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Article

Determination of Rosuvastatin at Picogram Level in Serum by Fluorimetric Derivatization with 9-Anthryldiazomethane using HPLC

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For the first time, a carboxyl group derivatization assay has been developed and validated for the determination of the cholesterol-lowering drug rosuvastatin in human serum at picogram level by high-performance liquid chromatography with fluorescence detection. The assay procedure involved a simple one-step liquid–liquid extraction of rosuvastatin with lovastatin as internal standard from serum with an ethyl acetate–methyl tertiary buthyl ether (1:1) mixture. After pre-column derivatization with 9-anthryldiazomethane at room temperature for one hour, the reaction mixture was injected onto a Phenomenex, Synergi C18 column (250 × 4.6 mm, 4 μ i.d.). The analytes were separated with a mobile phase composed of acetonitrile–water in gradient elution mode and detected at λ_{em} = 410 nm, exciting at 366 nm. Calibration curves were constructed in concentration range of 0.01–20.0 ng/mL and limit of detection and limit of quantification values were found to be 0.68 and 2.30 pg/mL, respectively. To test suitability of the developed methods for clinic use, the pharmacokinetics of rosuvastatin were investigated after oral administration of a 20 mg rosuvastatin film tablet to a healthy volunteer and maximum plasma concentration, time to reach that concentration and elimination half life were found to be 17.5 ng/mL, 3.5 h and 18.09 h, respectively.

Introduction

The group of medications referred to as statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, has been the most popular and widely prescribed for treating atherosclerosis. Statins are used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase that catalyzes the conversion of HMG-CoA to mevolanate, which plays a central role in the production of cholesterol in the liver (1–2).

Rosuvastatin (ROS), one of the newest members of the statin drug group, a synthetic lipid-lowering agent, is chemically *bis*[(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl-(methylsulfonyl)amino] pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoicacid] calcium salt (Figure 1) and was approved in the United States in 2003 for the treatment of dyslipidemia (3). ROS also reduces the level of low-density lipoprotein (LDL) in plasma, modestly increases the level of HDL cholesterol in the blood and is satisfactorily tolerated by the patients (4).

To date, all of the developed methods for the determination of ROS in biological fluids are based on liquid chromatography with either ultraviolet (UV) (5–10) or mass spectrometric (MS) (11–19) detection. These methods involved tedious steps for the pretreatment of the samples (10) and have inadequate sensitivity (5–9), which is critically important for determination of a lower amount of drugs and use of expensive detectors (e.g., tandem MS) (11–19) that are not available in most research laboratories. On the other hand, a low amount of ROS active ingredient in tablets and low plasma concentrations of ROS observed after oral administation require implementation of sensitive quantitation methods for ROS.

9-Anthryldiazomethane (ADAM) (Figure 1) (20–21) is an important label for fluorimetric (FL) detection of carboxylic acids. This reagent reacts with the carboxyl functional group under mild conditions at room temperature without an activation reagent, even in the presence of water. ADAM has been widely used as an FL derivatization reagent for carboxylic acids. The resulting derivatives permit pmol-level detection of these acids by reversed-phase high-performance liquid chromatography (HPLC).

In this study, ROS has been derivatized by the reaction of ADAM and determined in serum samples. The proposed derivatization reaction is shown in Figure 1. This work is the first derivatization method of the carboxylic group of ROS with ADAM reagent based on fluorimetric detection and provides low detection limits in the pg/mL range.

Experimental

Reagents and solutions

Rosuvastatin calcium was provided by Astra Zeneca (London, UK) and lovastatin (internal standard; IS) was provided by Merck Sharp and Dohme Corp. (Whitehouse Station, NJ). Crestor 20 mg tablets were purchased from a local drugstore. ADAM was purchased from Sigma-Aldrich (Oslo, Norway). Sodium acetate, acetonitrile, ethyl acetate (EA), methyl tertiary buthyl ether (MTBE), chloroform, glacial acetic acid and sodium sulphate anhydrous were purchased from Merck (Darmstadt, Germany). HPLC-grade ultrapure water was prepared by an aquaMAX (Younglin Instrument, Korea) water purification system.

