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ORIGINAL PAPER

Determination of Rosuvastatin in Urine by Spectrofluorimetry After Liquid–Liquid Extraction and Derivatization in Acidic Medium

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Abstract Statins are a class of drugs mostly used for treating hyperlipidemia, and rosuvastatin is the newest drug in the market belonging to this class. In this present work, a method was developed based on the molecular fluorescence technique, with the objective to quantify rosuvastatin in urine samples. For this purpose, the study of several parameters was made to achieve the maximum analytical signal (under reaction with sulfuric acid during 40 min). Also, a previous step to avoid matrix interference was carried out (liquid–liquid extraction). The limit of detection (LOD) and the limit of quantification (LOQ) were 0.38 and 1.28 mg L^{-1}, respectively. Linear relationship between rosuvastatin concentration and it's fluorescence intensity was found until 5.0 mg L^{-1}. The proposed method was tested in several samples spiked with rosuvastatin and recovery was found in the range of 90±10 %.

Keywords Rosuvastatin · Urine · Derivatization

Introduction

Cholesterol is essential for the proper workings of human organism, as longs as it plays a crucial role in maintaining the membrane cell integrity and helps in physiological functions of the body, including membrane physiology, nutrient absorption, reproductive systems, stress responses, salt and water balance, and calcium metabolism [1]. However, at high concentrations, cholesterol is responsible for the incidence of pathological conditions such as coronary artery disease [2] which is responsible for 15 million of deaths per year worldwide [3].

From the total cholesterol in human body, about 60 % is produced inside the liver and the rest come from food. The types of cholesterol are LDL (low-density lipoprotein), HDL (high-density lipoprotein) and VLDL (very low density lipoprotein).

The elevation of lipid concentration in plasma (hyperlipidemia) is the manifestation of a heterogeneous disorder usually characterized by an increased flux of free fatty acids, raised levels of triglycerides, LDL and apolipoprotein B, and reduced HDL concentration in plasma. In general, the causes of this disorder are metabolic effects, dietary and/or lifestyle habits [4].

The LDL and VLDL cholesterol are responsible for the deleterious effects coming from cholesterol, for this reason they are known as “bad cholesterol”, while HDL is known as “good cholesterol”, since it removes as much fat from the blood vessels as from the liver. From this knowledge, all the attempts to reduce cholesterol levels in blood were based on avoiding the ingestion of food that contain it, especially the ones rich in animal fats (such as fatty meats, fried foods, saturated fats, egg yolk, etc.).

When a special diet plan is not enough to reduce cholesterol levels, the use of drugs is necessary. The drugs class widely used for this purpose is the one of statins.

Statins are the most common drugs used for the treatment of hyperlipidemia in order to decrease the levels of lipoproteins (fat) rich in cholesterol and to reduce the risk of coronary artery disease [5]. Its mode of action is based on the inhibitory activity over 3-hydroxy-3-methylglutaryl coenzyme A, the enzyme that catalyzes the rate-controlling step in cholesterol biosynthesis [6]. This substances can be

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divided in natural and synthetics, and differ in terms of potency, pharmacokinetics and pharmacological profile.

The first statin discovered and commercially available was mevastatin, discovered in 1976 as a metabolite isolated from cultures of *Penicillium Citrium* [7]. It was found that it has an enzyme affinity about 10000 times greater than the substrate HMG-CoA. Later, lovastatin was isolated and had its marketing approved by FDA in 1987, at the time which mevastatin was abandoned due to its toxicity when administered to dogs and rats [5].

The introduction of synthetic statins in market began in 1996 and 1997 with the commercialization of atorvastatin (Lipitor) and cerivastatin (Baycon), respectively. However, in 2001, Bayer took cerivastatin out of market due to a great number of side effects, which led atorvastatin to become the most sold statin in the world, making US$ 8 billion in 2002.

In 2003, the newest synthetic statin rosuvastatin, available as Crestor-AstraZeneca (structure shown in Fig. 1), had its use approved by FDA. The manufacturer claimed that rosuvastatin decreases more efficiently LDL than any other statin in the market, and has the advantage of increasing HDL and reducing triglycerides.

