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Rapid liquid chromatographic -tandem mass spectrometric method for the quantification of pravastatin in human plasma

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ABSTRACT

A simple, rapid and sensitive liquid chromatography / tandem mass spectrometry method was developed and validated for the quantification of pravastatin in human plasma. Topiramate was used as an internal standard. The analytes were extracted from human plasma samples by liquid-liquid extraction technique. The reconstituted samples were chromatographed on a C_{18} column by using a 90:10, v/v mixture of acetonitrile and 5 mM ammonium acetate as the mobile phase at a flow rate of 1.0 mL/min. The calibration curve obtained was linear ($r \geq 0.99$) over the concentration range of 0.20-499.04 ng/mL. The results of the intra- and inter-day precision and accuracy studies were well within the acceptable limits. A run time of 1.5 min for each sample made it possible to analyze more than 400 plasma samples per day. The proposed method was found to be applicable to clinical studies.

Keywords: Pravastatin in human plasma, Liquid-liquid extraction, Liquid chromatography-tandem mass spectrometry, Pharmacokinetics.

INTRODUCTION

Pravastatin, hexahydro-6-hydroxy-2-methyl-8- (2-methylbutyryloxy)-1-naphthyl)-3, 5-dihydroxyheptanoate (Fig. 1), a competitive inhibitor of 3-hydroxy -3- methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis and is widely used to treat hypercholesterolemia [1, 2]. Its pharmacological action is due to the increase of low density lipoprotein receptors on hepatocytes as a consequence of decreased synthesis of

mevalonic acid by inhibition of HMG-CoA reductase, leading to and enhanced hepatic uptake of the plasma low density lipoprotein and to a decreased plasma cholesterol level. Since hyperlipidemia is a major contributing cause to the development of coronary heart disease, pravastatin has also been used to reduce the cardiovascular events [3], such as atherosclerosis and myocardial infarction, diabetes mellitus [4] and renal diseases [5].

In a few publications quantification of pravastatin with LC-MS is described [6–10], all these reported methods are based on solid phase extraction techniques which is more laborious, time consuming and required more skills. Mulvana et.al [6] reported liquid chromatographic – electrospray ionization tandem mass spectrometric (LC-MS/MS) method for the determination of pravastatin and its biotransformation products in serum. Zhu and Neirinck [7] reported a method with plasma concentration range of 0.25-300 ng/mL using 1.0 mL plasma sample volume. This method includes drying and reconstitution in 100 µL with an injection volume of 10 µL onto LC-MS/MS. This makes absolute on-column pravastatin loading. Kawabata et.al [8] reported an LC-MS/MS with APCI probe for the estimation pravastatin and its main metabolite R-416 using processing plasma volume of 1.0 mL, which is high and on-column loading, is again very high compared to the present work. Jain et al. [9] and Deng et.al [10] reported an LC-MS/MS method. This includes sensitivity not adequate for pharmacokinetic study and chromatographic run time was more than 3 min.

In the present investigation, we have developed a method having a shorter run time with simple liquid-liquid extraction technique. The following are the advantages of the proposed method over those reported earlier: (1) Because of the use of less plasma volume (250 µL), the volume of the sample to be collected per time point from an individual during the study is reduced significantly. This allows inclusion of additional points; (2) Employing a single step liquid-liquid extraction procedure minimizes the chances of errors, saves considerable time and simplifies the sample preparation procedure; (3) Greater sensitivity is achieved even with low plasma volumes and the method is well suited for pharmacokinetic analysis; (4) The rapid sample turnaround time of 1.5 min makes it an attractive procedure in high-throughput bioanalysis of pravastatin.

MATERIALS AND METHODS

Chemicals and reagents

Pravastatin reference standard (purity: 97.80%) was obtained from Ranbaxy Laboratories Limited (New Delhi, India). Topiramate (purity: 99.20%) was employed as an internal standard (IS) obtained from Neucon Pharma Limited, (Goa, India). Chemical structures are presented in Fig. 1. Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Acetonitrile and methanol were of HPLC grade and purchased from Merck (Darmstadt, Germany). Analytical grade ammonium acetate, formic acid and ethyl acetate were from Merck (Mumbai, India). The control human plasma sample was procured from Cauvery Diagnostics and Blood Bank (Secunderabad, India).

