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Simultaneous determination of atorvastatin, amlodipine, ramipril and benazepril in human plasma by LC-MS/MS and its application to a human pharmacokinetic study

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ABSTRACT: A rapid, simple, sensitive and specific LC-MS/MS method has been developed and validated for the simultaneous estimation of atorvastatin (ATO), amlodipine (AML), ramipril (RAM) and benazepril (BEN) using nevirapine as an internal standard (IS). The API-4000 LC-MS/MS was operated under the multiple-reaction monitoring mode using electrospray ionization. Analytes and IS were extracted from plasma by simple liquid–liquid extraction technique using ethyl acetate. The reconstituted samples were chromatographed on C_{18} column by pumping 0.1% formic acid–acetonitrile (15:85, v/v) at a flow rate of 1 mL/min. A detailed validation of the method was performed as per the FDA guidelines and the standard curves were found to be linear in the range of 0.26–210 ng/mL for ATO; 0.05–20.5 ng/mL for AML; 0.25–208 ng/mL for RAM and 0.74–607 ng/mL for BEN with mean correlation coefficient of ≥0.99 for each analyte. The intra-day and inter-day precision and accuracy results were well with in the acceptable limits. A run time of 2.5 min for each sample made it possible to analyze more than 400 human plasma samples per day. The developed assay method was successfully applied to a pharmacokinetic study in human male volunteers. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: atorvastatin; amlodipine; ramipril; benazepril; LC-MS/MS; method validation; human plasma; pharmacokinetics

Introduction

Hypercholesterolemia control is important for the prevention of coronary artery disease. Currently, 3-hydroxy-3-methylflutaryl-coenzyme A (HMG-CoA) reductase inhibitor is the first-choice therapeutic agent for patients with hypercholesterolemia (Shepherd, 1998; Williams and Feely, 2002; Matsubara *et al*., 2003). Hypercholesterolemia is often accompanied by hypertension, an associated risk factor for coronary artery disease (Sander and Giles, 2002; Kannel, 2000). Calcium channel blockers have been widely used in the treatment of hypertension and/or angina pectoris, and are often prescribed in association with a lipid-lowering agent such as atorvastatin (ATO) (Jukema *et al*., 1996, 1998; Abernethy, 1992). ATO is a synthetic HMG-CoA reductase inhibitor and induces a significant reduction in total cholesterol, low-density lipoproteins cholesterol and plasma triglycerides (Bakker-Arkema *et al*., 1996; Nawrocki *et al*., 1995). Amlodipine (AML) is a potent dihydropyridine calcium antagonist (Abernethy, 1989; Kungys *et al*., 2003) useful in the management of angina pectoris and hypertension (Reid *et al*., 1988). AML and ATO fixed dose combinations have been demonstrated in numerous clinical trails to be highly effective in lowering blood pressure and low-density lipoprotein cholesterol (Blank, 2006; Blank *et al*., 2007; McKeage *et al*., 2008; Preston *et al*., 2007). One such combination available in the market is Caduet® (www.caduet.com), which is a combination of amlodipine and atorvastatin in a single pill.

More than one anti-hypertensive agent is indicated for patients with multiple cardiovascular risk factors such as hypertension (Shikata *et al*., 2007). Combination therapy with calcium channel blockers and angiotensin-converting enzyme inhibitors provides enhanced antihypertensive activity (Chien *et al*., 2005). In 1995, the US Food and Drug Administration approved the use

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Abbreviations used: AML, amlodipine; ATO, atorvastatin; BEN, benazepril; HMG-CoA, 3-hydroxy-3-methylflutaryl-coenzyme A; RAM, ramipiril.

of a capsule formulation of combination amlodipine–benazepril for hypertension. One such preparation available in the market is Lotrel® (www.lotrel.com), which is combination of AML and benazepril (BEN) in a single capsule. Angiotensin-converting enzyme inhibitors and HMG-CoA reductase inhibitors combination therapy is recommended in cardiac syndrome (Pizzi *et al*., 2004). The combination of ATO and ramipril (RAM) formulation is available in the market (Atocor-R). RAM and BEN are prodrug-type angiotensin-converting enzyme inhibitors used in the treatment of hypertension and heart failure. Both the drugs are almost completely converted to their active metabolites ramiprilat and benazeprilat by hydrolytic cleavage of the ester linkage in the liver (Unger *et al*., 1986; Balfour and Goa, 1991; Campbell *et al*., 1993; Warner and Perry, 2002).

To the best of our knowledge no published method is available for the simultaneous quantification of ATO, AML, RAM and BEN in any of the matrices. We felt that this simultaneous estimation method will help the researchers as the four drugs used in this method were available in market with mixed combinations, viz., ATO + AML (Caudet), ATO + RAM (Atocor-R), AML + BEN (Lortel). In the present paper the authors report the development of a simple, rapid and reproducible method to estimate ATO, AML, RAM and BEN concentrations simultaneously in human plasma without compromising the sensitivity reported earlier for each drug. Indeed in the present paper we have achieved a higher sensitivity for AML. This method meets the requirements and provides a high degree of accuracy, sensitivity and specificity by simple liquid–liquid extraction technique using high-performance liquid chromatography and detection by electrospray tandem mass spectrometry. This method will be particularly useful to estimate the concentration of ATO in plasma samples collected from the patients on ATO in combination with AML or RAM or BEN. The application of this assay in a clinical pharmacokinetic study following oral administration of ATO is described.

Experimental

Chemicals and Materials

ATO (purity >94.80%, Fig. 1), AML (purity >99.40%, Fig. 1), RAM (purity >99.84%, Fig. 1) and BEN (purity >99.50%, Fig. 1) were procured from the Neucon Pharma Ltd, Goa, India. Nevirapine (IS) (purity >99.48%, Fig. 1) was purchased from IFPRESS (Indian Foundation for Pharmaceutical Reference Standard Substances). HPLC-grade methanol and acetonitrile, manufactured by J. T Baker, ethyl acetate HPLC-grade, manufactured by Merck Ltd, and formic acid GR-grade and glacial acetic acid GR-grade, manufactured by Merck Ltd, were purchased from Jignesh Agency, Mumbai, India. High-purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore Pvt. Limited, Bangalore, India. Blank K_2EDTA human plasma was obtained from Cauvery Diagnostics and Blood Bank, Secunderabad, India and stored at –20°C prior to use.