As it is a relatively new drug, few methods are reported in the current literature regarding rosuvastatin determination in different kinds of samples. Most of these methods are applied to plasma samples and are based on the separation in a liquid chromatographic system with tandem mass spectrometric detection. Hull et al. [8] proposed a method for rosuvastatin determination in human plasma after its removal by solid-phase extraction, followed by LC-MS/MS determination. The method suggested by Oudhoff et al. [9] compared different microbore high-performance liquid chromatography in combination with tandem mass spectrometry for rosuvastatin determination in human plasma; and also Trivedi [10], who published a method based on LC-MS/MS determination.

Despite these proposals of quantification in plasma matrices, Martin et al. [6] presented the study of pharmacokinetics of rosuvastatin in human volunteers, in which the monitoring of the concentration of analyte was made by HPLC-MS/MS in

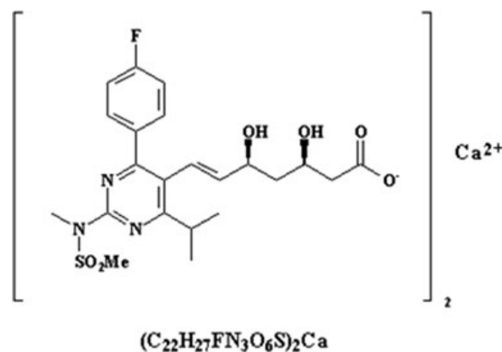

Fig. 1 Rosuvastatin calcium

urine samples. The author did so because he could prove that only 10 % of the drug is metabolized in human body (to the n-desmetyl form). From the remaining unchanged part, 90 % is eliminated in feces and the 10 % are in urine.

Martin et al. [6] also proved that, although the main part of the drug is eliminated in feces, complete pharmacokinetics information about it is only obtained by the study of the fraction eliminated in urine. Additionally, the monitoring of the drug through a non-invasive exam is a convenience that should always be considered.

It's remarkable that there are many studies published for the determination of rosuvastatin in plasma or urine matrices by LC-MS/MS, but the only spectrophotometric methods found so far in literature are based on UV–vis [11, 12], and it is able for determination of rosuvastatina just in pure form or in pharmaceutical formulations.

This present work proposes an alternative method for doing this monitoring by spectrofluorimetry. For doing so, a liquid–liquid extraction is performed to extract rosuvastatin from urine, followed by a derivatization of the analyte in acidic medium, made in order to increase the analytical signal.

Experimental

Apparatus

Fluorescence measurements were carried out with a Varian Cary Eclipse spectrofluorimeter (Mulgrave, Australia), with a 10-mm quartz cuvette purchased from the same company. The instrument was coupled to a personal computer for data acquisition and treatment.

All pH measurements were performed with a Digimed DM-22 pHmeter (São Paulo, Brazil) equipped with a combined glass electrode (Ag/AgCl as reference).

An analytical balance furnished by Shimadzu, model AY 220 (Tokyo, Japan), was employed throughout the experimental work.

Reagents and Solutions

All reagents were of analytical grade or better. Deionized water (18.2 MΩ cm) was produced with a water purification system furnished by Elga, model PureLab Classic (Bucks, UK).

Rosuvastatin standard (calcium salt) was supplied by Gamma (Beijing, China).

Argon (99,995 % grade) from Linde Gases (Macaé, Brazil) was employed in the experimental work.

Sulfuric acid and chloroform were supplied by Tedia (Fairfield, OH, USA). Methanol, boric acid, phosphoric acid, acetic acid and sodium hydroxide from Merck (Darmstadt, Germany) were used.

Procedures

Preparation of Standard Solutions

A stock solution of rosuvastatin with 25 mg L^{-1} concentration was prepared by dissolving 2.5 mg of the solid standard in exactly 100 mL of purified water. In order to help the rosuvastatin solubilization, the obtained solution was sonicated for 15 min in an ultrasonic bath. Working solutions were prepared, daily, by transferring convenient aliquots of the stock solution and 2.5 mL of concentrated sulfuric acid to volumetric flasks of 25 mL. Then, the volume was completed to the mark with purified water in order to obtain final rosuvastatin concentrations in the range of 0.5–5.0 mg L^{-1}. The solutions were shaken and then left on the table for 40 min before fluorescence measurements.

