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High-performance liquid chromatography coupled with negative ion tandem mass spectrometry for determination of pravastatin in human plasma

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Abstract

A new method, using high-performance liquid chromatography/ion electrospray (negative ion) mass spectrometry, has been developed for the determination of a hydrophilic liver-specific inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, pravastatin in human plasma. In this method, plasma samples were prepared by a solid-phase extraction on C_{18} Bond Elut cartridge. Chromatography was carried out with a Zorbax C_8 column. Simple isocratic chromatography conditions were used. The method has been validated in a linear range of 0.25–300 ng/ml with a coefficient of variation of 0.6–3.4%. The overall recovery was 90.5% for pravastatin and 90.8% for the internal standard β-hydroxy-lovastatin. The method is simple and reliable with a total run time of less than 2 min.

Keywords: Pravastatin

1. Introduction

Pravastatin is one of a class of lipid-lowering compounds, the HMG-CoA reductase inhibitors, which reduce cholesterol biosynthesis. These agents are competitive inhibitors of 3-hydroxy-3-methylglutaryl-co-enzyme a (HMG-CoA) reductase, the enzyme catalyzing the conversion of HMG-CoA to mevalonate an early rate-limiting step in cholesterol biosynthesis. A number of HMG-CoA reductase inhibitors have been introduced into clinical therapy since the introduction of the first substance, lovastatin. These compounds are used for the treatment of hypercholesterolemia. Many pharmacokinetic studies have been performed and different HMG-CoA reductase inhibitors have been compared. Pravastatin is characterized as one of the best, due to the hydroxyl group attached to its decalin ring, which results in a greater hydrophilicity than other HMG-CoA reductase inhibitors [1–3].

Several high-performance liquid chromatography methods have been published [4–7]. These methods enable the detection of pravastatin in human plasma at a level of 1–5 ng/ml. A method using high-performance liquid chromatography (HPLC)/atmospheric pressure chemical ionization mass spectrometry [8] and an HPLC/ion electrospray tandem mass spectrometry method [9] were published as

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well. These methods allow the detection of pravastatin in plasma at a lower level (0.5–0.625 ng/ml). However, due to lower doses of pravastatin in clinical studies, lower plasma concentrations are expected. In a typical single dose 40-mg dose study, after 16 h, the pravastatin level in plasma is expected to be below 0.5 ng/ml. Therefore, it is necessary to have a sensitive method to detect the lower concentrations of pravastatin in plasma. The objective of this work was to develop a method with a lower detection limit, a simple sample preparation procedure and a short run time. This would allow us to perform high throughput routine analysis of plasma samples.

2. Experiments

2.1. Materials and reagents

Pravastatin was in-house supply. The internal standard, lovastatin hydroxy acid ammonium salt was obtained from MDS (Montreal, QC, Canada). Acetonitrile, methanol, ammonium acetate, ammonium formate and acetic acid were purchased from Fisher (Nepean, ON, Canada). Formic acid was obtained from BDH (Toronto, ON, Canada). The solid-phase extraction cartridges (Bond-Elut, C_{18}, 3 cc/100 mg) were purchased from Varian Canada (Mississauga, ON, Canada). The analytical column (Zorbax XDB C_8, 50×2.1 mm) was purchased from Chromatography Specialties (Mississauga, ON, Canada). HPLC grade water was obtained from an in-house Nano-pure water purification system.

Drug-free human plasma used for the preparation of calibration standards and quality control samples was purchased from Biological Specialties (Colmar, PA, USA). All plasma samples were stored at −20 °C prior to use.

2.2. Solutions and calibration standards preparation

The stock solutions of pravastatin and the internal standard β-hydroxy-lovastatin were prepared separately in methanol at a concentration of 100 μg/ml, and the stock solutions were stored at −20 °C. The HPLC mobile phase was prepared by mixing two volumes of acetonitrile and one volume of 1 m*M* ammonium formate and the pH was adjusted to 3.3.