HPLC Operating Conditions

A Shimadzu LC-20 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of Shimadzu LC-20 AD HPLC pump, Shimadzu series DGU-20A5 Degasser and a Shimadzu SIL-HTC autosampler was used to inject 10 µL aliquots of the processed samples on a Zorbax-SB C_{18} column (50 × 4.6 mm, 5 µm, Agilent Technologies, California, USA), which was kept at ambient temperature (24 ± 2°C). The isocratic mobile phase, a mixture of 0.1% formic acid–acetonitrile (15:85, v/v) was filtered through a 0.45 µm membrane filter (XI5522050) (Millipore, USA or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 1 mL/min (with splitter 60:40) into the mass spectrometer electrospray ionization chamber.

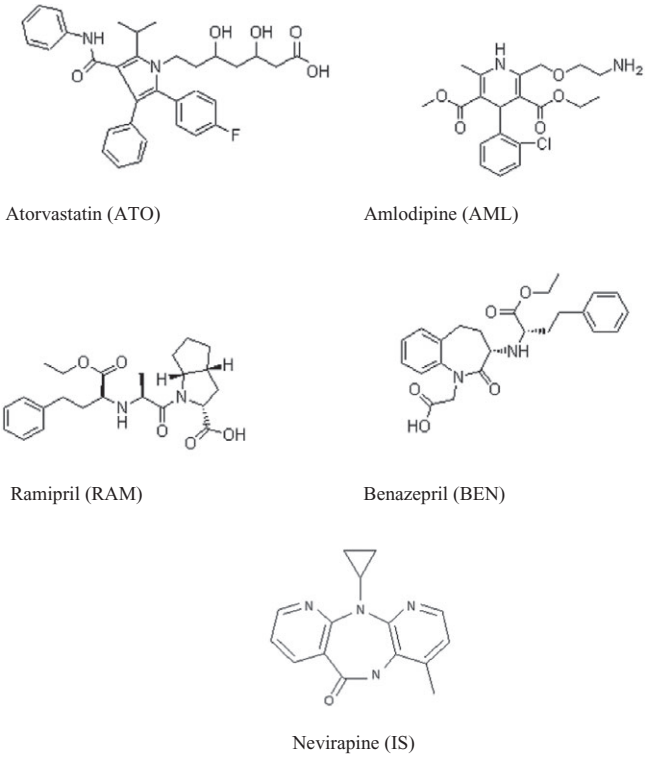

Figure 1. Chemical structures of atorvastatin (ATO), amlodipine (AML), ramipril (RAM), and benazepril (BEN) and nevirapine (IS).

Table 1. Optimized mass spectrometry parameters for analytes and IS

Analyte	DP (V)	CE (V)	CXP (V)
ATO	103	32	5
AML	40	15	5
RAM	73	31	5
BEN	80	32	5
IS	70	35	5

Mass Spectrometry Operating Conditions

Quantitation was achieved by MS/MS detection in positive ion mode for analytes and IS using a MDS Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 450°C. The common parameters, viz. curtain gas, nebulizer gas (GS1), heater gas (GS2) and CAD gas, were set at 15, 35, 30 and 5 psi, respectively. The compound parameters, viz. declustering potential (DP), collision energy (CE) and collision exit potential (CXP), for the analytes and IS are shown in Table 1. The ion voltage spray was set at 5000 V. Quantitation of ATO, AML, RAM, BEN and IS was achieved by monitoring the precursor (Q1)/product (Q3) ions at *m/z* 560.4/441.4, 409.3/238.0, 417.2/234.3, 425.1/351.2 and 267.1/226.1, respectively. The analytical data were processed using Analyst software (version 1.4.2).

Preparation of Stock Solutions of Analytes and IS

Primary stock solutions of ATO, AML, RAM and BEN for preparation of standard and quality control (QC) samples were prepared from separate weighings. Primary stock solutions of ATO (300 µg/mL), AML, RAM and BEN (all at 500 µg/mL) were prepared in methanol. Similarly, the primary stock solution of IS (500 µg/mL) was also prepared in methanol. The stock

solutions of ATO, AML, RAM, BEN and IS were stored at 2–8°C, and were found to be stable for 20 days (data not shown). They were then successively diluted with methanol–water (50:50, v/v) to prepare appropriate working solutions to prepare the calibration curve. Another set of working stock solutions of ATO, AML, RAM and BEN were made in methanol–water (50:50, v/v) (from primary stock) at appropriate dilutions for the preparation of QC samples. Working stock solutions were stored approximately at 2–8°C for a week (data not shown). A working IS solution (1044 ng/mL) was prepared in methanol–water (50:50, v/v). Working solutions of ATO, RAM and BEN were prepared in combination, whereas AML was prepared separately.

Preparation of Calibration Curve Standards and Quality Control Samples

Calibration samples were prepared by spiking 475 µL of control human plasma with the appropriate working solution of the each analyte (12.5 µL combined dilution of ATO, RAM, BEN and 12.5 µL of AML). The calibration curve standard was prepared, consisting of a set of nine non-zero concentrations of 0.26–210 ng/mL for ATO, 0.05–20.5 ng/mL for AML, 0.25–208 ng/mL for RAM and 0.74–607 ng/mL for BEN. Samples for the determination of precision and accuracy were prepared by spiking control human plasma in bulk with ATO, AML, RAM and BEN at appropriate concentrations and 500 µL plasma aliquots were distributed into different tubes. The QCs prepared for each analyte were: for ATR – 0.26 (LLOQ), 0.79 (LQC), 31.6 (MQC1), 105 (MQC2) and 176 ng/mL (HQC); for AML – 0.05 (LLOQ), 0.14 (LQC), 2.79 (MQC1), 9.31 (MQC2) and 18.6 ng/mL (HQC); for RAM – 0.26 (LLOQ), 0.80 (LQC), 31.9 (MQC1), 106 (MQC2) and 177 ng/mL (HQC); and for BEN – 0.76 ng/mL (LLOQ), 2.30 (LQC), 91.8 (MQC1), 306 (MQC2) and 510 ng/mL (HQC). All the samples were stored at –70 ± 5°C.

Recovery

The extraction efficiency of ATO, AML, RAM, BEN and IS extraction from human plasma was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from post-extracted plasma standard sample at equivalent concentrations by liquid–liquid extraction. Recoveries of each analyte were determined at low QC, medium QC and high QC concentrations (ATO: 0.79, 105 and 176 ng/mL; AML: 0.14, 9.31 and 18.6 ng/mL; RAM: 0.80, 106 and 177 ng/mL; and BEN: 2.30, 306 and 510 ng/mL), whereas the recovery of the IS was determined at a single concentration of 1044 ng/mL. The mean overall recoveries of the analytes and IS were determined by comparing the peak areas of extracted plasma standards with the peak areas of post extraction plasma samples spiked at corresponding concentration.