LC-MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Thermo Hypurity Advance C_{18} column (50 mm x 4.6 mm, 5 µm), a binary LC-20AD prominence pump, an auto sampler (SIL-HTc) and a solvent degasser (DGU-20A_3) was used for the study. Aliquot of 20 µL of the

processed samples were injected into the column, which was kept at 30 ºC. An isocratic mobile phase consisting of a mixture of acetonitrile-5mM ammonium acetate buffer (90:10, v/v) was used to separate the analyte from the endogenous components and delivered at a flow rate of 1.0 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS-MS detection in negative ion mode for the analyte and the internal standard using an MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 ºC. The ion spray voltage was set at 5000 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 40, 30, 20, and 10 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were –55, –45, –10, –7 V for pravastatin and –60, –50, –10, –10 V for topiramate. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 423.2 precursor ion to the *m/z* 101.0 product ion for pravastatin and *m/z* 338.0 precursor ion to the *m/z* 77.8 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data obtained were processed by Analyst software™ (version 1.4.2).

Preparation of plasma standards and quality controls

Standard stock solution of pravastatin and IS (1 mg/mL) was prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water-methanol (40:60, v/v; diluent). The IS working solution (0.3 µg/mL) was prepared by diluting its stock solution with diluent.

The above working solutions (50 µL) were added to drug-free plasma (950 µL) as a bulk, to obtain pravastatin concentration levels of 0.20, 0.40, 1.99, 9.96, 49.82, 103.80, 259.50, 399.23 and 499.04 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 0.20 (LLOQ), 0.60 (low), 207.76 (middle) and 415.53 ng/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at –70ºC until analyses.

Sample processing

A 250-µL volume of plasma sample was transferred to a 15-mL glass test tube, and then 25 µL of IS working solution (0.30 µg/mL) was spiked. Then 10µL of 10% formic acid was added. After vortexing for 30 s, 4-mL aliquot of extraction solvent, ethyl acetate was added using Dispensette Organic (Brand GmbH, Wertheim, Germany). The sample was shaken-mixed for 10 min using reciprocating shaker (Orbitek, Scigenics Biotech, Chennai, India) and centrifuged for 5 min at 3000 rpm using Heraeus Megafuse 2.0R, Japan. The organic layer (3.0 mL) was transferred to a 5-mL glass tube and evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40ºC under a stream of nitrogen. Then the dried extract was reconstituted in 500 µL of mobile phase and a 20-µL aliquot was injected into the chromatographic system.

Bioanalytical method validation

The validation of the above method was carried out as per US FDA guidelines [11]. The parameters determined were selectivity, specificity, matrix effect, linearity, precision, accuracy, recovery, stability and dilution integrity.

RESULTS AND DISCUSSION

Method development

Mass parameters were tuned in both positive and negative ionization modes for the analyte. Good response was found in negative ionization mode. The most sensitive mass transition was from m/z 423.2 to 101.0 for pravastatin and from m/z 338.0 to 77.8 for the IS. Data of the MRM mode was considered to get better selectivity. The MRM state file parameters were optimized to maximize the response for the analyte. The product ion mass spectra of pravastatin and IS were presented in the Figs 1A and 1B, respectively.

Separation was attempted using various combinations of acetonitrile and buffer with varying contents of each component on different columns like C_8 and C_{18} of different make like Chromolith, Hypersil, Hypurity advance, Zorbax, Kromasil and Intertsil etc. Use of a buffer (ammonium acetate) helped in achieving good response for MS detection in the negative ionization mode. A mobile phase consisting of acetonitrile and 5 mM ammonium acetate (90:10, v/v) was found suitable for the analyte and IS. Thermo Hypurity Advance C_{18} column (50 mm x 4.6 mm, 5 µm) gave a good peak shape and response even at LLOQ level. The high proportion of organic solvent coeluted both analyte and the IS at retention time of 0.70 min. A flow-rate of 1.0 mL/min produced a good peak shape and permitted a runtime to 1.5 min.

