Asian Journal of Chemistry; Vol. 24, No. 2 (2012), 584-588

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Ultra Performance Liquid Chromatography Determination of Pravastatin Sodium in Erythrocytes

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(*Received*: 27 January 2011; *Accepted*: 7 October 2011) AJC-10486

This work comprised a novel, rapid, accurate, precise and specific ultra performance liquid chromatography method for pravastatin determination in erythrocytes. Pravastatin sodium was extracted from pravastatin loaded erythrocytes by liquid/ liquid extraction. The method was performed on acquity® UPLC system, acquity® UPLC BEH C_{18} column (2.1 mm × 50 mm, 1.7 µm). The mobile phase was a mixture of 0.25 % formic acid in distilled water and acetonitrile (65:35, v/v), with flow rate of 0.5 mL/min and injection volume was 1 µL. Photodiode array (PDA) detector was set to acquire 3D data from 210 to 280 nm while the 2D channel was recording at 237 nm, the column temperature was kept at 50 ºC while sample temperature was kept ambient. Method was validated and found to be linear, selective, precise and accurate as per FDA guidelines with low limit of detection 0.1 µg/mL and low limit of quantitation 0.5 µg/mL.

Key Words: Ultra performance liquid chromatography, Pravastatin, Erythrocytes.

INTRODUCTION

The quantification of drugs in biological systems is playing an important role in the discovery of new drugs as well as drug targeting. The new analytical methodologies and their right extraction combination help to quantify analytes accurately, rapidly and with high sensitivity. The traditional methods of extraction of drugs from biological matrices are including liquid/liquid extraction or solid phase extraction procedures[1].

Pravastatin, [1-naphthalene-hepatanoic acid, 1,2,7,8,8a hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy)-monosodium salt], is a hydrophilic liver specific inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase inhibitors). It effectively lowers plasma cholesterol and low density lipoprotein (LDL) concentrations in humans and prevents the onset and the development of atherosclerosis[2,3]. Furthermore, pravastatin is now found useful in the treatment of the several diseases such as osteoporosis, alzheimer disease, cardiac diseases, organ transplantation, stroke and diabetes [4]. Fig. 1 displays the chemical structure of pravastatin.

A number of techniques are reported for pravastatin determination and quantification in biological fluids based on gas chromatography-mass spectrometry[5-7], high performance liquid chromatography (HPLC)[8-14] and liquid chromatography tandem mass spectrometry[15-19]. The disadvantages of the previous methods are complexity of chromatographic conditions and/or long retention times.

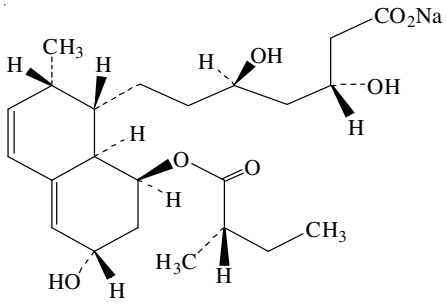

Fig. 1. Chemical structure of pravastatin sodium

Ultra performance liquid chromatography (UPLC) is considered a novel trend of liquid chromatography. Ultra performance liquid chromatography, as its first producer waters proclaims, means speed, resolution and sensitivity[20]. Ultra performance liquid chromatography has high separation efficiency because of using columns packed with ultra small particles with size less than 2 µm and so have huge surface area for chromatography and perform at high linear velocity[21]. The ability to increase resolution speed and efficiency is the major advantage of ultra performance liquid chromatography over conventional HPLC. Ultra performance liquid chromatography can achieve 5 to 10 times faster separations while maintaining or increasing peak resolution, thus resulting in higher throughput[22].

The ultra performance liquid chromatography systems are capable for withstanding the high pressure originated from the small void spaces even at low flow rates. The ultra performance liquid chromatography pump can operate at high pressures up to 15000 psi, which is more than the capabilities of traditional HPLC systems[20,22]. Also the ultra performance liquid chromatography detectors are modified to collect higher frequency data precisely due to the higher speed of ultra performance liquid chromatography chromatograms. The sharper and more concentrated peaks improve detection sensitivity as well. Ultra performance liquid chromatography needs lower solvent consumption due to the short run times and low flow rates, so it has a ecological favour by reducing waste disposal[23,24]. Owing to the benefits of ultra performance liquid chromatography in clinical, toxicological and forensic analysis, several researches have reported determination of different compounds in urine and rat or human plasma by ultra performance liquid chromatography[22].