Sample Preparation

A simple liquid–liquid extraction method was followed for extraction of ATO, AML, RAM and BEN from human plasma. To an aliquot of 500 µL plasma, IS solution (25 µL of 1044 ng/mL) and 400 µL of 100 mM sodium acetate adjusted to pH 5.0 ± 0.05 units using glacial acetic acid were added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 3 mL of ethyl acetate, the samples were placed on reciprocating shaker for 10 min at 200 rpm, followed by centrifugation for 5 min at 4000 rpm on a Multifuge 3_{SR} at 4°C (Heraus, Germany). The organic layer (2.4 mL) was separated and evaporated to dryness at 45°C using a gentle stream of nitrogen (Turbovap®, Zymark®, Kopkinton, MA, USA). The residue was reconstituted in 500 µL of the mobile phase and 10 µL was injected onto LC-MS/MS system.

Validation Parameters

A validation according to the FDA guidelines (US DHHS, FDA, CDER, 2001) was performed for the assay in human plasma.

Specificity and selectivity. The specificity of the method was evaluated by analyzing human plasma samples from at least six different lots to investigate the potential interferences at the LC peak region for analytes (ATO, AML, RAM and BEN) and IS.

Matrix effect. Matrix effect is investigated to ensure that precision, selectivity and sensitivity are not compromised by the matrix screened. Matrix effect was checked with the six different lots of EDTA plasma. Three replicates each of LQC and HQC were prepared from different lots of plasma.

Calibration curve. Linearity was assessed by weighted linear regression ($1/x^2$) of each analyte–IS peak area ratio based on five independent calibration curves prepared on each of five separate days using a nine-point calibration curve. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set at ±20% (US DHHS, FDA, CDER, 2001). The calibrators used for each analyte were: ATO – 0.26, 0.51, 10.2, 20.5, 40.9, 81.9, 126, 168 and 210 ng/mL; AML – 0.05, 0.10, 1.00, 2.00, 4.00, 8.01, 12.3, 16.4 and 20.5 ng/mL; RAM – 0.25, 0.51, 10.1, 20.3, 40.5, 81.0, 125, 166 and 208 ng/mL and BEN – 0.74, 1.48, 29.6, 59.1, 118, 237, 364, 485 and 607 ng/mL.

Precision and accuracy. The intra-assay precision and accuracy were estimated by analyzing six replicates containing ATO, AML, RAM and BEN at five different QC levels, viz. LLOQ, LQC, MQC1, MQC2 and HQC in human plasma. The inter-assay precision was determined by analyzing the five-level QC samples on five different runs. The criteria for acceptability of the data included accuracy within ±15% from the nominal values and a precision of within ±15% relative standard deviation (RSD) or CV% except for LLOQ, where it should not exceed ±20%. (US DHHS, FDA, CDER, 2001).

Stability experiments. The stability of ATO, AML, RAM, BEN and IS in the injection solvent was determined periodically by injecting replicate preparations of processed plasma samples for up to 48 h (in the autosampler at 10°C) after the sample loading. The re-injection stability of each analyte was determined by injecting the replicate preparations of processed plasma samples for up to 24 h after the first injection. Stability of each analyte (ATO, AML, RAM and BEN) in plasma during 10 h (bench-top) was determined at ambient temperature (24 ± 2°C) at two concentrations (LQC and HQC) in six replicates. Freezer stability of each analyte in human plasma was assessed by analyzing the LQC and HQC samples stored at –70 ± 5°C for 60 days. The stability of ATO, AML, RAM and BEN in human plasma following four freeze–thaw cycles was also assessed. The samples were stored at –70 ± 5°C between freeze–thaw cycles. The samples were thawed by allowing them to stand (unassisted) at room temperature for ~1.5 h. The samples were then returned to the freezer. The samples were processed using the same procedure as described in the Sample Preparation section. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. ±15%) and precision (i.e. ±15% RSD).

Pharmacokinetic Study

A pharmacokinetic study was performed in healthy ($n = 6$) male subjects. Blood samples were collected following oral administration of 40 mg tablet of ATR at pre dose, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 8, 12, 16, 24 and 48 h, using K_2EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ± 5°C until use. Plasma samples were spiked with IS and processed as described above. Along with clinical samples, QC samples at low, middle 1, middle 2 and high concentrations were assayed in triplicate and were distributed among the unknown samples in the analytical run; not more than 33% of the QC samples were greater than ±15% of the nominal