Sample Preparation

Urine samples were treated as follow before the spectrofluorimetric measurement of rosuvastatin. A convenient volume of sample (1–4 mL) and 4 mL of chloroform were added to a 15 mL polyethylene tube. Then, the two-phases mixture was vigorously shaken in a vortex apparatus to promote total penetration of one phase in the other and promote the liquid–liquid extraction of rosuvastatin. Afterwards, the mixture was left on the table until separation of the phases and the organic one (containing rosuvastatin) was taken and transferred to another polyethylene tube. The solvent was eliminated under argon flux and the remaining solid was dissolved in 2 mL of water. After that, 2.5 mL of concentrated sulfuric acid was added and the volume was made up to 25 mL with purified water. The resulting solution was left on the table for 40 min before fluorescence measurement.

The same procedure was adopted in the recovery test. However, in this case, 500 μL of a rosuvastatin solution containing known amount of the analyte was added to the urine sample in the beginning of the process.

Results and Discussion

Influence of pH on the Fluorescence Signal of Rosuvastatin

The initials tests showed that rosuvastatin had a native fluorescence, with two absorption peaks (227 and 320 nm) and just one of emission (370 nm, when excited in 227, no fluorescence peak was observed when excited in 320 nm) Fig. 2. The first test performed to achieve the maximum analytical signal of rosuvastatin was the study of the influence of the pH of the medium over the analytical signal, booth the intensity and the peaks position. For do so, different aqueous solutions with the same rosuvastatin concentration (0.5 mg L^{-1}) were prepared using 1 mL of Britton-Robbinson buffer (final concentration of 0.01 mol L^{-1}) in different pH values in the range of 2–10. Fluorescence signals were measured and intensity of the bands of absorption and fluorescence were recorded (Fig. 3).

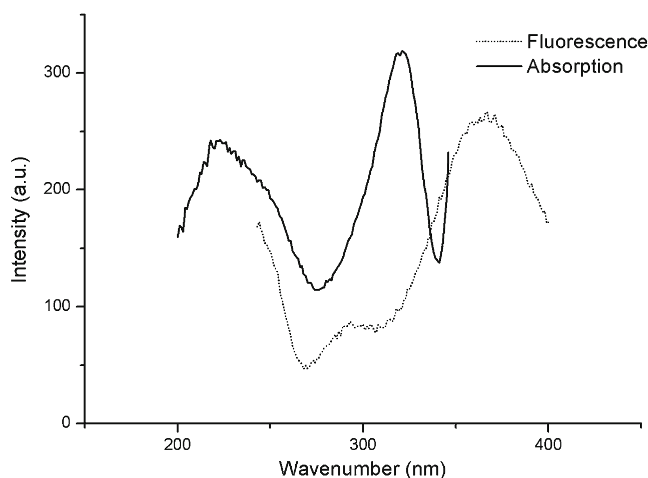

Fig. 2 Natural fluorescence of rosuvastatina in aqueous media. Rosuvastatin concentration = 0.50 mg L-1., slit width = 20 nm

Rosuvastatin contains in its structure acid and basic chemical groups such as the carboxylic and amine groups, respectively (Fig. 1). This characteristic confers an amphoteric behavior to the rosuvastatin in aqueous solutions. In basic solutions the rosuvastatin appears as an anion, due to the dissociation of the carboxylic group. In acid solutions, the carboxylic group appears in its associated form and the nitrogen atoms can be protonated. The dissociation (or not) of the carboxylic