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 analyses. Ethyl acetate was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the analyte from the plasma.

For an LC-MS/MS analysis, utilization of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. Isotope-labeled analyte was not obtainable to serve as IS, so, in the initial stages of this work, several compounds were investigated to find a suitable IS and finally topiramate was found to be best for the present purpose.

Linearity

The nine-point calibration curve was found to be linear over the concentration range of 0.20–499.04 ng/mL for pravastatin. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration-detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99.

Selectivity and chromatography
The selectivity of the method was examined by analyzing blank human plasma extract (Fig. 2A) and a blank plasma sample spiked with the IS (Fig. 2B). As shown in Fig. 2A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte and IS. Similarly, Fig. 2B shows the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 2C depicts a representative ion-chromatogram for the LLOQ sample (0.20 ng/mL).

Sensitivity
The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ. The precision and accuracy of analyte at LLOQ concentration was found to be 4.57% and 100.2%, respectively.

Matrix effect
No significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations. The precision and accuracy for pravastatin at LQC concentration was found to be 1.94% and 97.7%, and at HQC level they was found to be 3.73% and 98.3%, respectively.

Precision and accuracy
As shown in Table 1, the precision and accuracy of analyte in the intra-day and inter-day runs were within ± 15% at LQC, MQC1, MQC2 and HQC concentrations and within ± 20% at LLOQ QCs.

Extraction efficiency
Six replicates at low, medium and high quality control concentration for pravastatin was prepared for recovery determination. The mean overall recovery of pravastatin was 71.3± 2.16% with a precision of 3.03%. The recovery of IS was 83.0% with the precision range of 3.03-4.90%. Recoveries of the analyte and IS were high and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high throughput bioanalysis.

Dilution integrity
The upper concentration limit of pravastatin can be extended to 898.27 ng/mL for by 1/2 and 1/4 dilutions 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%.

Stability studies
In the different stability experiments carried out viz. bench top stability (15 h), autosampler stability (72 h), repeated freeze-thaw cycles (3 cycles), reinjection stability (48 h), wet extract stability (24 h at 2-8 °C) and long term stability at –70 °C for 60 days the mean % nominal values of the analyte was found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). Thus, the results were found to be within the acceptable limits during the entire validation.

Stock solutions of pravastatin and IS were found to be stable for 30 days at 2-8 °C. The percentage stability (with the precision range) of pravastatin and IS were 104.4% (3.74-4.13%) and 95.6% (3.91-4.51%), respectively.

Table 1. Precision and accuracy of the LC-MS/MS method for determining pravastatin concentrations in plasma samples

Concentration added (ng/ mL)	Intra-day precision and accuracy (*n*=12; 6 from each batch)			Inter-day precision and accuracy (*n*=30; 6 from each batch)		
	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD; ng/mL)	Precision (%)	Accuracy (%)
0.20	0.20 ± 0.02	10.97	97.2	0.20 ± 0.02	11.46	96.5
0.60	0.63 ± 0.06	8.92	104.6	0.63 ± 0.05	8.49	104.4
207.76	216.59 ± 17.57	8.11	104.2	208.44 ± 19.76	9.48	100.3
415.53	418.82 ± 20.64	4.93	100.8	417.42 ± 31.04	7.44	100.5

Table 2. Stability of pravastatin in human plasma

Sample concentration (ng/mL; n = 6)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Bench top stability for 15 h			
0.60	0.55 ± 0.04	7.68	91.0
415.53	366.97 ± 25.63	6.98	88.3
Three freeze-thaw cycles			
0.60	0.60 ± 0.05	8.92	100.5
415.53	446.08 ± 34.41	7.71	107.4
Autosampler stability for 72 h			
0.60	0.54 ± 0.04	7.55	90.2
415.53	375.91 ± 31.75	8.45	90.5
Long term stability for 60 days at -70°C			
0.60	0.57 ± 0.05	8.00	94.7
415.53	440.10 ± 32.00	7.27	105.9
Reinjection stability for 48 h			
0.60	0.66 ± 0.03	4.38	109.3
415.53	450.90 ± 28.80	6.39	108.5
Wet extract stability for 24 at 2-8 °C			
0.60	0.54 ± 0.04	8.12	89.6
415.53	364.78 ± 26.67	7.31	87.8