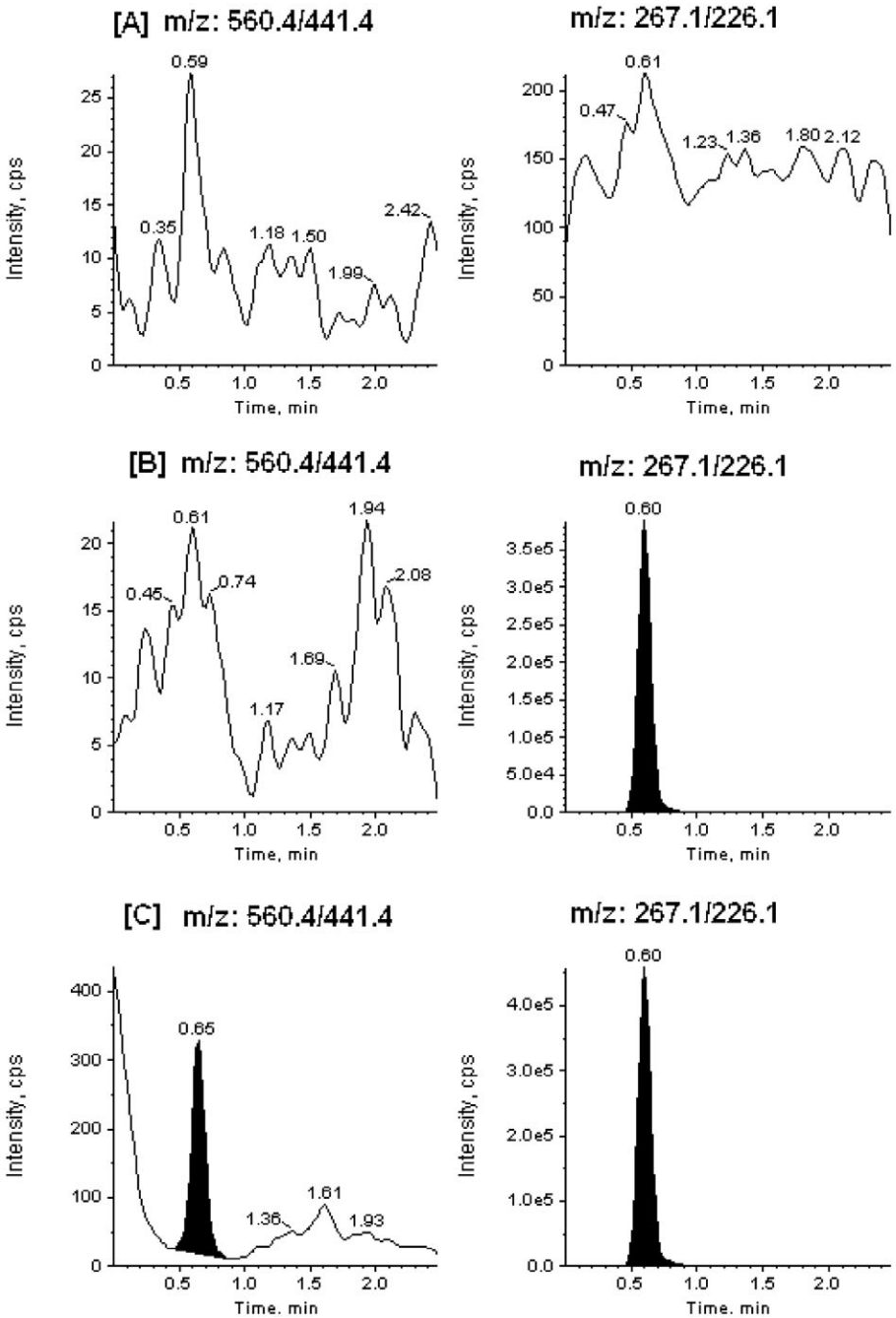

Figure 2. Typical MRM chromatograms of ATO (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS and (C) a 0.5 h plasma sample showing ATO peak obtained following an oral dose of ATO tablet to a healthy volunteer along with IS.

concentration. The plasma concentration–time profile of ATO was analyzed by non-compartmental method using WinNonlin Version 5.2 (Pharsight Corporation, Mountain View, CA, USA).

Results

Mass Spectrometry

To obtain optimum sensitivity and selectivity, ESI technique operated in the positive ion mode was used for the LC-MS/MS multiple reaction monitoring (MRM) analyses. The protonated form of each analyte and IS, $[M + H]^+$ ion, was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain the Q_3 product ion spectra. The compound optimized mass parameters, DP, CE and CXP, are presented in Table 1. The product ion mass spectrum of positively charged ATO (*m/z* 560.4) shows the formation of characteristic product ions at *m/z* 99.4, 175.1, 233.0, 291.1 423.1, 441.4, 467.4 and 543.7 (data not shown). The most sensitive mass transition was monitored from *m/z* 560.4 to 441.4. The product ion mass spectrum of AML (*m/z* 409.3) shows the forma-

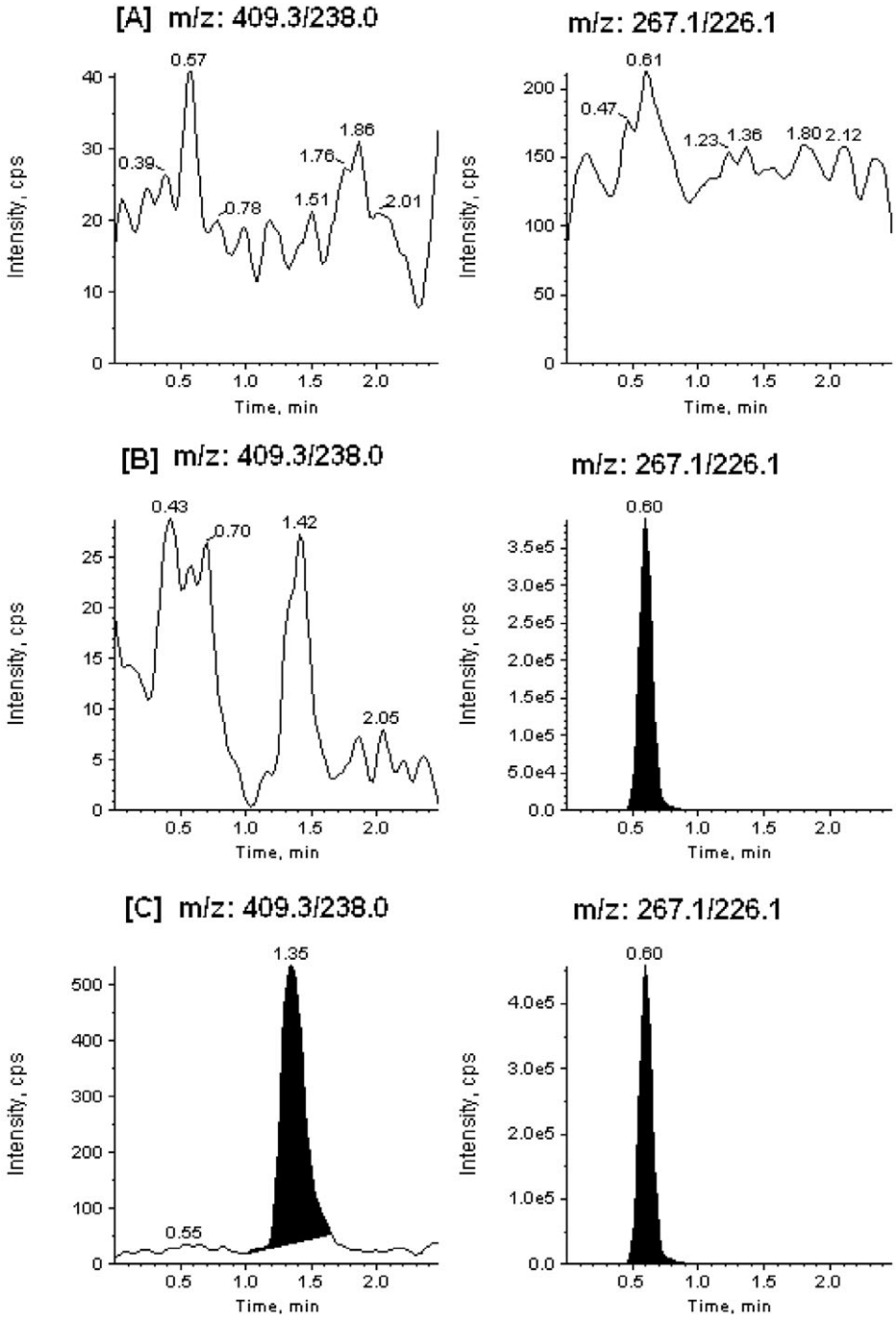

Figure 3. Typical MRM chromatograms of AML (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS and (C) a LLOQ sample along with IS.

