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Precision meets affordability: A highly sensitive HPLC-FLD technique for accurate pitavastatin quantification in human plasma†

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Abstract: High Performance Liquid Chromatography (HPLC) with ultraviolet/visible (UV/Vis) or diode array detection (DAD) is routinely used for drug quantification in R&D all around the world. However, it may lack the sensitivity required for bioanalytical studies. On the other hand, HPLC with fluorescence detection (FLD) is a cost-effective alternative that significantly increases drug signal, allowing the detection of compounds at very low concentrations. Pitavastatin is a lipid-lowering drug that contains the structure of quinoline, a highly fluorescent molecule. Recently, it has gained interest due to its pleiotropic effects on different conditions. Bearing this in mind, an HPLC-FLD method was herein developed and validated for the quantification of pitavastatin in human plasma. Overall, a signal gain of 54-70 times was achieved when using fluorescence vs UV detection. Sample preparation included a one-step protein precipitation with acetonitrile, followed by centrifugation and filtration prior to injection. Pitavastatin was separated from endogenous matrix interferents using a C18 column and applying a gradient elution. Atorvastatin was used as internal standard. Accordingly, the method was shown to be selective, specific, and sensitive, with the lower limit of quantification of 3 ng/mL, a complete absolute and relative recoveries higher than 94%. The method was linear over the wide concentration range of 3 – 900 ng/mL (R^2 = 0.998), accurate (bias < 7.15 %) and precise (RSD < 9.63 %). This method allows the therapeutic monitoring of patients treated with pitavastatin but can also support novel clinical studies of this drug in human plasma.

Keywords: Fluorescence detection; bioanalytical; pharmacokinetics; pitavastatin; human plasma

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1. Introduction

High Performance Liquid Chromatography (HPLC) with ultraviolet/visible (UV/Vis) or diode array detection (DAD) is routinely used in R&D facilities all around the world. This technique is extremely versatile, allowing the simultaneous quantification of a large pool of compounds differing in chromophores and absorption maxima in the UV-Vis range. Additionally, HPLC-DAD also provides structural information, giving insights on peak purity and identification. Notwithstanding, UV-Vis based detection may not guarantee the high sensitivity for bioanalytical studies, in which lower limits of quantification are required. On the contrary, fluorescence is a cost-affordable technique that takes

advantage of the emitted light by a certain compound following electron excitation at certain wavelengths. This can be observed in intrinsic fluorescent molecules or upon derivatization (e.g. o- o-phthalaldehyde (OPA) reacts with primary amines and forms fluorescent isoindole derivatives). As the fluorescent signals are usually stronger, FLD lowers the limits of quantification of a chromatographic technique, bringing them closer to those of LC with mass spectrometry.

Pitavastatin is a synthetic 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitor prescribed to treat hyperlipidemia. There is increasing evidence that expands the therapeutic effect of this statin to several other diseases: these pleiotropic effects go from decreasing inflammation and oxidative stress, to improving endothelial function, regulating angiogenesis, osteogenesis and reducing tumor progression [1].

There is only a limited number of chromatographic methods for pitavastatin quantification in human plasma, with the majority relying on mass detection [2-5]. One method uses UV [6] and two others fluorescence detection [7, 8]. Concerning the latter, Ojha et. al. use 1 mL of human plasma, liquid-liquid extraction and 85 μL of injection volume, while Zhou and colleagues use 100 μL of plasma, protein precipitation, 50 μL of injection volume but no internal standard.

This work focuses on the development of an HPLC-FLD method for the quantification of pitavastatin in human plasma samples, allowing the therapeutic drug monitoring and supporting future clinical and preclinical studies of this statin.

2. Materials and Methods

2.1. Chemicals and reagents

Pitavastatin calcium (CAS number: 147526-32-7) and atorvastatin calcium (CAS number: 134523-03-8) were kindly supplied by Hovione (Loures, Portugal) and the Tecnimede Group (Sintra, Portugal), respectively. The different drugs used for the specificity study were of analytical or HPLC grade. Acetonitrile and methanol HPLC gradient grade and glacial acetic acid were purchased from Honeywell and Carlo Erba Reagents (Milan, Italy), respectively. Water was ultrapurified (Ω = 18.2 MΩ.cm, TOC < 1.5 μg/L) and filtered (0.22 μm) before use (Sartorius arium pro, Gottingen, Germany). Human plasma was collected from healthy non-treated donors, supplied by the Centro de Sangue e da Transplantação de Coimbra, Portugal, and stored at -20 °C.