The drugs analysis in red blood cells (RBCs) plays an important in the field of drug delivery. Up till now there is no ultra performance liquid chromatography method for pravastatin analysis in erythrocytes. Therefore, the aim of this work is to develop a novel method for determination of pravastatin in red blood cells. Pravastatin is firstly, loaded on erythrocytes as a carrier system and then extract by liquid/liquid extraction. Afterward, the drug was assayed using ultra performance liquid chromatography with UV detection. The major concern is to develop rapid, simple, sensitive and reproducible method suitable to quantify pravastatin loaded in red blood cells.

EXPERIMENTAL

Pravastatin sodium was obtained as gift from SPIMACO, KSA while sodium chloride and potassium dihydrogen phosphate was supplied from Merck, Germany. Potassium chloride is provided from Fluka chemie AGCH, Switzerland. Disodium phosphate dodecahydrate, formic acid (98 %) HPLC grade acetonitrile and methanol acquired from (BDH, UK). Deionized water was obtained in-house by through ELGA® purification system, Vivendi Water Systems Ltd., UK) and was further filtered through a 0.2 μm membrane PTFE disposable syringe filter from Macherey-Nagel GmbH, Germany. Class A, volumetric flasks from E-Mil Boro, England and from Duran Schott, Germany.

Chromatography was performed on acquity® (ultra performance liquid chromatography) system equipped with binary solvent manger, automatic sample manager, column heater, photodiode array (PDA) eλ detector and acquity® ultra performance liquid chromatography BEH C_{18} column (2.1 × 50 mm,1.7 μm) obtained from waters (Waters Inc., Bedford, MA, USA). Analytical balance AJ150, from Mettler, Switzerland. Water bath SW22, from Julabo, Germany. Centrifuge EBA 20 and MIKRO20 were supplied from Hettich, Germany. Vortex mixer, VWR from Scientific Industries Inc., Bohemia, NY, USA. Micro pipettes, diamond were obtained from Globe Scientific, Winters Avenue Paramus, NJ, USA.

Standard solutions: Two pravastatin standard stock solutions were prepared in water to produce final concentrations 250 μg/mL and 2500 μg/mL. Each stock solution was then serially diluted to 14 working standard solutions. The low-concentrated stock solution was diluted to the first concentration range from 0.1 to 65 μg/mL, while the second range was obtained by diluting the high stock solution to obtain range from 1 to 600 μg/mL. The two curves were intersecting at five concentration levels to validate the dilution procedure as well as the analyst performances as there were two analysts involved in the method development. Furthermore, this wide concentration range was designed to compass the unexpected wide range of loading.

Extraction procedure of pravastatin sodium from loaded erythrocytes: The blood specimens were collected from apparently healthy donors not suffered from acute and chronic diseases. Blood samples were collected in heparinized vacutainers and centrifuged for 5 min at 5000 rpm. The plasma and the buffy coat were removed by aspiration. Erythrocytes were washed three times in cold phosphate buffer saline with centrifugation for 5 min at 5000 rpm[25].

The hematocrite was adjusted at 50 % and then erythrocyte suspension was incubated with pravastatin sodium for 1 h. Pravastatin was extracted from pravastatin loaded erythrocytes by liquid/liquid extraction. The loaded erythrocytes were hemolyzed by addition of equal volume of distilled water with vigorous shaking to ensure erythrocyte hemolysis. After adding 1 mL of methanol for proteins precipitation, the mixture was mixed well and vortexed for 15 min, centrifuged at 13000 rpm for 15 min. The supernatant is taken and filtered using 0.2 μm PTFE disposable filters; and then completed to 5 mL by water. The experimental protocol was approved by the research center ethics committee of King Saud University, College of Pharmacy, Riyadh, Saudi Arabia.

Chromatographic conditions: The mobile phase was a mixture of 0.25 % formic acid in distilled water and acetonitrile (65:35, v/v), with flow rate of 0.5 mL/min and injection volume of 1 μL. PDA detector was set to acquire 3D data from 210 to 280 nm while the 2D channel was recording at 237 nm. The column temperature was kept at 50 ºC while sample temperature was kept ambient.

Method validation: The validation experiments were designed to test several performance parameters as selectivity, linearity, limit of detection (LOD), lower limit of quantitation (LLOQ), accuracy and precision according to FDA guidelines[26].