tion of characteristic product ions at *m/z* 238.0, 293.9, 377.1 and 392.0 (data not shown) and the most sensitive mass transition was observed from *m/z* 409.3 to 238.0. The product ion mass spectrum of RAM (*m/z* 417.2) shows the formation of characteristic product ions at *m/z* 156.4, 234.3 and 343.1 (data not shown) and the most sensitive mass transition was observed from *m/z* 417.2 to 234.3. The product ion mass spectrum of BEN (*m/z* 425.1) shows the formation of characteristic product ion at *m/z* 146.0, 190.1 and 351.2 (data not shown) and the most sensitive mass transition was observed from *m/z* 425.1 to 351.2. Similarly, the product ion mass spectrum of IS (nevirapine, *m/z* 267.1) shows the formation of characteristic product ions at *m/z* 120.9, 107.2, 144.2, 161.3, 173.0, 211.1, 226.1 and 249.3. The most sensitive mass transition was from *m/z* 267.1 to 226.1, which led to the production of the product ion (data not shown). As the earlier publications have discussed extensively the fragmentation pattern of ATO (Nirogi *et al*., 2006), AML (Massaroti *et al*., 2005), RAM (Yuan *et al*., 2008), BEN (Vonaparti *et al*., 2006) and IS (nevirapine, Chi *et al*., 2003), we are not presenting the data pertaining to this.

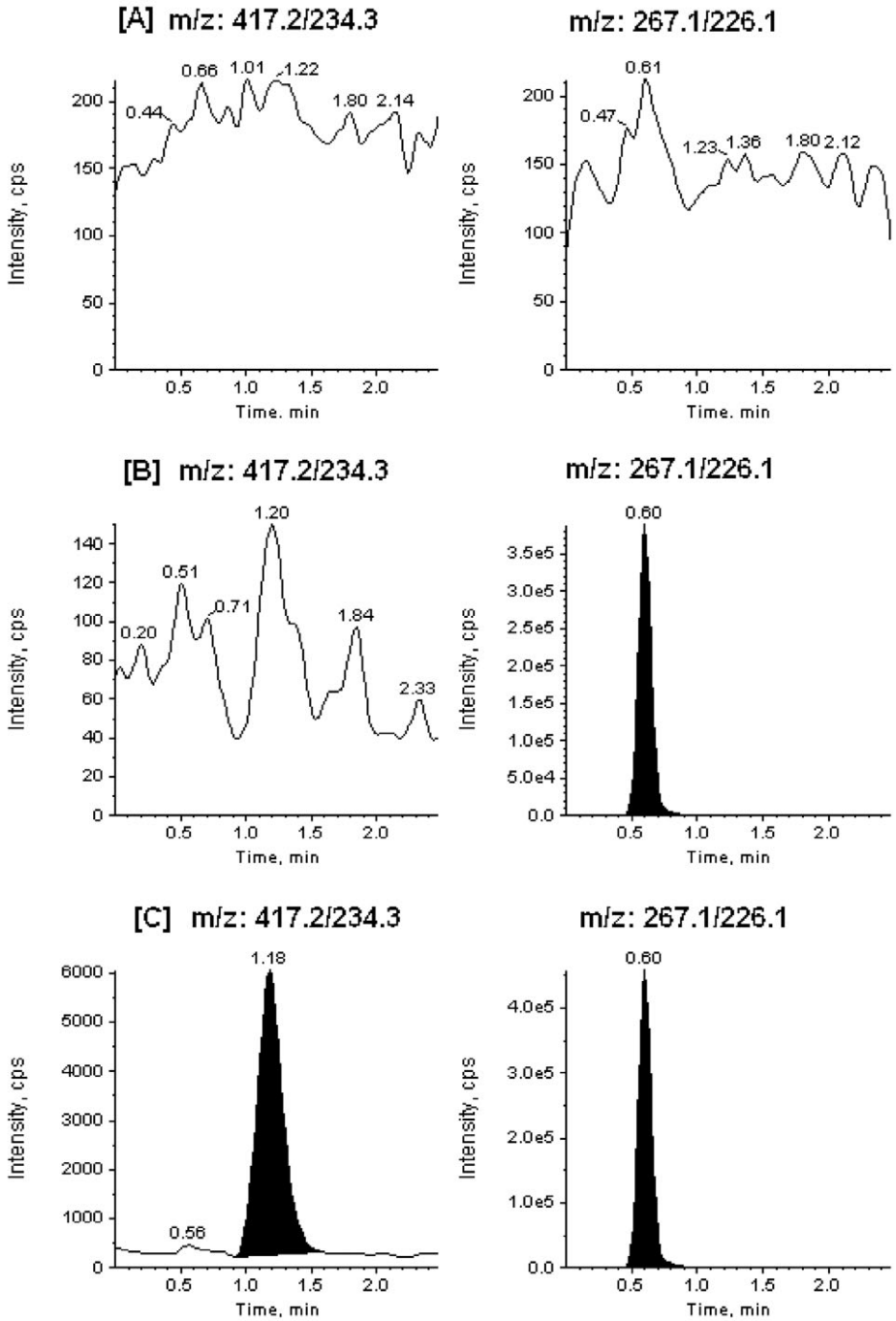

Figure 4. Typical MRM chromatograms of RAM (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS and (C) an LLOQ sample along with IS.

Method Development

Method development starts with the tuning of molecules. MS parameters were tuned in both positive and negative ionization modes for ATO, AML, RAM, BEN and IS. However, a good response was found in positive ionization mode. Data acquisition was performed in MRM mode to obtain more selectivity.