Instrumentation and chromatographic conditions

A Shimadzu (Kyoto, Japan) LC 20A liquid chromatograph was used, consisting of a model LC 20 AT solvent delivery system, a DGU-20A5 degasser and a SIL-20AC autosampler. The RF 10 AXL fluorescence detector was set at an excitation wavelength of 366 nm and an emission wavelength of 410 nm. Data acquisition was performed using LC Solution system software. Separations were performed on a Phenomenex Synergi C18 column (4 μm, 250 × 4.6 mm i.d.) with a Phenomenex guard column (4 × 3 mm i.d.) packed with the same material. Chromatographic separation was achieved at 30°C by using an

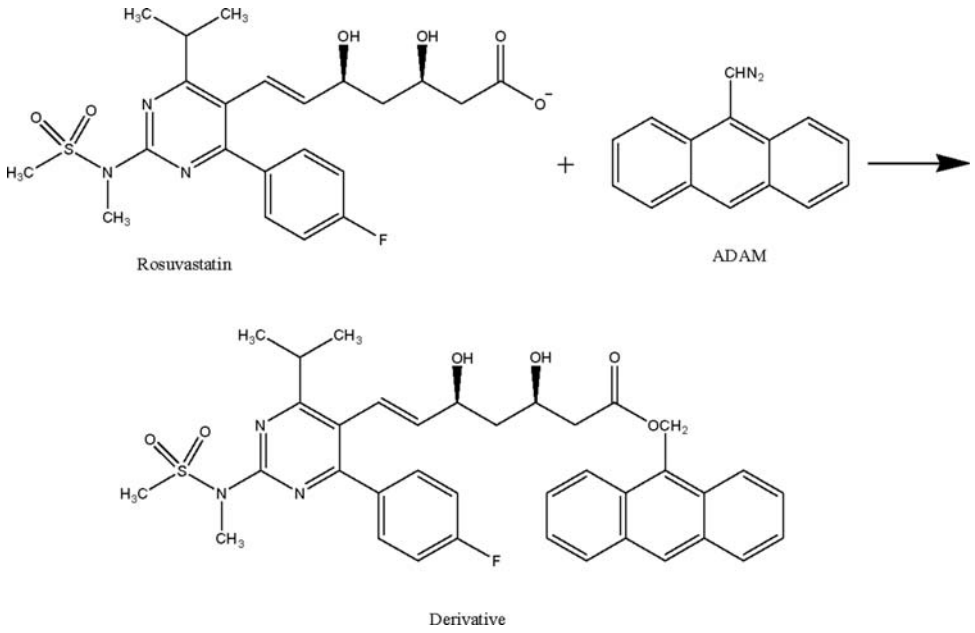

Figure 1. The proposed reaction between ROS and ADAM.

Table I
Gradient Elution Program

Time (min)	Acetonitrile (%)	Water (%)
0.01	40	60
10	80	20
15	80	20
15.01	40	60
20	40	60

acetonitrile–water mixture as mobile phase in gradient elution mode at a flow rate of 1.0 mL/min. The gradient elution program (Table I) was run for 20 min.

Preparation of standard solutions

The stock solution of ROS was prepared by dissolving an amount corresponding to 1.0 mg/mL concentration of rosuvastatin calcium in acetonitrile and then diluted with acetonitrile to give a 1 μg/mL concentration. Working Solutions I and II of ROS were prepared in an acetonitrile–water (1:3) mixture to give 25 and 1 ng/mL concentrations, respectively. ADAM and IS solutions were prepared in acetonitrile and chloroform, respectively, to give 100 μg/mL concentrations. Reagent solutions prepared freshly every day and kept shielded from light. Acetate buffer solution was prepared by dissolving an appropriate amount sodium acetate in water to give 0.1 M concentration and pH was adjusted to 4.0 with glacial acetic acid (22).