A seven-point standard curve of pravastatin ranging from 0.25 to 300 ng/ml was prepared by adding appropriate amounts of pravastatin into drug-free human plasma. Quality control samples were prepared in the same manner as the standards but at three concentration levels that contained 0.75, 5 or 150 ng/ml of pravastatin. The spiking solutions for both standards and quality control samples were prepared in acetonitrile/water (90:10) solution. The spiking solutions were prepared daily. The standards and quality control samples were prepared in a pool and transferred in aliquots into polypropylene tubes after the preparation, then stored at −20 °C in a freezer until use. Drug-free human plasma sample was screened prior to spiking to ensure it was free of endogenous interference at the retention times of pravastatin and the internal standard. A weighted linear regression was used for quantitation in this study.

2.3. Sample preparation

The solid-phase extraction cartridge (Bond Elut C_{18}) was conditioned with 1 ml of acetonitrile and 1 ml of 50 m*M* ammonium formate buffer prior to the sample loading. The plasma samples were prepared by mixing 1-ml plasma samples with 100 μl of internal standard solution that contained 200 ng/ml β-hydroxy-lovastatin and 1 ml 50 m*M* ammonium formate buffer. The sample mixture was loaded on to the conditioned cartridge and then washed with 2 ml of de-ionized water and 2 ml of methanol/water (1:4). The cartridge was then eluted with 1 ml of elution solution. The eluent was dried under nitrogen and then reconstituted with 100 μl of reconsititution solution which consisted of acetonitrile and 1 m*M* ammonium acetate at 1:1 ratio; 10 μl of reconstituted sample was injected for analysis.

2.4. Instrumentation

Chromatographic separation was carried out on an Agilent 1100 HPLC. Chromatography was achieved with isocratic conditions using a flow-rate of 0.210

ml/min. The column temperature was set at 45 °C. The auto-sampler was set at 4 °C due to the stability of pravastatin. Under these conditions, the pravastatin eluted at 0.8 min and the internal standard eluted at 1.3 min. The total run time for each sample analysis was 1.8 min.

Mass spectra were obtained with a Sciex API 2000 mass spectrometer (Concord, Canada) equipped with a turbo ion-spray source. The data were acquired with Analyst software, version 1.1. Ions were monitored in a negative mode. The mass ion-pair measured was 423.0→101.2 for pravastatin and 421.0→101.2 for the internal standard. Quantification of pravastatin in human plasma was based on the peak area ratios of pravastatin versus the internal standard.

2.5. Validation

The method has been validated for accuracy, precision, selectivity, linearity, recovery and stability. The accuracy was measured by replicate analysis of samples containing known amounts of pravastatin. The intra-assay precision and accuracy were determined by analyzing six replicates of the LOQ and quality control samples at each level from the same batch. The inter-assay precision and accuracy were measured by analyzing the quality control samples that were tested on five different occasions. The inter-assay and intra-assay precision and accuracy were evaluated using back calculated concentrations.

Selectivity was determined by analyzing blank samples from different donors to test for interference with the retention time of pravastatin and the internal standard.

The recovery of pravastatin and the internal standard was determined by comparing the response of the peak area of extracted analytes with the peak area response of the solutions that were prepared at the same concentration.

As part of the validation, extracted plasma sample stability, bench top stability, in-process stability,