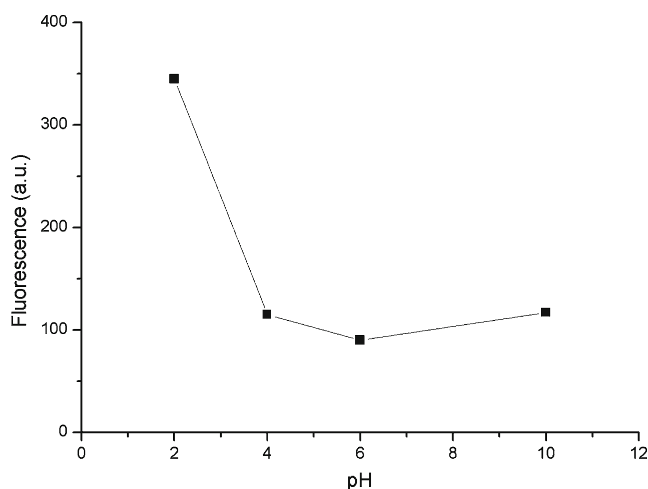

Fig. 3 Influence of the pH on the fluorescence signal of the rosuvastatin in aqueous medium. Rosuvastatin concentration = 0.50 mg L^{-1}, slit width = 20 nm

Table 1 Influence of the ionic strength over a fluorescence analytical signal of a rosuvastatin (2.0 mg L^{-1}) solution in water

[NaCl] (mol L^{-1})	Fluorescence (a.u.)
0	154.0
0.1	98.3
0.2	61.7

group seems not to be important in the fluorescent behavior of the rosuvastatin, since the fluorescence of this molecule is probably associated to the presence of the aromatic group, which is not significantly affected by dissociation (or not) of the carboxylic group. On the other hand, the protonation of the nitrogen atoms can affect the resonance structures of the rosuvastatin, thus affecting its fluorescence intensity.

This hypothesis is corroborated by the results observed in the study of the effect of the pH. In more acidic conditions (pH=2.0), the fluorescence of rosuvastatin is enhanced (Fig. 3). Compounds containing one or more heterocyclic nitrogen atoms have low-lying n $\rightarrow\pi^*$ transitions, which explains their relatively low fluorescence quantum yields [13], the formation of bounds between this nitrogen and hydrogen could performance a inversion of the lowest-lying n–π and π–π* states, what could increase the quantum yeld. In alkaline conditions the nitrogen atoms are not protonated, which causes a decrease of the fluorescence intensity, even with the dissociation of the carboxylic group that does not participate of this process.

This provokes an acid base equilibrium between species according to the pH of rosuvastatin solutions that may have different quantum yield.

In fact, results in Fig. 3 show that the fluorescence signal of rosuvastatin vary depending on the pH of the medium. However, when the signals of all solutions which had their pH adjusted are compared to the one of the solution prepared without pH adjustment (pH value of 5.8, marked as “water” in Table 1), it is seen that the last one is greater. This signal loss can be attributed to the some change in the refractive index of the solution (due the increase in the ionic strength), which prevented a signal gain due to the pH adjustment. To confirm this hypotheses, a study of ionic strength was made (Table 1), this result confirm a decrease in the analytical signal with the increase of ionic strength.

Opposite to the analytical signal, the wavelength of maximum intensity of the peaks of excitation and emission varied minimally in function of pH adjustment.

Table 2 Influence of the solvent system over the analytical signal of rosuvastatin solution 0.50 mg L^{-1}

Solvent	Fluorescence signal	λ (nm)
Water	530	217/340
Acetone–water 1/1	767	314/411
Methanol–water 1/1	956	210/284
Methanol–water 9/1	971	212/285
Ethanol–water 9/1	720	210/284
Ethanol–water 1/1	975	210/280

Table 3 Analytical parameters

Parameter	value
Angular coefficient	294.17 (L/mg)
linear coefficient	200.02(L/mg)
r^2	0.9934
L.D.	0.38 mg L^{-1}
L.Q.	1.28 mg L^{-1}
Linear range	5.00 mg L^{-1}
Repeatability (0.5 mg L^{-1})	2.5 % (DPR)

Influence of Solvent System Over the Analytical Signal

Another test carried out with the objective to increase the analytical signal was the study of the solvent system. Different combinations of water/ethanol, water/methanol and water/acetone were studied to find the best analytical condition. These results are shown in Table 2

Data presented in Table 3 indicates that the ethanol-water 1/1 system provides more intense analytical signal in comparison to the other ones studied.

It is also remarkable that the wavelength of maximum intensity of excitation and emission vary significantly with the solvent change, different to what was observed during the study of the pH influence.

