clinical practice alone or in combination for many years [4], and the pharmacokinetic properties of the two drugs have been studied as an individual agent in earlier years. Recent studies [7–10] have demonstrated that there is no significant effect on either pharmacokinetic or pharmacodynamic property, indeed, there is some evidence that there might be some pharmacodynamic benefits associated with combining these agents [4].

AT is given in an inactive form of calcium salt and is converted into active atorvastatin acid which is highly soluble and permeable [11]. However, atorvastatin acid is subject to extensive first-pass metabolism in both the gut and liver [11]. Its metabolic pathway is complex, involving cytochrome P450 (CYP450) 3A4-mediated oxidation, β-oxidation, lactonization, hydrolysis and upper gastrointestinal-mediated glucuronidation [12]. CYP3A4 is responsible for the formation of at least two active metabolites [13], ortho-hydroxy atorvastatin (o-AT) which is the dominant

* Corresponding author. Tel.: +86 27 85726063; fax: +86 27 85726685.
E-mail address: lweiyong2002@yahoo.com.cn (W. Li).

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metabolite detected in plasma and para-hydroxy atorvastatin (p-AT) whose plasma concentration is very low [14], which remains a challenge for pharmacokinetic study.

Many analytical methods are available for separate determination of AM or AT as well as simultaneous determination of AT and its active metabolites o-AT and p-AT in individual drugs [11–15]. Recently, as the development of combination tablets, analytical methods like UV spectrophotometric [15], HPLC [16,17], HPTLC [18], LC–MS [14], electrochemical, radioimmunoassay and micellar electro kinetic chromatography [19] have been developed and validated for simultaneous determination of AM and AT in combination tablets. However, so far no method has yet been reported for simultaneous determination of AM, AT, o-AT and p-AT in combination tablets. It is necessary to determine the four compounds simultaneously because of the activities of the metabolites.

In the present study a sensitive, specific, simple and rapid HPLC ESI-MS/MS method was developed for the simultaneous determination of the four agents in human plasma. Advantages of this method included shorter run time, simple sample preparation procedure and expanded low limit of quantitation. It has been fully validated and successfully applied to the bioequivalence study of combination tablets containing AM and AT with coadministered individual drugs in 50 healthy Chinese male volunteers.

2. Experimental

2.1. Chemicals and reagents

Amlodipine besylate standard (purity: 99.4%, Batch No. 100374-200903), atorvastatin calcium standard (purity: 95%, Batch No. 100590-200501) and lansoprazole standard (purity: 99.7%, Batch No.100709-201103) were purchased from National Institutes for Food and Drug Control (Beijing, PR China). Ortho-hydroxy atorvastatin sodium standard (purity: 95%, Batch No. 10-PSB-53-1) was obtained from Synthetic Toronto Chemicals Inc. (North York, Canada). para-Hydroxy atorvastatin calcium salt standard (purity: 98%, Batch No. 1215-058 A1) was obtained from TLC Pharma Chemicals Inc. (Ontario, Canada).

The test drug was combined tablets of amlodipine besylate/atorvastatin calcium (5 mg/10 mg, Batch No. 101201), which was supplied by Shandong New Time Pharmaceutical Co., Ltd. (Linyi, PR China). The reference drugs were co-administered individual drugs of amlodipine besylate (5 mg, Batch No. 1005068) and atorvastatin calcium (10 mg, Batch No. 031610K), which were obtained from Pfizer Limited (New York, USA).

HPLC grade methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Analytic grade formic acid and ammonium acetate were purchased from Guangzhou Dumas (Guangzhou, PR China). Analytic grade methyl *tert*-butyl ether, and ethyl acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, PR China). Ultrapure water was used throughout the study. Blank human plasma was provided by Union Hospital, Tongji Medical College, Huazhong University of Science & Technology (Wuhan, PR China).

2.2. Chromatographic and mass spectrometric conditions

An Agilent 1200 High Performance Liquid Chromatography System (Agilent, Waldbronn, Germany) equipped with quaternary pump, degasser, autosampler and column oven was used in the study. The chromatographic separation was achieved on a CAPCELLPAK CR 1:4 column (5 μm, 150 mm × 2.0 mm i.d., Shimadzu, Kyoto, Japan) at temperature of 30 °C with isocratic elution. The mobile phase consisted of acetonitrile and ammonium acetate buffer (20 mM) containing 0.3% formic acid (50:50, v/v) at a flow rate of 0.45 ml/min. The total run time was within 6.0 min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (Applied Biosystems, Concord, Ontario, Canada). The mass spectrometer was operated in the electro spray ionization mode with positive ion detection in multiple reaction monitoring (MRM) mode. Data acquisition was performed with Analyst 1.5.1 software (Applied Biosystems, Concord, Ontario, Canada).

Table 1
Concentrations of standard solutions for each analyte.

C (ng/ml)	AM	AT	o-AT	p-AT
C1	100	250	100	75
QCH	80	200	80	50
C2	40	75	40	25
C3	15	25	15	8.0
C4(QCM)	5.0	7.5	5.0	3.0
C5	2.0	2.5	1.5	1.0
C6(QCL)	1.0	1.0	0.5	0.4
C7	0.35	0.35	0.20	0.15

2.3. Preparation of stock and working solutions

Primary stock solutions of analytes were prepared by dissolving accurately weighed reference substances in methanol and then diluted with methanol to obtain working solutions with corresponding concentrations, as shown in Table 1. The internal standard (IS, lansoprazole) stock solution was prepared by the same way and was further diluted to a concentration of 50 ng/mL, which was used as IS working solution for all analyses. All solutions were preserved at −20 °C.

2.4. Plasma sample preparation

An aliquot of 0.5 mL plasma sample was mixed with 50 μL of the IS solution (50 ng/mL) and then 5 mL of methyl *tert*-butyl ether-ethyl acetate (50/50, v/v) was added into the mixture. After vortexing for 10 min, the mixture was centrifuged at 3000 rpm/min (4 °C) for another 10 min. The supernatant was evaporated to dryness under nitrogen stream in a water bath at 35 °C. The extract reconstituted with 100 μL of a mixture of methanol-water (50/50, v/v) was vortexed and centrifuged for 2 and 5 min, respectively, and then 80 μL of the supernatant was transferred into autosampler vials.

2.5. Calibration curves and quality control sample preparation

A mixture of AM, AT, o-AT and p-AT (each 50 μL of the corresponding standard solution) was added into a 10 mL plastic tube and then evaporated to dryness under nitrogen stream in a water bath at 35 °C. 500 μL drug-free human plasma was added to obtain samples with concentrations of 35, 100, 200, 500, 1500, 4000 and 10,000 pg/mL for AM, concentrations of 35, 100, 250, 750, 2500, 7500 and 25,000 pg/mL for AT, concentrations of 20, 50, 150, 500, 1500, 4000 and 10,000 pg/mL for o-AT and concentrations of 15, 40, 150, 300, 800, 2500 and 7500 pg/mL for p-AT, respectively. The quality control (QC) samples were prepared in the same way with concentrations of 100, 500 and 8000 pg/mL for AM, concentrations of 100, 750 and 20,000 pg/mL for AT, concentrations of 50, 500 and 8000 pg/mL for o-AT and concentrations of 40, 300 and 5000 pg/mL for p-AT, respectively, and processed according to Section 2.4, then 5 μL of the supernatant was injected for LC–MS/MS analysis.