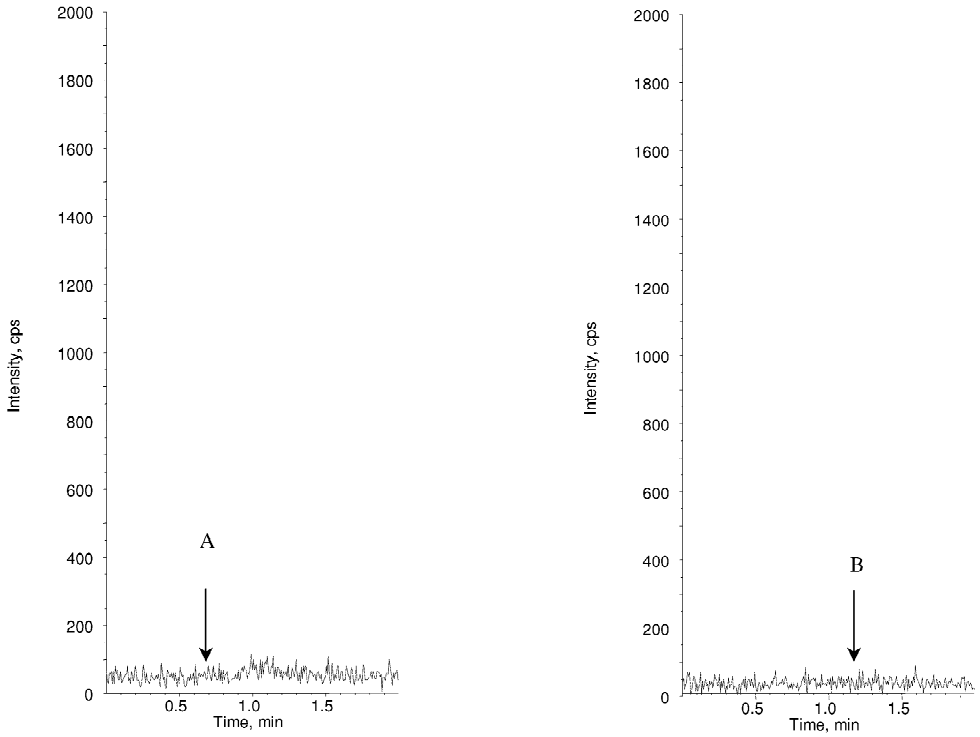

Fig. 1. Representative chromatogram of extracted blank plasma sample.

freeze–thaw stability and frozen plasma sample stability were evaluated. All stability evaluations were based on back-calculated concentrations.

3. Results and discussion

The isocratic conditions used in this method allowed the analysis time to be less than 2 min. Typical chromatograms of an extracted drug-free human plasma sample, an extracted plasma sample containing 0.25 ng/ml of pravastatin (lowest standard), and an extracted plasma sample containing 150 ng/ml pravastatin (highest quality control sample) are presented in Figs. 1–3. The signal/noise ratio of the LOQ was around 6.

The method was validated with five inter-assays and one intra-assay for testing the linearity, precision and accuracy. The results proved that the method was linear in the range of 0.25–300 ng/ml. The average correlation coefficient was 0.9987. During this validation, six different sources of drug-free human plasma samples were screened and no endogenous interference was observed at the retention times of pravastatin and the internal standard. Seven-point calibration standards were prepared and analyzed on five different days. The C.V.% of calibration standards were in the range of 0.6–3.4%. The detailed results of the calibration samples are presented in Table 1. The inter-assay precision and accuracy were determined by analysis of data from quality control samples that were extracted on five different occasions. The C.V.% obtained was 4.1–5.3% as indicated in Table 2. The intra-assay precision of the method was assessed with six replicates of LOQ and quality control samples at three different concentration levels. The results for intra-assay precision and accuracy are listed in Table 3. For the quality control samples at concentrations of 0.75, 5 and 150 ng/ml, intra-assay precision was between −2.2 and 3.2%. The intra-assay precision for samples at the LOQ level (0.25 ng/ml) was 1.9%.