Selectivity: Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Selectivity was assessed to test the matrix effect on the samples. For selectivity, an analysis of blank samples of the appropriate erythrocytes was tested for interference at the same retention time with lower limit of quantitation.

Calibration and control samples: Suitable volumes of pravastatin sodium working standard solutions were conducted preparing non zero standard drug concentrations ranged from (0.1-500.0 μg/mL) and six quality control quality control concentrations covering the same range and including zero concentration (quality control zero). A calibration curve was constructed from samples covering the total range, including lower limit of quantitation. Injection of calibration samples were from low to high concentration at the beginning of each run.

Recovery studies: The recovery of a quality control sample is the response obtained from the amount of the analyte added and extracted from the biological matrix, in comparing to the response obtained for the true concentration of the pure authentic standard.

The absolute recovery of pravastatin sodium was evaluated by comparing drug peak areas of the quality control samples to that of aqueous standard solution which has been injected directly into an ultra performance liquid chromatography system. The assay absolute recovery at each concentration was computed using the following equation:

Absolute recovery = (peak area of extract/mean peak area of direct injection) × 100. While the relative recovery was calculated by the following equation:

Relative recovery = (conc. of extract/theoretical conc.) × 100.

Accuracy and precision: The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analyses of samples containing known amounts of the analyte. Accuracy was measured using six determinations per concentration.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. The intra-day precision was determined by six replicate analysis of samples at different concentrations including lower limit of quantitation while, the inter-day precision was determined by comparing quality control analysis performed on three different days repeatedly as sixtets of each quality control level. The precision was calculated as the relative standard deviation (RSD).

Statistical analysis: The data were analyzed by one way ANOVA followed by the bonferroni multiple comparison test, using PASW statistics 18 software, v. 5.01 (SPSS Software, Inc.).

RESULTS AND DISCUSSION

The method has been validated for linearity, selectivity, recovery, accuracy and precision and found to be within acceptable limits as per FDA guidelines for bioanalytical method validation[26].

Separation and selectivity: Specificity was tested in six different randomly selected samples of drug free human erythrocytes used for analyses. Figs. 2 and 3 show the representative chromatograms of blank solution and quality control zero while Fig. 4 shows the chromatogram of pravastatin loaded erythrocytes at lower limit of quantitation level. The analytes were well separated from erythrocytes samples after subjecting to the same chromatographic conditions at retention times of 0.6 min. The total run time was 1.2 min. The pravastatin peak (Rt = 0.61 min) was of good shape (tailing factor T= 1.07 and completely resolved from the nearest background peaks (relative retention factor r = 3.6) as in Fig. 5.

Linearity: The peak area of pravastatin sodium was linear with respect the analyte concentration over the range 0.5-480.0 µg/mL. The mean linear regression equation of calibration curve for analyte is y = 90.6545 (± 0.1786) × + 168.181 (± 24.721). The correlation factor (R^2) is 0.9987± 0.0001 over this wide concentration range (Fig. 6).

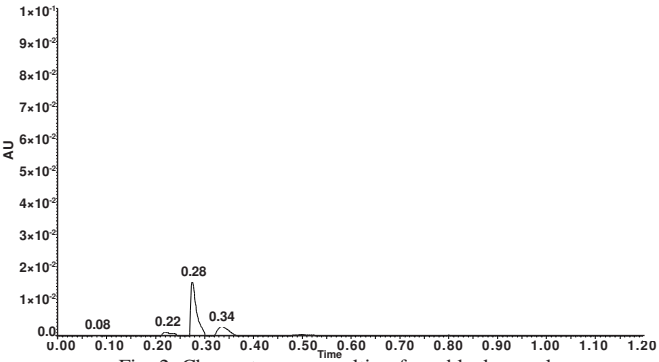

Fig. 2. Chromatogram resulting from blank sample.

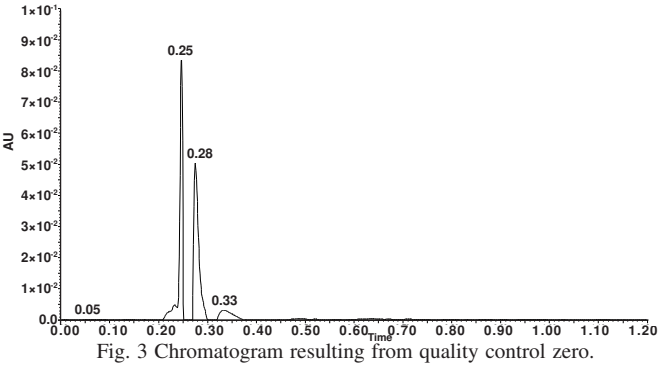

Fig. 3 Chromatogram resulting from quality control zero.