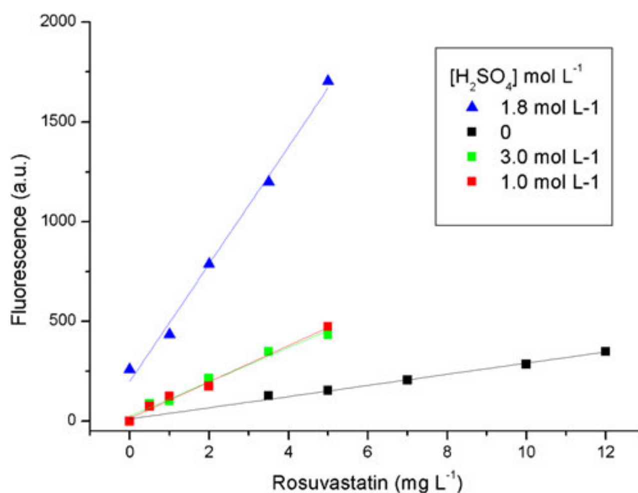

Fig. 4 Analytical curves for rosuvastatin in different sulfuric acid concentrations. Analytical conditions: reaction time of 30 min, 60 °C and slit of 20

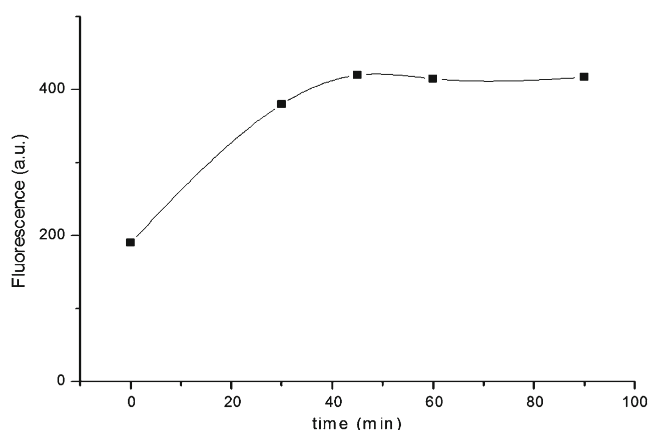

Fig. 5 Influence of reaction time over rosuvastatin fluorescence. Analytical conditions: [RSV]=4.0 mg L^{-1}, 60 °C, [H_2SO_4]=1.8 mol L^{-1}, slit 10

Influence of Concentration of Sulfuric Acid Over the Analytical Signal

In the fluorescence technique, it's relatively common to make a derivative step to obtain a product with better quantum yield. There are several reagents used for this purpose, like dansyl chloride [14, 15], fluorescein isothiocyanate [16, 17], rhodamines [18], sulfonyl chlorides [19], etc. Another approach to induce or increase fluorescence is to make a derivative step with strong base or acid, which can induce hydrolysis or another kind of reaction with the analyte [20].

Tests with NaOH and H_2SO_4 were performed and any alteration in the analytical signal was observed for the base addition, independent of the several concentrations tested. Nevertheless, an increase in rosuvastatin fluorescence signal was obtained when using sulfuric acid.

As a consequence of this observation, more accurate studies were performed to understand the influence of concentration of sulfuric acid over rosuvastatin fluorescence. Different analytical curves were prepared by adding different volumes of concentrated sulfuric acid. Figure 4 shows these curves. It is known that the time of reaction and temperature of the solution may also affect the analytical signal, and then the reaction time was fixed in 30 min and the temperature of the reaction medium in 60 °C.

By Fig. 4 it is possible to see that an increase in the analytical signal of 12 times is obtained with sulfuric acid concentration of 1.8 mol L^{-1}, in comparison to the analytical curve without any acid treatment.

This behavior could be explained by the same reason that made the variation of pH in the reaction medium not to influence the analytical signal. As it was said before, the ionic strength was the reason why the analytical fluorescence signal was higher for the solution without pH adjustment. Then, it is possible to conclude that the reaction of hydrolysis might be still happening until the point of concentration of sulfuric acid of 1.8 mol L^{-1}. Beyond this point, the acid's role turns into simply increase the ionic strength of the solution and, as a consequence, it decreases the analytical signal.