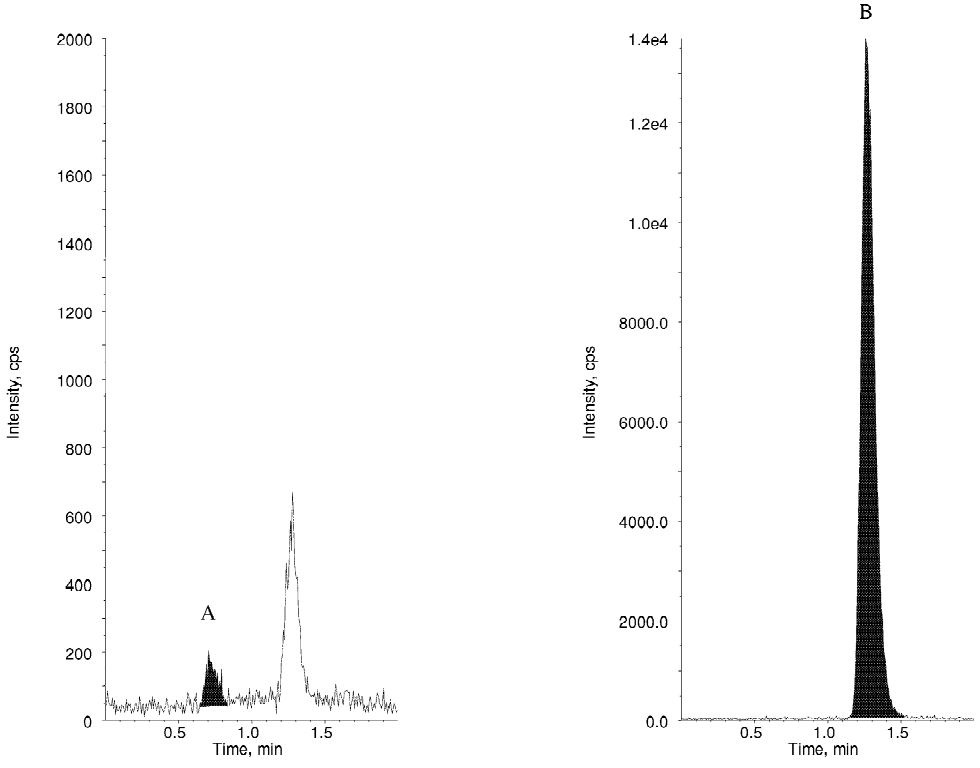

Fig. 2. Representative chromatogram of extracted plasma sample (LOQ).

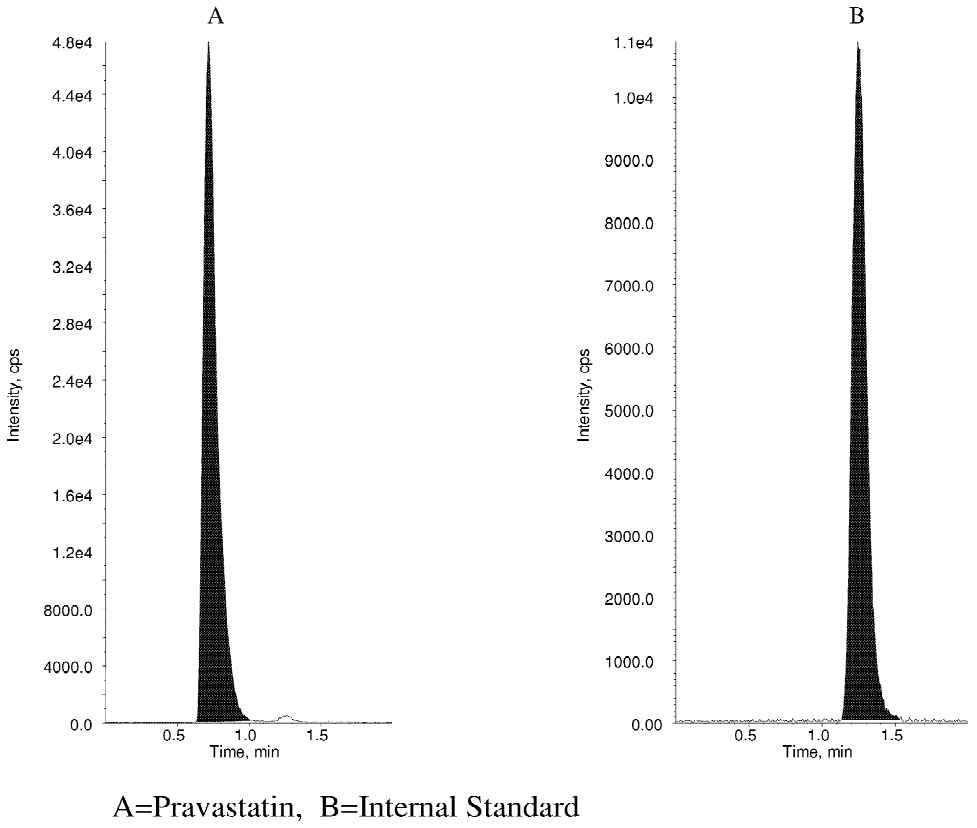

Fig. 3. Representative chromatogram of extracted plasma sample (high QC). (A) Pravastatin; B, internal standard.