2.2. Chromatographic equipment and conditions

Pitavastatin was quantified in a Shimadzu Prominence Modular system equipped with a quaternary pump, autosampler, oven and two sequential detectors: SPD-M20A for UV/Vis (photodiode array) and RF-20A XS for fluorescence detection. Analyte separation was conducted using a LiChroCART® Purospher Star reversed-phase column with 3 μm particle size, 4 mm of internal diameter and 55 mm length (Merck KGaA, Darmstadt, Germany). The analysis was performed with acetic acid (2% v/v):methanol in gradient mode at 1 mL/min, at 35 °C under the following conditions: 4 min (55:45), 8 min (35:65), 12 min (35:65), 15 min (55:45), 20 min (55:45). Analyte detection was set at λ_{ex} = 257 nm, λ_{em} = 421 nm for fluorescence and λ_{max} = 250 nm for UV/Vis.

2.3. Preparation of solutions, calibration standards and quality control samples

Stock solutions of each statin were prepared in methanol (1 mg/mL). Pitavastatin working solutions were then prepared by dilution at different concentrations: 0.03, 0.06, 1, 3, 6 and 9 μg/mL. Quality control (QC) solutions were prepared independently at the lower limit of quantification (QC_{LLOQ}), low (QC_1), medium (QC_2) and high (QC_3) concentration levels: 0.03, 0.09, 4.5 and 7.5 μg/mL, respectively. The atorvastatin working solution was prepared at 50 μg/mL.

2.4. Sample preparation and extraction procedure

Each aliquot (90 µL) of plasma was spiked with 10 µL of a working/QC solution and IS. Following the addition of 200 µL of acetonitrile, samples were vortexed for 2.5 min and centrifuged at 14 000 rpm, at 4 °C. The supernatant was then filtered through a PTFE membrane (0.22 µm) before HPLC quantification.

2.5. Method Validation

The chromatographic method was validated according to the ICH M10 guideline on bioanalytical method validation and study sample analysis [9].

2.5.1. Selectivity and Specificity

Selectivity and specificity were evaluated through the analysis of blank and spiked chromatograms from six different plasma donors, in terms of potential interferents at the retention times of the statins. A pool of thirty approved drugs in the clinic, potentially co-administered, were also inspected under the optimized chromatographic conditions.

2.5.2. Linearity

Calibration standards were prepared daily for six days using six plasma samples spiked with the working calibration solutions, as well as with the IS. The peak area ratio between pitavastatin and atorvastatin was plotted against the corresponding nominal plasma concentrations. Data heterocesdaticity was corrected using the weighting factor with the lowest sum of absolute error amongst all the standards.

2.5.3. Accuracy and precision

Accuracy and precision were evaluated at the intra and interday levels by analysing six replicates of each QC sample on a single day, as well as on three consecutive days, respectively. The acceptance criteria was defined at a bias ≤ 15 % and coeficient of variation (CV) ≤ 15 % for QC_1, QC_2 and QC_3, and a bias ≤ 20 % and CV ≤ 20 % for QC_{LLOQ}.

2.5.4. Recovery and carryover

The recovery of each statin from the matrix was determined in six samples at each QC level, by comparing the concentrations from extracted samples with the equivalent extracts from post-extraction spiked samples. Carryover was inspected through the injection of blank samples after the calibration standard of the upper limit of quantification, and considering the 5% and 20% limits for IS and LLOQ, respectively.

2.5.5. Stability

Sample stability (spiked plasma and processed samples) was assessed in six replicates at the different quality control levels (QC_{LLOQ}, QC_1, QC_2 and QC_3), in four different experimental conditions, mimicking sample handling and storage time during the analyses.

3. Results and Discussion

3.1. Method Development

The chromatographic method was developed aiming at quantifying pitavastatin in human plasma. For that reason, an internal standard was introduced to guarantee the reproducibility of sample preparation. A gradient elution was implemented and refined in order to reduce the analysis time without compromising any chromatographic parameters (peak symmetry, retention and resolution), due to the distinct behaviour under isocratic conditions of both statins while using acetic acid (2% v/v) and methanol. Under the optimized conditions, pitavastatin elutes at 5.62 min while atorvastatin elutes at 13.65 min in a 20 min analysis.