The two other parameters (temperature and time) that could affect the reaction and the analytical signal were also studied.

Influence of Reaction Time and Temperature Over the Analytical Signal

In order to evaluate the influence of reaction time, several solutions of the same rosuvastatin concentration (4.0 mg L^{-1}) and sulfuric acid (1.8 mol L^{-1}) were prepared. The temperature of the solutions was maintained in 60 °C, as the fluorescence signals were measured in different moments (Fig. 5). In all experiments the control of temperature was made in a bath with temperature control.

By Fig. 5 it is possible to see that for 40 min there was an increase in rosuvastatin fluorescence, beyond this point, no alteration was observed. This fact shows that it is necessary to leave the solutions at rest for 40 min before taking measurements.

The influence of temperature was studied by varying this parameter in identical solutions. A solution with no adjustment (not placed in the bath) was also used. It was observed that the increase of temperature due the dissolution of sulfuric acid was high enough to complete the reaction. Then, no temperature control was used.

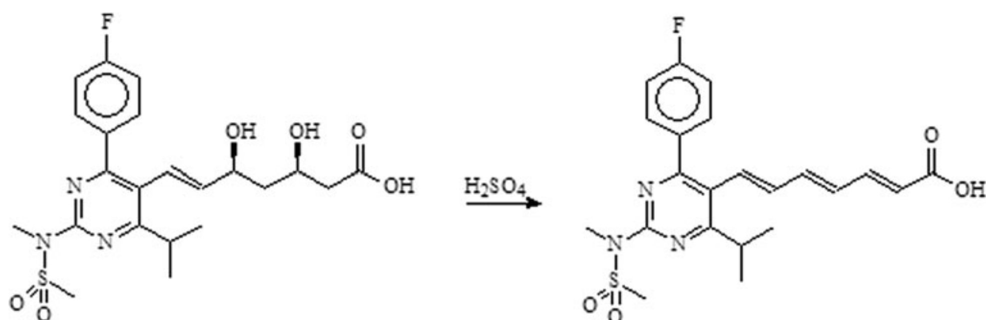

Fig. 6 Dehydration of rosuvastatin by sulfuric acid

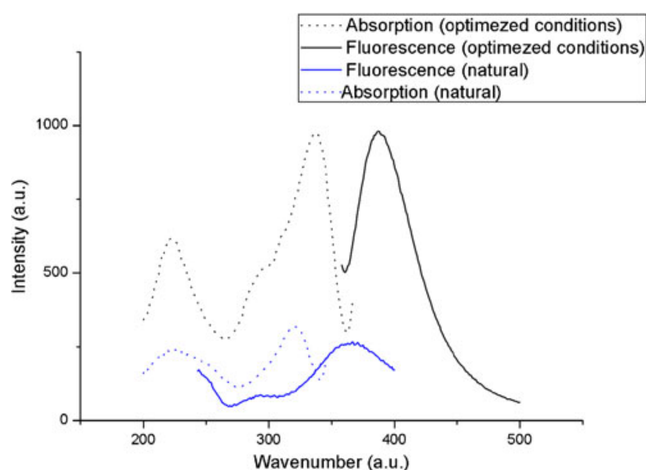

Fig. 7 Fluorescence spectrum from rosuvastatin in water without any treatment "a" and in the optimized conditions "b", RSV concentration of 2.0 mg L^{-1}, slit of 10

Reaction Proposal

The main idea when using acid or base to increase fluorescence is to induce the reaction of hydrolysis of the analyte, producing a fragment of smaller size, more rigid than the original molecule, which is able to increase fluorescence by reduction of external conversion. If the fragment eliminated don't have any chromophore group, the increase is achieved with no loss of the molecule's ability to absorb light, what increases even more it's fluorescence.

Figure 6 shows the reaction of dehydration of rosuvastatin by sulfuric acid. If it happens indeed, the new conjugated bonds increase the molecule's capacity to absorb electromagnetic radiation and also the molecule rigidity. Both effects increase fluorescence.