The ROS base solutions were prepared to remove calcium salt from the drug. For this purpose, a simple one-step liquid–liquid extraction (LLE) procedure was completed. Appropriate volumes of Working Solution II and 50 μL of a 10 μg/mL concentration of IS were transferred to a stoppered tube and diluted to 1 mL with water. Then, 1 mL acetate buffer and 5 mL EA–MTBE (1:1) were added and vortexed for 5 min. After centrifugation, the organic layer was separated in a test tube and dried using anhydrous Na_2SO_4, and 4 mL of anhydrous supernatant was evaporated to dryness under nitrogen at 40°C. The residues were further subjected to the derivatization procedure.

Sample preparation

Venous blood samples (4–5 mL) were placed into glass tubes then centrifuged (within 2 h from collection) at 1,400 × g for 15 min; the supernatant (serum) was then transferred into polypropylene test tubes and stored at −20°C until HPLC analysis.

For the extraction of ROS from serum samples, the same method was performed with the ROS base solutions preparation. To 100 μL of serum, 0.4–800 μL volumes of Working Solution II and 5 μL of 100 μg/mL IS solution were added and mixed for approximately 10 s; following that, 1 mL of acetate buffer and 5 mL EA–MTBE (1:1) were added and vortexed for 5 min. After centrifugation, the organic layer was separated in a test tube and dried using anhydrous Na_2SO_4, and then 4 mL of anhydrous supernatant was evaporated to dryness under nitrogen at 40°C. The residues were further subjected to the derivatization procedure.

Derivatization

The dried residues obtained from the serum sample pretreatment (or the dried residue of ROS base) was reacted with 125 μL of a 100 μg/mL concentration ADAM solution, the volume was adjusted 1 mL with acetonitrile and the solution was kept in the dark for 1 h at ambient temperature. Finally, 20 μL of the reaction mixture was injected into the HPLC system.

Method validation

The developed analytical method was validated according to international guidelines with respect to certain parameters

such as linearity, accuracy, precision, selectivity, limit of detection (LOD), limit of quantification (LOQ) and stability (23).

Serum calibration standards were prepared at six levels by spiking 100 μL of human serum with the 0.01–20 ng/mL concentration of ROS and a constant concentration of IS (500 ng/mL). The obtained mixture was subjected to the previously described LLE procedure, derivatized with ADAM, kept in the dark for 1 h at ambient temperature and injected into the HPLC system. The procedure was carried out in triplicate for each concentration. The analyte/IS peak area ratios obtained were plotted against the corresponding concentrations of the analytes (as ng/mL) and the calibration curves set up by means of the least-square method. Linear regression was applied and slope (*a*), intercept (*b*), correlation coefficient (*r*), standard deviation (SD) and relative standard deviation (RSD) were determined.

For the extraction recovery, blank human serum samples were spiked with the analytes at three different concentration levels (1, 5 and 15 ng/mL) of ROS while keeping the IS concentration constant (500 ng/mL). The serum was extracted with the previously described procedure, derivatized with ADAM and injected to the HPLC system in triplicate. Extraction recoveries were evaluated by using the calibration curve prepared with aqueous drug solutions.

Method precision and accuracy were determined in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision). To determine precision and accuracy, serum samples spiked at three different concentration levels (1, 5 and 15 ng/mL) of ROS and a constant concentration (500 ng/mL) of IS were analyzed six times a day in triplicate injections over six consecutive days in two months. The serum saples were extracted with the previously described procedure, derivatized with ADAM, injected into the HPLC system in triplicate and expressed as mean ± SD and %RSD calculated from the obtained data.

LOD and LOQ were determined through the intercept and slope of the calibration curve according to the following formula:

$$\mathrm{LOD} = \sigma/m, \qquad \mathrm{LOQ} = 10\,\sigma/m$$

where σ is the SD of the calibration curve's intercept and m is the slope of the calibration curve.

The stability studies of ROS and IS spiked serum samples derivatized with ADAM were conducted with 100 μL of Working ROS Solution II, which was subjected to the previously described derivatization procedure in six replications. Resulting derivatives were kept in the dark at ambient temperature and at +4°C for 12, 24, 48, 72 and 96 h and analyzed in HPLC system.