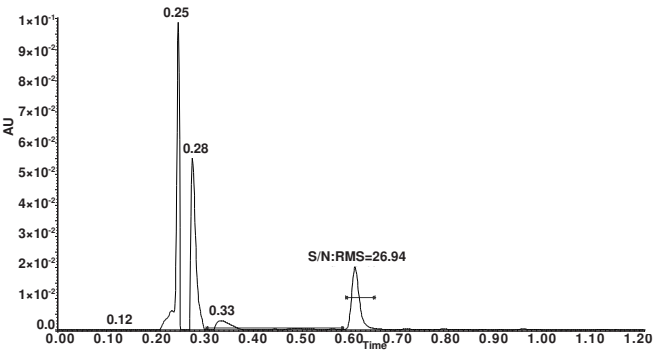

Fig. 4. LLOQ chromatogram resulting from human erythrocytes spiked with 0.5µg/mL pravastatin sodium.

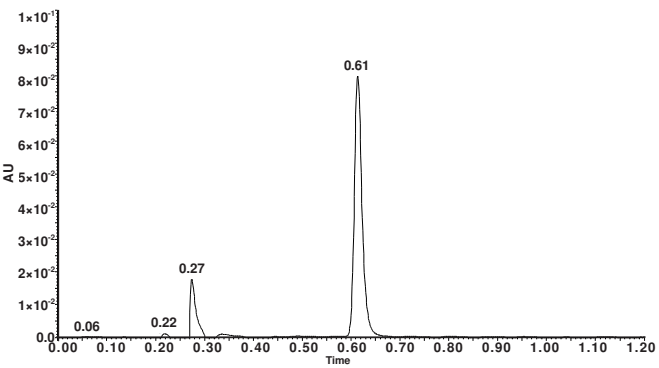

Fig. 5. Chromatogram shows shape, separation and resolution of pravastatin sodium peak.

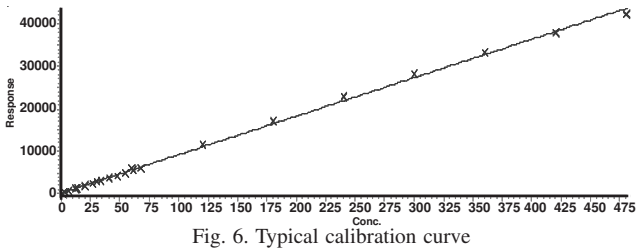

Fig. 6. Typical calibration curve

Limit of detection and lower limit of quantification: The lower limit of detection was estimated to be 0.1 µg/mL with S/N ratio of 15.26 as shown in Fig. 7. The lower limit of quantitation is lowest concentration of the standard curve, which can be measured with acceptable accuracy and precision for the analyte from loaded erythrocytes. Lower limit of quantitation was considered to be 0.5 µg/mL with signal to noise ratio (S/N ratio) of 26.94 and with recovery 108.18 % and precision of 2.75 %. Table-1 summarizes the back calculation of pravastatin sodium calibration standards.

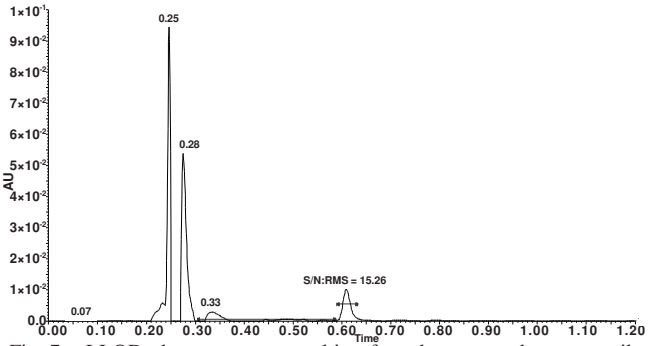

Fig. 7. LLOD chromatogram resulting from human erythrocytes spiked with 0.1µg/mL pravastatin sodium

TABLE-1
DATA OF BACK CALCULATED PRAVASTATIN SODIUM CONCENTRATION OF CALIBRATION STANDARDS

Nominal concentration (µG/ML)	Mean* (µg/mL)	Precision (RSD %)	Accuracy (%)
0.501	0.50	6.43	98.93
5.012	5.02	6.11	100.17
13.572	13.85	1.31	102.02
20.358	20.43	1.24	100.36
33.930	33.59	0.88	99.01
47.502	46.63	0.78	98.17
54.288	53.63	0.46	98.79
180.150	184.86	0.41	102.62
300.250	305.80	0.54	101.85
360.300	361.99	0.31	100.47
420.350	411.63	0.39	97.93

* Average of six determinations.