The recovery of pravastatin and the internal standard were evaluated by comparing the peak area response of extracted analyte with that of the reference quality control solution at the same concentration levels and reconstituted into blank plasma extracts. Six replicates of quality control samples at

Table 1
Summary of pravastatin calibration standards

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E. (%)	C.V. (%)	*n*
0.25	0.245±0.005	−1.9	1.8	5
0.5	0.516±0.02	3.2	3.4	5
2	2.06±0.01	2.3	0.6	5
10	9.90±0.28	−1.0	2.9	5
50	49.4±1.05	−1.2	2.1	5
200	201.5±4.05	0.7	2.0	5
300	293.5±6.23	−2.2	2.1	5

R.E., relative error; C.V., coefficient of variation.

Table 2
Inter-assay precision of pravastatin in human plasma

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E. (%)	C.V. (%)	*n*
0.75	0.748±0.04	−0.3	5.3	18
5	4.74±0.19	−5.3	4.1	18
150	144.9±6.1	−3.4	4.2	18

R.E., relative error; C.V., coefficient of variation.

Table 3
Intra-assay precision of pravastatin in human plasma

Concentration added (ng/ml)	Concentration found (ng/ml)	R.E. (%)	C.V. (%)	*n*
0.25	0.255±0.004	1.9	1.6	6
0.75	0.733±0.04	−2.3	5.9	6
5.0	4.6±0.22	−7.9	4.8	6
150	147.1±7.0	−1.9	4.8	6

R.E., relative error; C.V., coefficient of variation.

Table 4
Extraction yield of pravastatin and internal standard

Description	% Recovery	C.V. (%)	*n*
Low QC (0.75 ng/ml)	89.8±4.7	4.8	6
Mid QC (5 ng/ml)	88.9±3.7	3.8	6
High QC (150 ng/ml)	92.7±3.9	3.8	6
Internal standard	90.8±1.9	2.0	18

C.V., coefficient of variation.

0.75, 5 and 150 ng/ml were prepared for recovery determination and the overall recovery of pravastatin was 90.5% while the recovery of internal standard (β-hydroxy-lovasatin) was 90.8%. The recovery results are given in Table 4.

The stability of extracted pravastatin and internal standard in the mobile phase (processed sample stability) was tested and it was concluded that the processed samples are stable at 4 °C for at least 70 h. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0) with the samples that were re-injected 70 h after sitting in the auto-sampler at 4 °C. Evaluation was based on back-calculated concentrations. The extracted sample stability data are presented in Table 5.

The freeze–thaw stability was evaluated by comparing the stability of samples that had been frozen and thawed three times, with plasma samples that were thawed only once. Results demonstrated that pravastatin in human plasma was stable for at least three freeze–thaw cycles. Plasma sample stability at room temperature (2 h) and in-process sample stability (after addition of internal standard and buffer then placed at room temperature for 2 h) were tested as well and the results showed that plasma samples containing pravastatin are stable under the conditions described. Bench top and in-process stability results are listed in Table 5. The freeze–thaw stability results are presented in Table 6.

Frozen plasma sample stability evaluation involved an analysis of the low, mid and high range quality control samples that were stored at −70 °C for 62 days, together with a freshly spiked calibration standard, and quality control samples. The peak height ratio of pravastatin/internal standard was used for the evaluation. Less than 4% difference was observed under the conditions described which indicated that human plasma samples containing pravastatin are stable after 62 days of storage at −70 °C. The details of the results are presented in Table 5. The pravastatin and internal standard stock solution stability was also tested. Results show that both solutions are stable for 8 weeks at −20 °C.