The increase in the analytical signal after the acid treatment was accompanied by a shift in the wavelength of the fluorescence, from 217/340 nm to 337/387 nm, and the presence of a small peak at 290 nm (absorption spectra). This shift supports the proposed reaction's idea, as long as it's a consequence of the increase in the chromophore system, and the creation of new conjugated bonds could explain the creation of a new absorption peak. In Fig. 7 is possible to see the difference in the fluorescence spectrum obtained with the acidic treatment.

Comparing the Acid Treatment with the Solvent System Applied

Several rosuvastatin solutions 4.0 mg L^{-1} were prepared, some under acid treatment (sulfuric acid 1.8 mol L^{-1}, 40 min of reaction time), some in ethanol/water 1:1 and some only in water.

The gain in sensibility by the use of acid treatment was 12 times higher compared to the solutions prepared in water without such treatment. Also, the gain in sensibility achieved for the change in the solvent system from water to ethanol/water 1:1 was just 2 times better. For this reason only the acid treatment was applied.

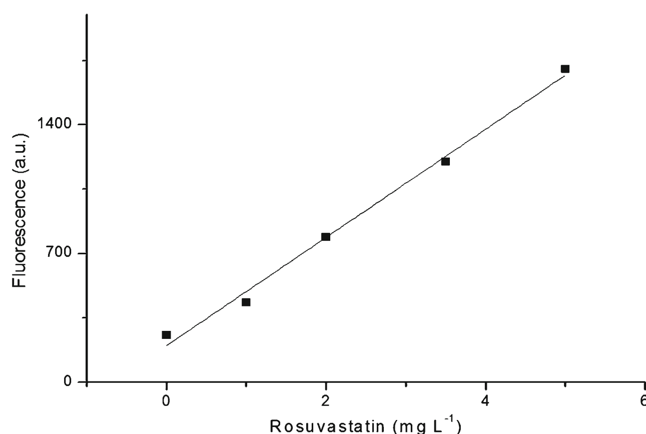

Fig. 8 Analytical curve of rosuvastatin

Analytical Parameters of Merit

Once the best conditions to obtain the maximum analytical signal were achieved, analytical curves were made to obtain the parameters of merit. One of this curves is represented in Fig. 8.

The analytical curve was prepared by taking around three measurements at each concentration, after reacting the analyte with sulfuric acid in the optimized conditions. The best curve was fitted by the method of least squares.

The linearity of the method was evaluated through linear coefficient correlation (r) and determination coefficient (r^2). Most of the technical literature about validation, like INMETRO [21], define that linearity is achieved when both coefficients are greater than 0.90. At the current case, both coefficients were greater than 0.99.

The criterion used to define the limit of detection (LOD) was

$$LOD = \frac{3S_b}{a} [21]$$

Where S_b is the standard deviation for ten blanks, and a is the angular coefficient of the analytical curve.

Table 4 Recovery tests in urine samples

[Added rosuvastatin] (mg L^{-1})	[Experimental concentration] (mg L^{-1})	Recovery (%)
3.12	3.3±0.2	106±5
3.12	2.9±0.2	92±8
3.12	3.5±0.3	112±8

To calculate the limit of quantification (LOQ), the criterion used was

$$LOQ = \frac{10S_b}{a} [21]$$

According to this, the limit of detection was estimated in 0.38 mg L^{-1} and the limit of quantification in 1.28 mg L^{-1}.

The precision degree of a method provides an estimate of the dispersion of results obtained for the same sample under defined conditions. Typically, these results are expressed by repeatability, intermediate precision and reproducibility [22]. In this work, the repeatability and intermediate precision were calculated.

For repeatability calculation, seven solutions of rosuvastatin were prepared in two concentrations levels (0.5 mg L^{-1} and 3.0 mg L^{-1}) under the optimized conditions. The result was expressed in terms of relative standard deviation: 2.5 % and 0.9 %, respectively.

The intermediate precision is calculated when one of several conditions of analysis is changed: the operator, temperature, day, etc. In this work, the intermediate precision was calculated by measuring the signal of seven rosuvastatin solutions 1.0 mg L^{-1}, prepared by the described acid treatment in different days and by different analysts. The difference relative standard deviation of the analytical signal was 2.4 %.