Recovery: Relative recovery of the analytes was calculated by comparing the concentration of the extracted drug to the theoretical concentration. Results are summarized in Table- 2. The average recoveries of pravastatin sodium extracted from loaded erythrocytes were between (108.18 and 96.09 %) with coefficient of variation between (0.32 and 11.51 %). These values were acceptable.

Precision and accuracy: Inter and intraday accuracy and precision at lower limit of quantitation as well as range containing low, medium and high concentrations of pravastatin sodium in loaded erythrocytes were within acceptable limits. Intraday and inter-day accuracy range are (97.93-102.61 %) and (96.91-104.75 %) respectively. Precision data (RSD) within days are less than 6.43 and 0.31; it is found to be less than 0.29 and 7.11 in between three days. These results are described in Tables 2 and 3.

TABLE-2
DATA OF RELATIVE RECOVERY OF PRAVASTATIN SODIUM FROM LOADED ERYTHROCYTES

Sample no.	Nominal concentration (µg/mL)	Mean found concentration* (µg/mL)	Recovery (%)	Precision
QC-1	0.50	0.54	108.18	2.75
QC-2	2.13	2.15	101.06	11.51
QC-3	4.12	3.96	96.09	2.31
QC-4	38.13	36.67	96.16	0.69
QC-5	75.46	73.38	97.25	1.47
QC-6	283.32	278.68	98.36	2.79
QC-7	301.72	300.88	99.72	0.58
QC-8	390.42	376.20	96.36	3.87

*Average of six determinations.

Conclusion

The new developed ultra performance liquid chromatography method of pravastatin sodium analysis in erythrocytes described is simple, accurate, fast and reproducible. The sensitivity and specificity of assay are sufficient for measurement of pravastatin sodium after extraction from drug-loaded erythrocytes by a liquid/liquid extraction. This analytical procedure provides several improvements over the previous HPLC methods in terms of sensitivity, specificity, analytical time and column stability. An additional improvement in the assay is an almost complete extraction recovery of the drug.

ACKNOWLEDGEMENTS

The work funded by SABIC graduate student fund (MED-30-41). The authors are grateful to the Research Center, College of Pharmacy, King Saud University and Deanship of Scientific Research, King Saud University for their support in this work.

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TABLE-3
DATA OF INTER-DAY ACCURACY AND PRECISION OF PRAVASTATIN SODIUM QC SAMPLES

Day of analysis	Pravastatin sodium concentration measure in erythrocytes (µg/mL)						
	LLOQ	QC-2	QC-3	QC-4	QC-5	QC-6	QC-7
	0.500	1.230	2.130	38.130	75.460	283.320	390.420
Day 1	0.51	1.21	2.19	36.98	72.97	280.25	392.01
	0.45	1.21	1.91	36.58	72.42	280.95	390.58
	0.46	1.31	1.98	36.81	72.57	282.70	391.89
	0.48	1.47	2.04	36.83	72.85	283.39	392.83
	0.47	1.46	2.02	36.27	73.39	283.70	393.76
Day 2	0.59	1.45	2.05	37.80	76.27	278.44	388.86
	0.50	1.23	2.00	37.68	74.81	280.53	382.64
	0.44	1.08	2.13	37.99	73.72	282.25	388.71
	0.43	1.05	1.77	38.25	74.60	282.22	394.68
	0.49	1.21	2.13	36.98	72.97	280.25	391.96
Day 3	0.49	1.21	2.13	36.98	72.97	280.25	391.96
	0.49	1.21	2.09	36.58	72.42	280.95	363.72
	0.53	1.31	1.98	36.67	75.29	265.13	391.77
	0.60	1.47	2.54	36.83	72.85	283.39	366.38
	0.59	1.46	2.02	36.27	73.39	283.70	367.18
Mean (µg/mL)	0.50	1.29	2.06	37.03	73.57	280.54	385.93
Accuracy (%)	100.39	104.75	96.91	97.12	97.49	99.02	98.85
Precision (RSD %)	6.07	6.19	7.11	1.01	1.44	1.27	1.76

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