As part of routine laboratory procedure, the system suitability was tested prior to the validation. The plasma matrix effect was tested as well. The results

Table 5
Stability of pravastatin in human plasma

		70 h extracted sample stability		62 days stability stored at −20 °C		2 h stability at room temperature		2 h in-process stability at room temperature	
		Back calculated conc. (ng/ml)	Diff. (%)	Back calculated conc. (ng/ml)	Diff. (%)	Back calculated conc. (ng/ml)	Diff. (%)	Back calculated conc. (ng/ml)	Diff. (%)
Low QC 0.75 ng/ml	Comparison sample (*t*=0 h) *n*=3	0.741±0.016	–	0.760±0.019	–	0.735±0.038	–	0.747±0.027	–
	Stability sample *n*=3	0.755±0.014	1.9	0.724±0.025	−4.7	0.743±0.000	1.1	0.739±0.027	−1.1
Mid QC 5.0 ng/ml	Comparison sample (*t*=0 h) *n*=3	4.74±0.04	–	4.79±0.06	–	4.89±0.04	–	4.74±0.21	–
	Stability sample *n*=3	4.71±0.07	−0.6	4.72±0.26	1.4	4.93±0.08	0.8	4.75±0.08	0.2
High QC 150 ng/ml	Comparison sample (*t*=0 h) *n*=3	142.2±0.6	–	139.0±4.1	–	145.2±1.7	–	140.9±2.8	–
	Stability sample *n*=3	143.5±1.1	0.9	141.3±4.1	1.5	147.2±2.6	1.4	147.8±1.5	4.6

Table 6
Pravastatin freeze–thaw stability in human plasma

		Back-calculated concentration (ng/ml)	Mean (ng/ml)	C.V.%	Difference (%)
Low QC (0.75 ng/ml)	Freshly thawed sample	0.823 0.733 0.780	0.779±0.037	4.7	–
	Freeze–thaw 3rd cycle	0.792 0.770 0.739	0.767±0.022	2.8	−1.5
Mid QC (5.0 ng/ml)	Freshly thawed sample	4.95 4.70 4.74	4.80±0.11	2.3	–
	Freeze–thaw 3rd cycle	4.55 4.55 4.80	4.63±0.12	2.5	−3.3
High QC (150 ng/ml)	Freshly thawed sample	153.5 150.4 146.3	150.1±2.9	2.0	–
	Freeze–thaw 3rd cycle	145.1 143.2 153.8	147.4±4.6	3.1	−1.8

of plasma samples from six different donors indicated no significant difference (<3%) in the peak area between plasma samples for both pravastatin and internal standard. We had also tested ion suppression, by comparing the peak area count of pravastatin and the internal standard prepared in the mobile phase with that prepared in plasma extract; 20% of ion suppression was noted for pravastatin and no suppression was noted for the internal standard.

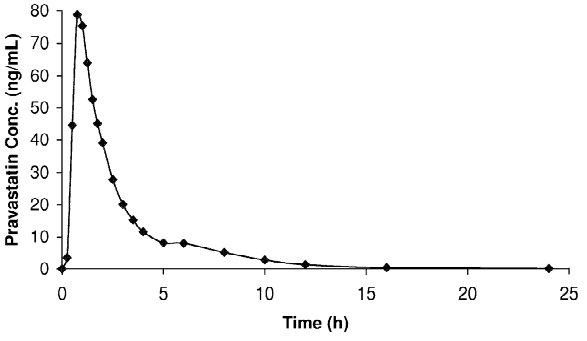

Fig. 4. Average plasma concentration of pravastatin following 40-mg oral dose to healthy human volunteers.

This method has been applied to a bioavailability study involving 18 healthy volunteers. The blood samples were collected from the volunteers over a 24-h period following an oral dose of 40 mg pravastatin. The plasma samples from the study were stored at −20 °C until analysis. The average pravastatin plasma concentration versus sampling time profile is presented in Fig. 4. The results show that after a single oral dose of 40 mg pravastatin, the average C_{max} is about 90 ng/ml and T_{max} is around 0.75 h.

4. Conclusions

A sensitive and accurate analytical method for the determination of pravasatin in human plasma is described in this paper. This method uses high-performance liquid chromatography coupled with a negative ion tandem mass spectrometry (LC–MS–MS) technique that allows the detection of pravastatin at a very low level. The method consists of a simple sample preparation procedure, and isocratic chromatography conditions. The precision and ac-

curacy of this method were demonstrated in the validation data. The method provides excellent specificity and sensitivity with a total run time of less than 2 min. The method was very sensitive (limit of quantitation at 0.25 ng/ml) and it had excellent reproducibility. In addition, the method is simple and easy to operate which is ideal for high throughput analysis.

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