We aimed to develop a simple separation chromatographic method with a short run time. Separation was tried using various combinations of acetonitrile and buffer with varying contents of each component on a variety of columns, such as C_8 and C_{18} of different makes like Chromolith, Hypersil, Hypurity advance, Zorbax, Kromasil and Intertsil. The use of formic acid buffer at concentration of 0.1% helped to achieve a good response for MS detection in the positive ionization mode. To get a good chromatographic separation with the desired response it was observed that mobile phase as well as selection of column is an important criterion. It was found that an isocratic mobile phase system consisting of 0.1% formic acid and acetonitrile (15:85, v/v) could achieve this purpose and was finally adopted as mobile phase. The high proportion of organic solvent (85% of acetonitrile) eluted ATO, AML, RAM, BEN and IS at retention times of

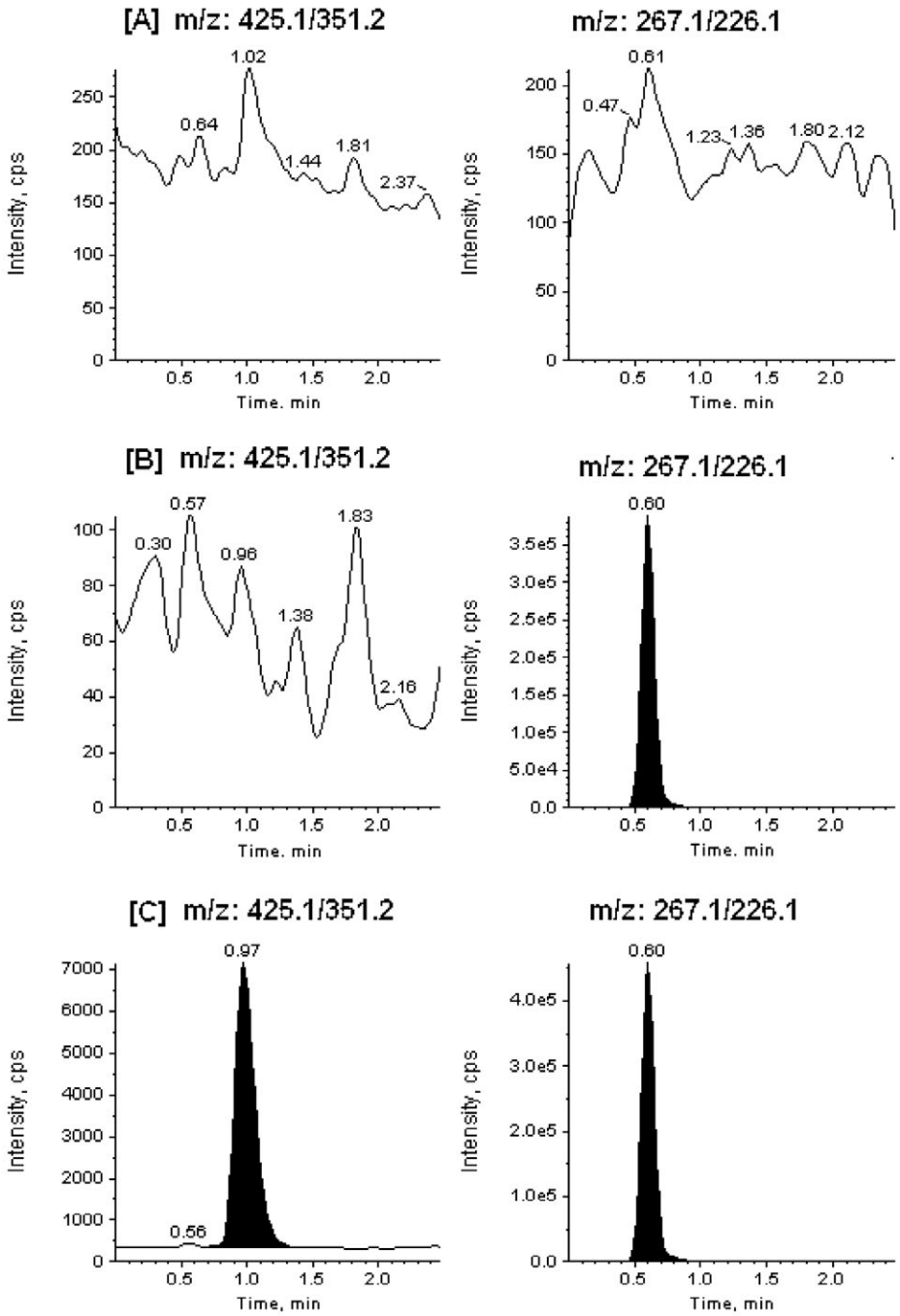

Figure 5. Typical MRM chromatograms of BEN (left panel) and IS (right panel) in (A) human blank plasma, (B) human plasma spiked with IS and (C) a LLOQ sample along with IS.

about 0.65, 1.35, 1.20, 1.00 and 0.60 min, respectively. A flow rate of 1 mL/min produced good peak shapes and permitted a run time of only 2.5 min. A good IS must mimic the analytes during extraction and compensate for any analyte on the column, especially with LC-MS/MS, where matrix effects can lead to poor analytical results. In the initial stages of this work, several compounds were investigated to find a suitable IS and finally nevirapine was found to be best for the present purpose. Consistent extraction efficiency using liquid–liquid extraction technique was found for all the analytes and selected IS.

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system and also minimized the experimental cost. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS-MS. Several solvents were checked alone and in combination; ethyl acetate was found to be optimal, which can produce a clean chromatogram for a blank sample and yields good, reproducible recovery for analytes from the plasma.

Selectivity and Chromatography

The degree of interference by endogeneous plasma constituents with analytes and IS was assessed by inspection of chromato-

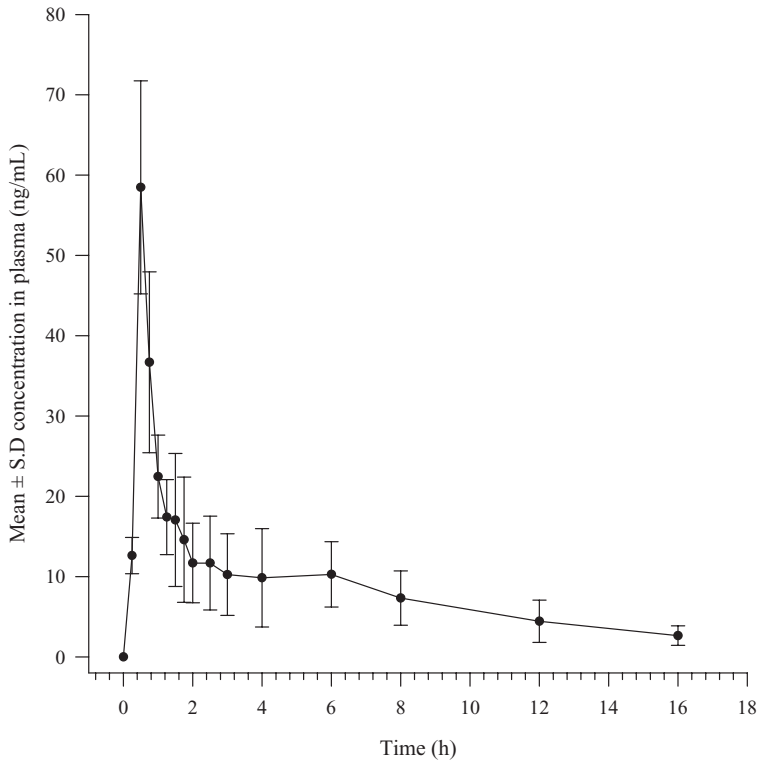

Figure 6. Time–plasma concentration (mean ± SD) profile of ATO in healthy human volunteers following oral dosing of ATO tablet (40 mg).

grams derived from processed blank plasma sample. As shown in Figs 2–5, no significant interferences in the blank human plasma traces were found from endogeneous components in drug-free human plasma at the retention times of the analytes and IS.