Pharmacokinetic study

A pharmacokinetic study was performed with a healthy volunteer (29 years old, female) by administrating a single oral dose of 20 mg of ROS. The venous blood samples were collected into tubes at 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 24, 36, 48 and 72 h postdosing. The serum samples were obtained from blood after the prosess described previously. The samples were stored at −20°C until analysis.

Pharmacokinetic analysis was performed using standard methods (24) and parameters were calculated using the TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis system (25).

Results

Derivatization procedure

Because ROS has a carboxylic acid group, fluorescent derivatization was chosen to obtain the sensitivity needed for the analysis in human serum. Among the different possible fluorescent reagents to react with carboxylic acid groups, ADAM is particularly attractive because it is relatively easy to synthesize from readily available compounds and undergoes fast reaction at room temperature in the dark to give high yields of the ester conjugates. Anhydrous reaction conditions, catalyst and heating are not required; the resulting derivative can be injected to HPLC system without any further purification or extraction.

Preliminary assays showed that the reaction proceeds in the dark with 10 min–2.5 h reaction time; the effect of temperature on reaction rates and reaction time were studied in the 25–60°C and 10–90 min range in the dark, respectively. A 1 h reaction time at ambient temperature in the dark gives satisfactory results for the derivatization reaction. The effect of reagent mol ratio was tested in the 40–800-fold reagent mol ratios and 55-fold was found sufficient.

Chromatographic conditions

Different types of analytical columns were tested, including C8, CN and C18 with different particle sizes, and the best resolution was obtained with a Phenomenex, Synergi C18 column (4 μm, 250 × 4.6 mm i.d.). The composition of mobile phase in particular was optimized to achieve good sensitivity and peak shapes for the ROS and IS, as well as a short run time. An acetonitrile–water mixture was chosen as mobile phase mixture and a gradient elution program (Table I) was applied at a flow rate of 1.0 mL/min. The fluorescence detector was set an excitation wavelength of 366 nm and an emission wavelength of 410 nm.

Method validation

The retention times of ROS–ADAM and IS–ADAM derivatives in this chromatographic system were 13.6 and 15.6 min, respectively. Representative chromatograms are given in Figure 2. The total run time of the chromatogram is 20 min. No interference peaks were detected for ROS and IS from the serum samples.

The ROS–IS peak area ratios were plotted against the corresponding concentrations of ROS (as ng/mL) and the calibration curves were set up in concentration range of 0.01–20.0 ng/mL for ROS in human serum. The mean linear regression equation of the calibration curves was $y = 0.2237\ x + 0.1017$ ($r = 0.9971$), where y represents the ratios of ROS peak area to that of IS and x represents the serum concentrations of ROS. The correlation coefficients of the calibration curves ranged from 0.9955 to 0.9988, which demonstrates good linearity. The linearity parameters of ROS in serum samples are presented in Table II.