3.2. Method Sensitivity

The detection of pitavastatin was optimized in terms of signal intensity (peak area), at the maximum experimental wavelenghts (λ_{ex} = 257 nm, λ_{em} = 421 nm for fluorescence and λ_{max} = 250 nm for UV). For that, a total of four calibration curves were plotted through simple linear regression in spiked plasma samples (**Figure 1A**). Overall, FLD allows a peak area gain ranging from 54 to 70, as well as a substantially lower limit of detection and quantification (0.003 vs 0.100 μg/mL). Reference plasma concentrations for pitavastatin range from 0.031 – 0.081 μg/mL, thus highlighting the relevance of the FLD method [10].

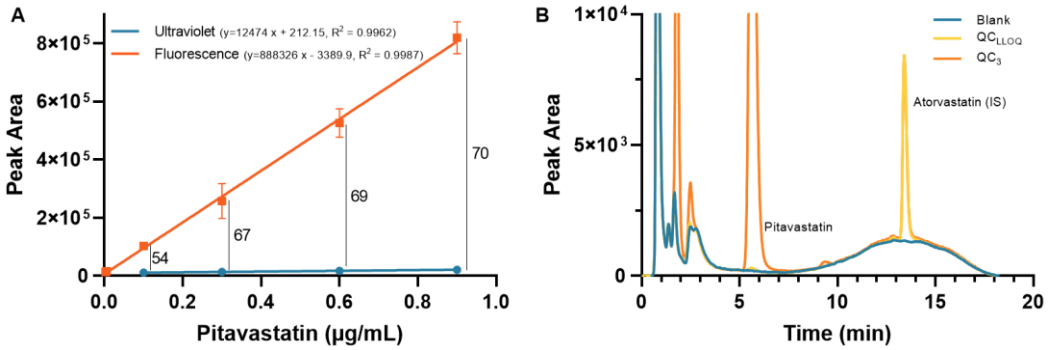

Figure 1. **A.** Detection sensitivity between fluorescence and photodiode array for pitavastatin (0.003 to 0.9 μg/mL); **B**. Representative chromatogram of blank and spiked plasma samples, showing absence of peaks at the retention time of pitavastatin and atorvastatin in the former one.

3.3. Selectivity and Specificity

Selectivity was evaluated through the visual comparison between six blank plasma samples of different donors and spiked samples (QC_{LLOQ} and QC_3). No interferents were found at the retention times of pitavastatin and IS (**Figure 1B**). Furthermore, the method was applied to a pool of thirty different drugs commonly used in the clinic, to infer on potential peaks that may impair the quantification of the analyte. Several compounds can be detected under the optimized conditions, displaying different retention times (**Table 1**). However, there are no drugs that co-elute with pitavastatin or IS, thereby guaranteeing specificity and supporting the potential use of the method in the clinic.

Table 1. Method specificity analysis considering thirty commonly prescribed drugs in the clinic.

	Retention Time			Retention Time			Retention Time	
Drug	**UV/Vis**	**FLD**	**Drug**	**UV UV/Vis**	**FLD**	**Drug**	**UV UV/Vis**	**FLD**
Acetaminophen	0.73	ND	**Furosemide**	2.03	2.15	**Perampanel**	7.94	8.05
Amitriptyline	1.00	ND	**Lacosamide**	ND	ND	**Propranolol**	1.91	2.02
Antipyrine	0.63	0.72	**Levetiracetam**	ND	ND	**Quinidine**	0.81	0.91
Apixaban	3.24	3.36	**Levofloxacin**	0.67	0.79	**Rivaroxaban**	2.62	ND
Cimetidine	ND	ND	**Metformin**	ND	ND	**Sertraline**	ND	ND
Ciprofloxacin	0.83	0.71	**Metoprolol**	ND	ND	**Sulfasalazine**	5.64	ND
Dabigatran	0.63	0.73	**Moxifloxacin**	0.94	1.04	**Theophylline**	0.79	1.12
Digoxin	9.57	ND	**Naproxen**	9.66	9.78	**Trazodone**	1.27	1.38
Enalapril	ND	ND	**Nifedipine**	6.74	ND	**Verapamil**	2.96	3.05
Escitalopram	1.91	2.02	**Paroxetine**	4.28	4.39	**Zonisamide**	0.97	ND

ND: not detected.