Sensitivity

The lowest limit of reliable quantification for analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 11.9 and 103%; 5.19 and 108%; 9.76 and 110%; and 8.58 and 89.8% for ATO, AML, RAM and BEN, respectively.

Extraction Efficiency

A simple LLE with ethyl acetate proved to be robust and provided the cleanest samples. The recoveries of analytes and IS were good and reproducible. The mean overall recoveries (with the precision range) of ATO, AML, RAM and BEN were 67.1 ± 4.19% (1.81–6.66%), 53.6 ± 3.05% (1.14–7.58%), 70.0 ± 2.88% (4.33–7.56%) and 74.8 ± 4.23% (2.41–5.11%), respectively. The recovery of IS was 79.0 ± 3.28% with the precision range of 4.08–5.08%.

Matrix Effect

No significant matrix effect was observed in all six batches of human plasma for the analytes at LQC and HQC concentrations. The precision and accuracy for ATO, AML, RAM and BEN at LQC concentration were found to be 5.72 and 92.8%; 5.26 and 101%; 7.13 and 94.4%; 7.17 and 102%, respectively. Similarly, the precision and accuracy for ATO, AML, RAM and BEN at HQC concentration were found to be 5.86 and 98.8%; 6.77 and 100%; 7.24 and 99.3%; 5.38 and 95.9%, respectively.

Linearity

After comparing the two weighting models, $1/x$ and $1/x^2$, a regression equation with a weighting factor of $1/x^2$ of drug to IS concentration was found to produce the best fit for the concentration–detector response relationship for all the analytes in human plasma. By using the recommended $1/x^2$ model, values for correlation coefficient (r^2) were obtained which indicate linearity over the whole calibration range for all the analytes. Also, the mean value of r^2 was greater than 0.99 in the concentration ranges of 0.26–210, 0.05–20.5, 0.25–208 and 0.74–607 ng/mL for ATO, AML, RAM and BEN, respectively.

Precision and Accuracy

Accuracy and precision data for intra- and inter-day plasma samples for ATO, AML, RAM and BEN are presented in Table 2. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

Dilution Integrity

The upper concentration limits can be extended to 367 ng/mL for ATO; 36.7 ng/mL for AML; 363 ng/mL for RAM and 1062 ng/mL for BEN by a 1:2 or 1:4 dilution with screened human blank plasma. The mean back-calculated concentrations for 1:2 and 1:4 dilution samples were within 85–115% of their nominal value. The coefficients of variation (%CV) for 1:2 and 1:4 dilution samples were less than 10% for all the analytes.

Table 2. Intra- and inter-day precision of determination of ATO, AML, RAM and BEN in human plasma

Quality control	Run	Measured concentration of ATO (ng/mL)				Measured concentration of AML (ng/mL)				Measured concentration of RAM (ng/mL)				Measured concentration of BEN (ng/mL)			
		Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)
Intra-day variation (six replicates at each concentration)																	
LLOQ[a]	1	0.28	0.02	8.88	107	0.05	0.00	8.03	98.7	0.23	0.02	8.79	89.1	0.74	0.03	4.21	97.1
	2	0.27	0.02	8.17	102	0.04	0.00	4.48	87.8	0.23	0.03	12.5	88.1	0.74	0.04	5.11	97.0
	3	0.29	0.03	8.85	111	0.05	0.00	7.48	97.5	0.31	0.02	7.76	118	0.81	0.06	7.84	107
	4	0.24	0.02	8.08	90.9	0.05	0.00	5.69	107	0.28	0.02	5.65	105	0.70	0.08	10.7	92.9
	5	0.23	0.02	7.05	90.1	0.05	0.00	10.1	96.5	0.27	0.02	7.32	102	0.80	0.11	13.2	105
LQC[b]	1	0.82	0.04	4.81	104	0.15	0.01	6.81	108	0.84	0.09	10.8	106	2.40	0.33	13.6	105
	2	0.71	0.05	6.76	90.2	0.15	0.02	10.4	106	0.84	0.06	7.17	106	2.24	0.10	4.45	97.4
	3	0.81	0.04	5.54	102	0.16	0.02	11.3	113	0.83	0.08	9.89	104	2.44	0.30	12.1	106
	4	0.89	0.09	9.55	113	0.14	0.01	10.7	98.3	0.77	0.03	3.39	97.0	2.61	0.32	12.3	114
	5	0.86	0.07	8.61	108	0.15	0.01	5.76	108	0.84	0.08	9.23	106	2.51	0.28	11.0	109
MQC1[c]	1	33.1	1.76	5.32	105	2.77	0.19	6.92	99.2	29.2	2.46	8.44	91.5	95.1	6.59	6.92	104
	2	30.4	2.37	7.79	96.0	2.61	0.16	5.99	93.5	29.6	2.35	7.95	92.8	95.7	3.53	3.69	104
	3	28.5	2.37	8.31	90.1	2.64	0.25	9.36	94.4	34.2	1.35	3.96	107	95.1	4.79	5.03	104
	4	29.0	1.60	5.52	91.8	2.93	0.07	2.55	105	31.9	1.21	3.78	100	95.2	9.01	9.46	104
	5	33.2	2.38	7.16	105	2.56	0.19	7.53	91.8	33.0	3.44	10.4	104	102	4.81	4.71	111
MQC2[d]	1	106	7.71	7.28	100	10.1	0.77	7.65	109	94.3	9.66	10.2	88.7	311	13.8	4.45	102
	2	108	6.03	5.59	102	9.04	0.44	4.85	97.1	105	7.96	7.61	98.4	307	9.58	3.12	100
	3	116	6.67	5.75	110	9.87	0.54	5.51	106	115	5.18	4.51	108	296	19.0	6.39	96.8
	4	96.1	4.71	4.90	91.1	10.4	0.55	5.24	112	95.2	3.30	3.47	89.5	313	17.1	5.48	102
	5	113	11.8	10.5	107	8.73	0.44	5.01	93.7	96.2	4.15	4.31	90.5	305	22.4	7.34	99.7
HQC[e]	1	165	8.77	5.33	93.7	19.6	2.23	11.4	105	177	11.1	6.27	100	508	7.54	1.49	99.5
	2	187	6.75	3.60	107	19.5	0.89	4.57	105	174	10.4	5.97	98.0	486	20.2	4.16	95.3
	3	176	4.93	2.80	100	77.7	1.00	5.68	94.9	179	16.5	9.21	101	512	36.2	7.07	100
	4	159	11.6	7.30	90.5	18.3	0.67	3.66	98.1	185	9.19	4.97	105	529	18.0	3.40	104
	5	185	5.68	3.06	106	18.3	0.85	4.64	98.5	172	9.99	5.82	96.9	505	17.2	3.41	98.9
Inter-day variation (30 replicates at each concentration)																	
LLOQ		0.26	0.03	11.6	100	0.05	0.00	9.43	97.5	0.26	0.04	13.8	101	0.76	0.08	10.0	99.9
LQC		0.82	0.08	10.2	104	0.15	0.01	9.74	106	0.83	0.07	8.67	104	2.44	0.29	11.7	106
MQC1		30.8	2.81	9.13	97.5	2.70	0.22	8.00	96.7	31.6	2.92	9.24	99.1	96.7	6.30	6.52	105
MQC2		108	10.0	9.31	102	9.64	0.84	8.72	104	101	9.96	9.87	95.0	307	16.8	5.49	100
HQC		175	13.5	7.75	99.3	18.7	1.40	7.49	100	117	11.9	6.70	100	508	24.7	4.87	99.6