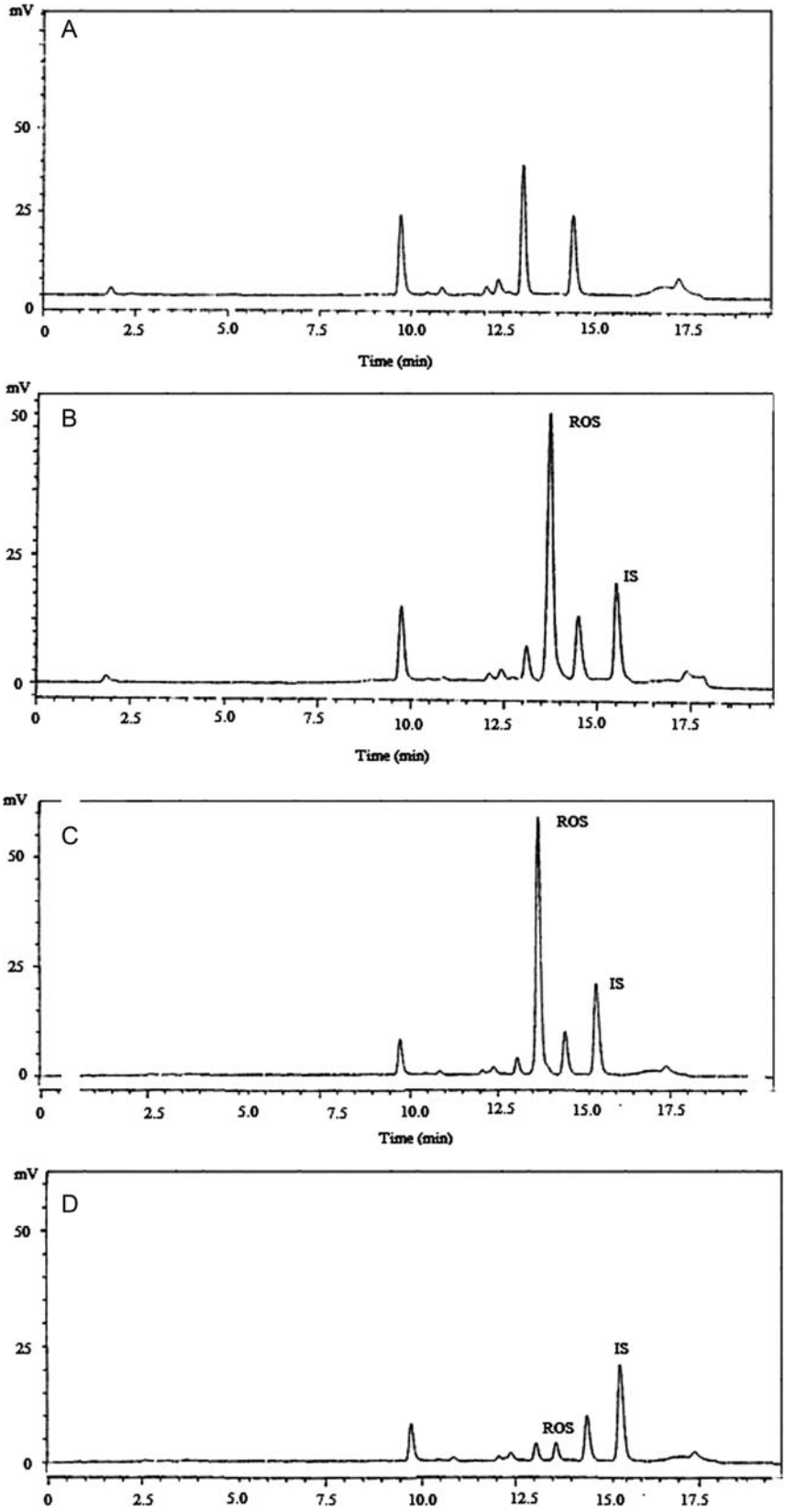

Figure 2. Drug-free human serum (A); a serum sample spiked with ROS and IS (15 and 500 ng/mL, respectively) (B); a serum sample obtained from a volunteer 4 h after oral administration (C); a serum sample obtained from a volunteer 72 h after oral administration (D).

Table II
Linearity Parameters for ROS in Serum Samples

Parameters	
Calibration range (ng/mL)	0.01–20.0
Slope	0.2237
Intercept	0.1017
Correlation coefficient	0.9971
LOD (pg/mL)	0.68
LOQ (pg/mL)	2.30

Table III
Extraction Recovery

Concentration added (ng/mL)	Concentration found (ng/mL)	%RSD	Extraction yield (%)
1	0.80 ± 0.023	4.220	81.4
5	3.98 ± 0.043	1.551	80.0
15	11.94 ± 0.101	1.061	79.8

Table IV
Inter-Intra Day Precision Values on Serum Samples

Concentration added (ng/mL)	Concentration found (ng/mL)					
	Intra-day (mean ± SD)	%RSD	%RME	Inter-day (mean ± SD)	%RSD	%RME
1	0.96 ± 0.0381	3.966	−4.01	0.89 ± 0.0451	4.713	−4.41
5	4.92 ± 0.0608	1.236	−1.61	4.91 ± 0.0374	0.761	−1.62
15	14.95 ± 0.0532	0.355	−0.31	14.93 ± 0.0308	0.206	−0.46