Application of the Proposed Methodology in Urine Samples

When the proposed methodology was applied for rosuvastatin determination in urine, a spectral interference was observed in rosuvastatin wavelength that prevents its determination. To solve this problem a pre-treatment step was developed based on liquid–liquid extraction, followed by elimination of the organic solvent, with the purpose to separate rosuvastatin and redissolve it in water, to perform later the same acid treatment. The best solvent to extract rosuvastatin from the ones testes was chloroform.

After optimizations, the method was applied in urine samples spiked with rosuvastatin, as it was described previously in item 2.3.2.

By following this procedure and using the typical analytical curve it was possible to make recovery tests to confirm the efficiency of the method. These results of the media of three determinations, made in three different days are shown in Table 4.

Conclusions

From the previous results it can be seen that the analytical approach developed in this work for rosuvastatin determination in urine is efficient, precise and exact. This new methodology, based on the luminescence properties of the analyte, was enhanced by sulfuric acid treatment. In order to accomplish the quantification, an extractive step (in the present case, a liquid–liquid extraction, using chloroform as solvent) must be done to avoid matrix interference.

A reaction was proposed between sulfuric acid and rosuvastatin, which could explain the great improvement in the analytical signal. Also, the conditions of its maximum were studied. Under this optimized conditions, the limit of detection (LOD) for the method was 0.38 mg L^{-1} and the limit of quantification (LOQ) 1.28 mg L^{-1}.

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References

1. Tabas I (2002) J Clin Investig 110:583–590
2. Hardman JG, Gilman AG, Limbird LE (1996) Goodman and Gilman's. The pharmacological basis of therapeutics. McGraw-Hill, New York
3. The Pontifical Academy of Sciences (2010) Working group on atherosclerosis: the 21st century epidemic. Vaticano: s.n.
4. Micallef MA, Garg ML (2009) J Nutr Biochem 20:927–939
5. Campo VL, Carvalho I (2007) Quím Nova 30:425–430
6. Martin PD et al (2003) Clin Ther 25(11):2822–2835
7. Corsini A, Maggi FM, Catapano AL (1995) Pharmacol Res 31(1):9–27
8. Hull CK, Penman AD, Smith CK, Martin PD (2002) J Chromatogr B 772:219–228
9. Oudhoff KA et al (2006) J Chromatogr B 832:191–196
10. Trivedi RK et al (2005) J Pharm Biomed Anal 39:661–669
11. Krishna MV, Sankar DG (2007) E-J Chem 4(1):46–49
12. Gupta A, Mishra P, Shah K (2009) E-J Chem 6:89–92
13. Valeur B (2001) Molecular fluorescence: principles and applications. Wiley-VCH Verlag GmbH
14. Bartzatt R (2001) J Pharmacol Toxicol Methods 45:247–253
15. Naassner M, Mergler M, Wolf K, Schuphan I (2002) J Chromatogr A 945:133–138
16. Wilderspin AF, Green NM (1983) Anal Biochem 132:449–455
17. Hoffmann C, Leroy-Dudal J, Patel S, Gallet O, Pauthe E (2008) Anal Biochem 372:62–71
18. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin de Jong J (1985) J Immunol Methods 80:1–6
19. Suliman FEO, Al-Kindi SS, Al-Kindy SMZ, Al-Lawati HAJ (2006) J Chromatogr A 1101:179–184
20. Instituto Nacional de Metrologia Nomarlização e Qualidade Industrial. Orientações sobre validação de métodos de ensaios químicos. Rio de Janeiro: s.n., 2003 Vols. Vol. DOQ-CGCRE-008
21. Instituto Nacional de Metrologia Nomarlização e Qualidade Industrial. Vocabulário Internacional de termos fundamentais e gerais de metrologia (VIM). *Portaria INMETRO nº 102/88 2007.* Vols. Instituto Nacional de Metrologia Nomarlização e Qualidade Industrial in Vocabulário Internacional de termos fundamentais e gerais de metrologia (VIM),, Vol. Portaria INMETRO nº 102/88 2007
22. Nozaki O, Ohata T, Ohba Y, Moriyama H, Kato Y (1991) J Chromatogr B: Biomed Sci Appl 570:1–11