[a] LLOQ for ATO, AML, RAM and BEN is 0.26, 0.05, 0.26 and 0.76 ng/mL, respectively.
[b] LQC for ATO, AML, RAM and BEN is 0.79, 0.14, 0.80 and 2.30 ng/mL, respectively.
[c] MQC1 for ATO, AML, RAM and BEN is 31.6, 2.79, 31.9 and 91.8 ng/mL, respectively.
[d] MQC2 for ATO, AML, RAM and BEN is 105, 9.31, 106 and 306 ng/mL, respectively.
[e] HQC for ATO, AML, RAM and BEN is 176, 18.6, 177 and 510 ng/mL, respectively.

Table 3. Stability data of ATO, AML, RAM and BEN quality controls in human plasma

QC	Stability	Measured concentration of ATO (ng/mL)				Measured concentration of AML (ng/mL)				Measured concentration of RAM (ng/mL)				Measured concentration of BEN (ng/mL)			
		Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)
LQC[a]	In injector (48 h)	0.83	0.08	9.36	105	0.15	0.01	9.79	105	0.73	0.09	12.3	92.0	2.28	0.26	11.3	99.2
	Bench top (10 h)	0.83	0.09	11.4	105	0.13	0.02	12.3	94.1	0.82	0.09	10.9	103	2.43	0.34	13.8	106
	Fourth freeze–thaw	0.76	0.08	11.1	96.5	0.14	0.02	11.4	99.3	0.74	0.08	11.4	92.6	2.16	0.22	9.98	94.0
	Re-injector (24 h)	0.72	0.03	4.48	90.6	0.15	0.02	10.9	110	0.85	0.05	6.43	107	2.32	0.21	9.13	101
	60 days at –70°C	0.80	0.11	13.7	101	0.14	0.01	10.8	98.9	0.75	0.06	8.44	93.8	2.47	0.27	10.9	108
HQC[b]	In injector (48 h)	171	4.07	2.38	97.4	17.6	1.51	8.58	94.5	179	13.1	7.29	101	488	23.6	4.84	95.7
	Bench-top (10 h)	171	20.0	11.7	97.3	18.3	1.73	9.48	98.1	192	11.6	6.07	108	519	19.6	3.77	102
	Fourth freeze–thaw	170	15.1	8.88	96.5	18.0	1.38	7.65	96.9	185	5.36	2.90	104	496	23.4	4.72	97.3
	Re-injector (24 h)	189	6.98	3.70	107	19.9	1.69	8.49	107	175	9.41	5.38	98.7	494	20.8	4.22	96.9
	60 days at –70°C	170	14.2	8.37	96.8	20.2	1.88	9.30	109	166	11.2	6.76	93.6	502	7.54	1.50	98.3

[a] LQC for ATO, AML, RAM and BEN is 0.79, 0.14, 0.80 and 2.30 ng/mL, respectively.
[b] HQC for ATO, AML, RAM and BEN is 176, 18.6, 177 and 510 ng./mL, respectively.

Stability Studies

The stability studies of four analytes (ATO, AML, RAM and BEN) in human plasma over four freeze–thaw cycles indicate that the analytes are stable in human plasma, when stored at below –70 ± 5°C and thawed at room temperature. Results of bench-top (10 h), autosampler (48 h), re-injection stability (24 h) and freeze–thaw stability are presented in Table 3. The long-term stability of the analytes in human plasma stored for a period of 60 days at –70 ± 5°C showed reliable stability behavior. The results of the tested samples were within the acceptance criteria.

Pharmacokinetic Study

In order to verify the sensitivity and selectivity of this method in a real-world situation, the present method was used to test for ATO in human plasma samples collected from healthy male volunteers ($n = 6$). The mean plasma concentrations vs time profiles of atorvastatin are shown in Fig. 6. The maximum concentration in plasma (C_{max}) 58.7 ± 12.8 was achieved at 0.54 ± 0.10 (T_{max}). The area under the plasma concentration–time curve from zero hour to infinity ($AUC_{0-\infty}$) and half-life ($t_{1/2}$) for ATO were found to be 163 ± 59.7 and 4.93 ± 0.50, respectively.

Discussion

So far there have been no published methods available for the simultaneous quantification of ATO, AML, RAM and BEN in any of the matrices. To the best of knowledge, this is the first time all four analytes have been estimated simultaneously in any matrix without compromising the reported sensitivity of each analyte. The validated method is simple, rugged and rapid due to utilization of a short run time of 2.5 min for each sample analysis. The method uses single IS with a very simple sample preparation method using ethyl acetate (LLE). The cost-effectiveness, simplicity of the assay and shorter run time make it an attractive procedure in high-throughput bioanalysis of ATO, AML, RAM and BEN.

Conclusions

In summary, we have developed and validated a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantify ATO, AML, RAM and BEN simultaneously using single IS. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioequivalence studies and routine therapeutic drug monitoring with desired precision and accuracy. The established method was successfully applied to an atorvastatin human pharmacokinetic study.

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