Blank human serum was spiked with the analytes at three different concentration levels (1, 5 and 15 ng/mL) of ROS and keeping the IS concentration constant (500 ng/mL). The recoveries of ROS extracted from serum samples were 81.4, 80.0 and 79.8% at concentrations of 0.80 ± 0.023, 3.98 ± 0.043 and 11.94 ± 0.101 ng/mL, respectively, and mean recovery was found to be 80.4%. The results are presented in Table III.

The inter-day and intra-day precision and accuracy values are summarized in Table IV for ROS in serum samples. In this assay, RSD values were found within the range of 0.35–3.96 and 0.20–4.7 for inter-day and intra day precision, respectively. Relative mean error (RME) values were found within the range of 0.31–4.01 and 0.46–4.41 for inter-day and intra day assay, respectively. Because the RSD and RME values were within the acceptable range, the method has been demonstrated to possess satisfactory precision and accuracy.

LOD and LOQ values were found to be 0.68 and 2.3 pg/mL, respectively, which means the method was sensitive enough to characterize the pharmacokinetic profiles. The LOD and LOQ values are shown in Table II.

As a result of stability studies, ROS–ADAM derivatives in serum samples were found to be stable when kept in the dark at +4°C for 96 h. ROS stock solution was also found to be stable in acetonitrile at +4°C for 1 month.

Pharmacokinetic study

The proposed method was applied to the determination of ROS in serum for a pharmacokinetic study of the administration of single oral dose of 20 mg of ROS to a healthy volunteer (29 years old, female). The pharmacokinetic profiles of the mean serum concentration versus time are shown in Figure 3. The corresponding pharmacokinetic parameters (C_{max}, t_{max}, $t_{1/2}$, AUC_{0-t} and $AUC_{0-\infty}$) are presented in Table V. These pharmacokinetic parameters are in agreement with those found previously (3–5).

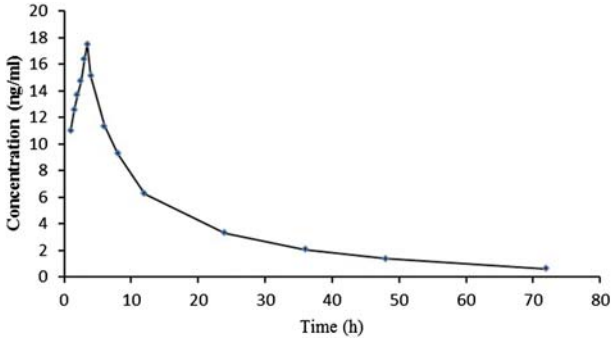

Figure 3. Mean serum concentration–time curve of ROS after a single oral dose of 20 mg.

Table V
Pharmacokinetic Parameters after a Single Oral Dose of a Crestor 20 mg Tablet

Parameters	
C_{max} (ng/mL)	17.5
t_{max} (h)	3.5
$t_{1/2}$	18.09
$AUC_{(0-\infty)}$ (ng/h/mL)	286.06

Discussions

A highly sensitive, selective, economic and simple assay was developed to determine ROS in serum for the purpose of using the method in pharmacokinetic studies or therapeutic drug monitoring. Because ROS has a carboxylic acid group, fluorescent derivatization was chosen to obtain the sensitivity needed for the analysis in human serum. Among the different possible fluorescent reagents that react with carboxylic acid groups, ADAM is particularly attractive because it undergoes fast reaction at room temperature in the dark to give high yields of the ester conjugates. Anhydrous reaction conditions, catalyst and heating are not required; the resulting derivative can be injected directly into the HPLC system without any further purification or extraction.