3.4. Linearity

Despite being linear, there is a significant variance within the calibration standards due to the wide range of the curve. Larger deviations at higher concentrations have a profound impact on the regression line, thus decreasing accuracy for the lower concentration levels. Calibration curves were corrected using different weighting factors, with $1/x^2$ resulting in the lowest sum of absolute error (380.0 ($1/x^2$) vs 393.7 ($1/x$); 396.6 ($1/y^2$); 399.9 ($1/y$); 455.2 ($1/\sqrt{y}$); 457.2 ($1/\sqrt{x}$) and 866.8 (non-corrected)). The method was shown to be linear over the range 0.003 – 0.9 μg/mL, with [Pita] = 8.439 peak area ratio + 0.024 and R^2 = 0.998.

3.5. Accuracy and precision

Data obtained from the intra- and interday accuracy and precision for QC_{LLOQ}, QC_1, QC_2 and QC_3 are reported in Table 2. For all QC levels, accuracy ranged from -6.7 to 5.97 % and precision varied between 0.88 to 9.63 %. All these results are in agreement with the ICH M10 guideline, confirming the method is both accurate and precise in quantifying pitavastatin in human plasma.

Table 2. Intraday and interday accuracy and precision.

Conc. (μg/mL)	Intraday (n=6)		Interday (n=18)	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.003	- 1.2	8.8	- 6.6	8.8
0.009	2.5	4.5	- 1.1	6.5
0.45	7.2	0.9	3.5	9.6
0.75	6.0	2.5	- 2.7	8.41

3.6. Recovery and carryover

The absolute recovery of pitavastatin was found to be 94.5 ± 10.7 % (QCL_{LOQ}), 94.9 ± 3.8 % (QC_1), 96.1 ± 1.2 % (QC_2) and 100.7 ± 5.3 % (QC_3) (n = 6). The absolute recovery for the IS was found to be 99.7 ± 4.7 % (n = 18). This can be explained by the simple sample preparation step, in wich the statins are fully released from the plasma sample and injected in the chromatographic system. No carryover effect was denoted in the consecutive injections of blank samples following the injection of an ULOQ sample, in terms of peaks at the retention times of the statins.

3.7. Stability

Sample stability was evaluated using spiked plasma samples, as well as processed ones in four different conditions (spiked plasma: room temperature, 4 h and - 80 °C, 15 days; processed: autosampler, 24 h and - 20 °C, 7 days). No stability concerns were raised for both pitavastatin and the IS, as accuracy and precision < 15 % for all QC levels.

Table 3. Stability studies of quality control samples (n = 6) in different conditions.

Conc. (μg/mL)	Spiked plasma (unprocessed)				Processed Samples			
	Room temperature, 4h		-80 °*C*, 15 days		Autosampler, 24 h		-20 °C, 7 days	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
0.003	7.8	10.7	10.6	6.8	- 0.8	3.7	5.5	9.6
0.009	2.3	5.8	6.0	5.0	7.3	5.4	3.5	3.5
0.45	- 3.2	1.3	- 3.1	1.2	- 0.8	1.3	- 3.2	1.4
0.75	2.4	8.2	0.2	6.7	2.3	7.3	- 0.8	7.4

4. Conclusions

A highly sensitive and accurate HPLC-FLD method for pitavastatin quantification was developed and fully validated, allowing the quantification of this statin in human plasma. The separation of pitavastatin and the internal standard (atorvastatin) was achieved after a single step protein precipitation with centrifugation and filtration. The quantification of pitavastatin by HPLC highly benefits from fluorescence detection: a 54-70-fold increase in peak area is achieved in comparison to a standard UV determination. Consequently, there is a remarkable reduction in the limit of quantification from 0.1 (HPLC-UV/Vis) to 0.003 μg/mL, without compromising the accuracy and precision of the results. The method can be used to monitor plasma levels of pitavastatin in the clinic, but can also be at the base of novel clinical and preclinical studies involving this drug.

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Conflicts of Interest: The authors declare no conflict of interest.

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