Application

In order to verify the sensitivity and selectivity of this method in a real-time situation, the present method was used to test for pravastatin in human plasma samples collected from healthy male volunteers (n = 6). Blood samples were collected following oral administration of 40 mg tablet of pravastatin at pre-dose and 0.17, 0.33, 0.5, 0.66, 0.83, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 8, 10, 12, 16 and 24 h, in EDTA 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 °C till their use. The mean plasma concentration vs time profile of pravastatin is shown in Fig. 3. Following oral administration, the maximum concentration (C_{max}) in plasma (210.1±71.18 ng/mL) was attained at 0.92±0.09 h (t_{max}). The plasma concentration–time curve from zero hour to the last measurable concentration (AUC_{0-t}) and area under the plasma

concentration–time curve from zero hour to infinity ($AUC_{0\text{-}inf}$) for pravastatin were 372.1±83.3 and 375.6±82.9 ng h/mL, respectively. The terminal half-life ($t_{½}$) was found to be 1.97±0.36 h. The results confirm that the assay is suitable for clinical pharmacokinetic studies of pravastatin.

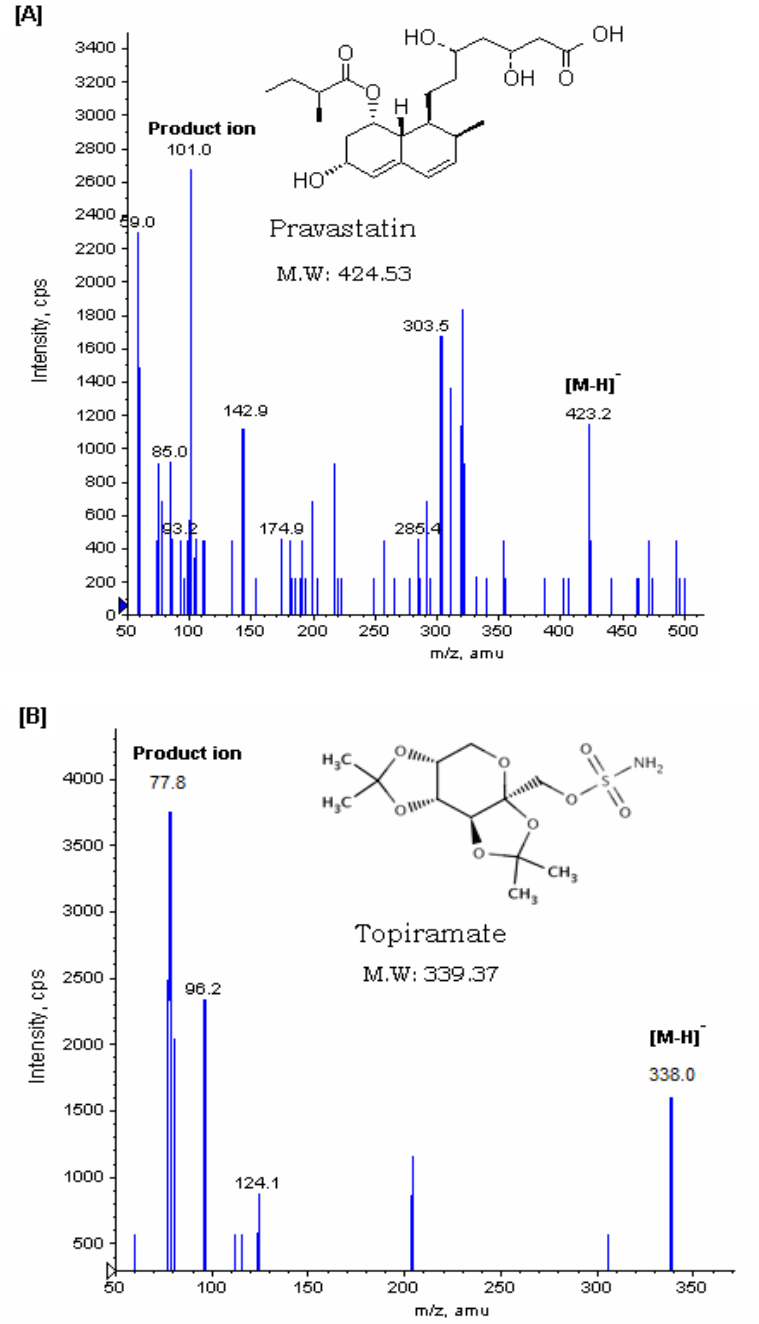

Fig. 1. Full scan negative mode product ion mass spectrum of [A] pravastatin and [B] topiramate (IS).