A relatively cheap detector has been used for the determination of ROS in human serum, and a fluorescence detector can easily be found in any laboratory, unlike MS detectors (11–19). Also, the method is the first derivatization method of ROS with ADAM reagent based on fluorimetric detection and provides low detection limits in the pg/mL range. All previously published methods based on UV detection (5–9) do not have sufficient sensitivity for the determination of ROS for pharmacokinetic studies. Only one method (16) has adequate sensitivity, but the current method is more sensitive than this method, which has an LOQ value of 2.3 pg/mL. Also, the proposed method is more sensitive than other published methods based on MS detection (11–19). Moreover, RSD values of the

method, at any of the studied concentrations, never exceeded 4.71%, which indicates assay precision that is better than those of other HPLC methods based on UV or MS detection (5–19). Additionally, mean percent recovery of the method was found to be 80.4%, which is better than some other publications (14–19). Therefore, the proposed method has significant advantages in sensitivity, applicability and cost with respect to those available in the literature; it has been successfully applied to the analysis of ROS in serum samples from a single dose treatment.

Concluding Remarks

In this study, a highly sensitive, selective, economic and simple HPLC method with fluorescence detection was developed for the determination of ROS in human serum. The developed method can be used as an alternative method for pharmacokinetic studies and therapeutic drug monitoring of ROS.

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References

1. Williams, K.J., Tabas, I.; The response-to-retention hypothesis of early atherogenesis; *Atherosclerosis Thrombosis Vascular Biology*, (1995); 15: 551.
2. Lewington, S., Whitlock, G., Clarke, R.; Blood cholesterol and vascular mortality by age, sex, and blood pressure: A meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths; *Lancet*, (2008); 370: 1829–39.
3. Martin, P.D., Mitchell, P.D., Schneck, D.W.; Pharmacodynamic effects and pharmacokinetics of a new HMG-CoA reductase inhibitor, rosuvastatin, after morning or evening administration in healthy volunteers; *Journal of Clinical Pharmacology*, (2002); 54: 472–477.
4. Carswell, C., Plosker, L., Jarvis, B.; Rosuvastatin; *Drugs*, (2002); 62: 2075–2085.
5. Kumar, T.R., Shitut, N.R., Kumar, P.K., Vinu, M.C.A., Kumar, V.P., Mullangi, R., *et al.*; Determination of rosuvastatin in rat plasma by HPLC: Validation and its application to pharmacokinetic studies; *Biomedical Chromatography*, (2006); 20: 881–887.
6. Sultana, N., Arayne, M. S., Shahzad, W.; Simultaneous determination of ceftriaxone sodium and statin drugs in pharmaceutical formulations and human serum by RP-HPLC; *Journal of the Chilean Chemical Society*, (2010); 55: 193–198.
7. Ma, Y.H., Peng, Y.F., Xu, Y.R, Yang, G.Y., Hu, Q.F.; Determination of atenolol, rosuvastatin, spironolactone, glibenclamide and naproxen in blood by rapid analysis liquid chromatography; *Asian Journal of Chemistry*, (2010); 22: 1136–1140.
8. Sultana, N., Arayne, M. S., Iftikhar, B.; Simultaneous determination of atenolol, rosuvastatin, spironolactone, glibenclamide and naproxen sodium in pharmaceutical formulations and human plasma by RP-HPLC; *Journal of the Chinese Chemical Society*, (2008); 55: 1022–1029.
9. Vittal, S., Shitut, N.R., Kumar, T.R., Vinu, M.C.A., Mullangi, R., Srinivas, N.R.; Simultaneous quantitation of rosuvastatin and gemfibrozil in human plasma by high-performance liquid chromatography and its application to a pharmacokinetic study; *Biomedical Chromatography*, (2006); 20: 1252–1259.
10. Shah, Y., Ikbal, Z., Ahmad, L., Khan, A., Khan, M.I., Nazir, S., *et al.*; Simultaneous determination of rosuvastatin and atorvastatin in human serum using RP-HPLC/UV detection: Method development, validation and optimization of various experimental parameters; *Journal of Chromatography B*, (2011); 879: 557–563.
11. Hussain, S., Patel, H., Tan, A.; Automated liquid-liquid extraction method for high-throughput analysis of rosuvastatin in human EDTA K(2) plasma by LC-MS/MS; *Bioanalysis*, (2009); 1: 529–535.
12. Kallem, R.R., Karthik, A., Chakradhar, L., Mullangl, R., Srinivas, N.; Development and validation of a highly sensitive and robust LC-MS/MS with electrospray ionization method for quantification of rosuvastatin in small volume human plasma samples and its application to a clinical study; *Arzneimittel Forschung*, (2007); 57: 705–711.
13. Hulla, C.K., Penman, A.D., Smith, C.K., Martin, P.D.; Quantification of rosuvastatin in human plasma by automated solid phase extraction using tandem mass spectrometric detection; *Journal of Chromatography B*, (2002); 772: 219–228.
14. Gao, J., Zhong, D., Duan, X., Chen, X.; Liquid chromatography/negative ion electrospray tandem mass spectrometry method for the quantification of rosuvastatin in human plasma: Application to a pharmacokinetic study; *Journal of Chromatography B*, (2007); 856: 35–40.
15. Lan, K., Jiang, X., Li, Y., Wang, L., Zhou, J., Jiang, Q., *et al.*; Quantitative determination of rosuvastatin in human plasma by ion pair liquid-liquid extraction using liquid chromatography with electrospray ionization tandem mass spectrometry; *Journal of Pharmaceutical and Biomedical Analysis*, (2007); 44: 540–546.
16. Xu, D.H., Ruan, Z.R., Zhou, Q., Yuan, H., Jiang, B.; Quantitative determination of rosuvastatin in human plasma by liquid chromatography with electrospray ionization tandem mass spectrometry; *Rapid Communications in Mass Spectrometry*, (2006); 20: 2369–2375.
17. Oudhoff, K.A., Sangster, T., Thomas, E., Wilson, I.D.; Application of microbore HPLC in combination with tandem MS for the quantification of rosuvastatin in human plasma; *Journal of Chromatography B*, (2006); 832: 191–196.
18. Singh, S.S., Sharma, K., Patel, H., Jain, M., Shah, H., Gupta, S., *et al.*; Estimation of rosuvastatin in human plasma by HPLC tandem mass spectroscopic method and its application to bioequivalence study; *Journal of the Brazilian Chemical Society*, (2005); 16: 944–950.
19. Trivedi, R.K., Kallem, R.R., Mullangi, R., Srinivas, N.R.; Simultaneous determination of rosuvastatin and fenofibric acid in human plasma by LC-MS/MS with electrospray ionization: Assay development, validation and application to a clinical study; *Journal of Pharmaceutical and Biomedical Analysis*, (2005); 39: 661–669.
20. Yamaguchi, Y., Tomita, T., Senda, M., Hirai, A., Terano, T., Tamura, Y., *et al.*; High-performance liquid chromatographic analysis of arachidonic acid metabolites by pre-column derivatization using 9-anthryldiazomethane; *Journal of Chromatography*, (1986); 357: 199–201.
21. Hatsumi, M., Kimata, S., Hirosawa, K.; 9-Anthryldiazomethane derivatives of prostaglandins for high performance liquid chromatographie analysis; *Journal of Chromatography*, (1982); 253: 271–275.
22. United States Pharmacopeial Convention (USP) 26, NF21 Supplement 2, Buffer Solutions; Rockville, MD, (2003).
23. Internnational Conference on Harmonization (ICH) Q2 (R1), Validation of Analytical Procedures: Text and Methodology, (2005). http://www.ich.org/LOB/media/MEDIA417.pdf (23 December 2010).
24. Rowland, M., Tozer, T.N.; Clinical pharmacokinetics: Concept and applications, 3rd ed., Lippincott Williams & Wilkins, Philadelphia, PA (1995); 211–212.
25. TOPFITT [Computer program]. Version 2.0. Pharmacokinetic and Pharmacodynamic Data Analysis System for the PC, Gustav-Fisher, Stuttgart, (1993).