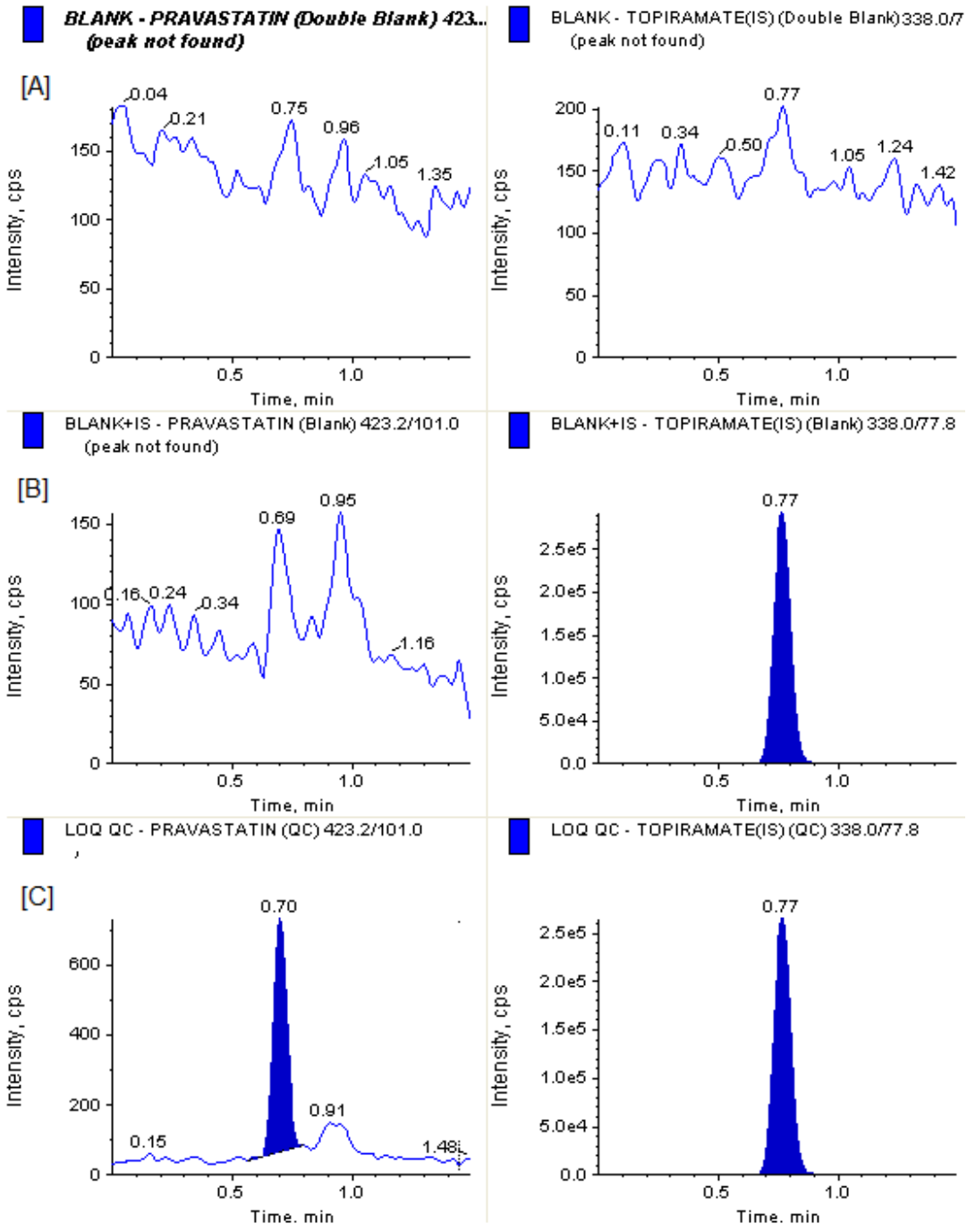

Fig. 2. Typical MRM chromatograms of pravastatin (left panel) and IS (right panel) in (A) human blank plasma (B) human plasma spiked with IS (C) a LLOQ sample along with IS.

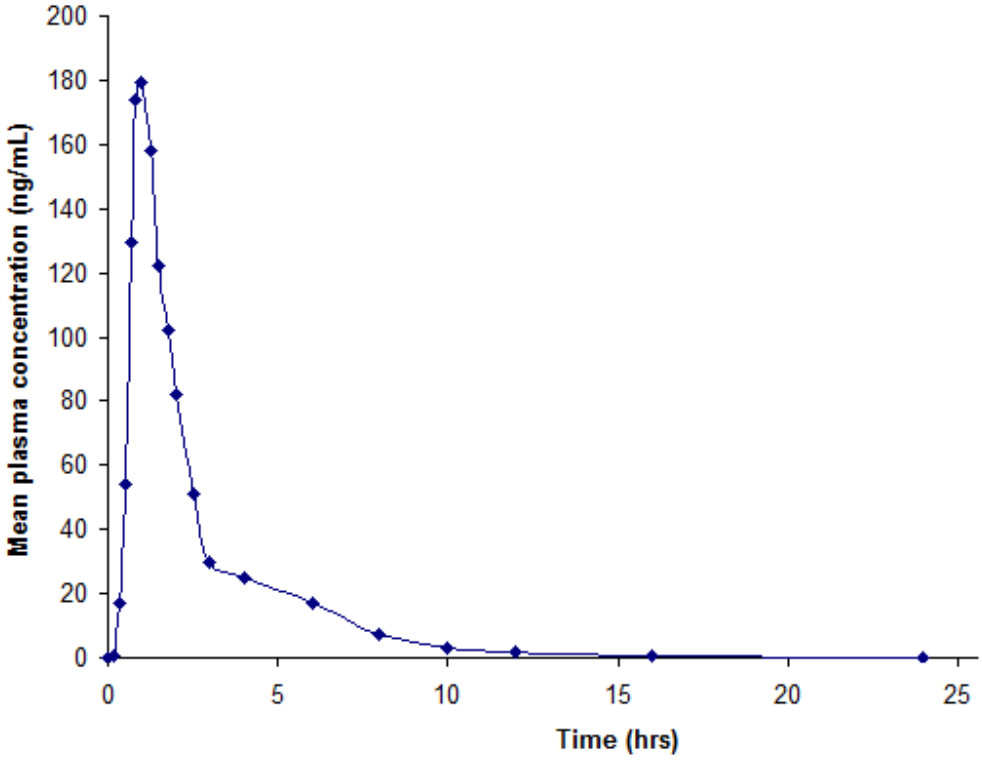

Fig. 3. Concentration-time profile of pravastatin in human plasma after a single oral dose of 40 mg

CONCLUSION

In summary, the method is described for the quantification of pravastatin from human plasma by LC-MS/MS in negative ionization mode using multiple reaction monitoring. The current method has shown acceptable precision and adequate sensitivity for the quantification of pravastatin in human plasma samples obtained for preliminary pharmacokinetic studies. The method described is simple, rapid, sensitive, selective and fully validated according to commonly accepted criteria [11]. The cost-effectiveness, simplicity of the assay and using rapid liquid-liquid extraction, and sample turnover rate of 1.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of pravastatin.

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