applied sciences

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A Validated HPLC-MS/MS Method for Quantification of Fingolimod and Fingolimod-Phosphate in Human Plasma: Application to Patients with Relapsing–Remitting Multiple Sclerosis

Claudia Fracasso [1], Alice Passoni [2], Laura Brambilla [3], Renato Mantegazza [3], Silvia Rossi [3], Marco Gobbi [1] and Jacopo Lucchetti [1,*]

[1] Department of Biochemistry and Molecular Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, 20156 Milan, Italy; claudia.fracasso@marionegri.it (C.F.); marco.gobbi@marionegri.it (M.G.)
[2] Department of Environmental Health Sciences, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, 20156 Milan, Italy; alice.passoni@marionegri.it
[3] Neuromuscular and Neuroimmunology Unit, Foundation IRCCS Neurological Institute Carlo Besta, 20133 Milan, Italy; laura.brambilla@istituto-besta.it (L.B.); renato.mantegazza@istituto-besta.it (R.M.); silvia.rossi@istituto-besta.it (S.R.)
* Correspondence: jacopo.lucchetti@marionegri.it

Featured Application: The analytical method described in this work can be applied to evaluate the inter-individual differences in the bioavailability of Fingolimod and its metabolite Fingolimod-phosphate, and to possibly relate these differences to inter-individual variability in clinical responses.

Abstract: Fingolimod is a sphingosine 1-phosphate-receptor modulator approved for the oral treatment of relapsing–remitting multiple sclerosis (RRMS), a form of MS characterized by a pattern of exacerbation of neurological symptoms followed by recovery. Here, we validated a simple and rapid liquid chromatography–tandem mass spectrometry method for the measurement of the concentrations of Fingolimod and its active metabolite Fingolimod-Phosphate (Fingolimod-P) in human plasma. The lower limits of quantification were set at 0.3 and 1.5 ng/mL for Fingolimod and Fingolimod-P, respectively, and the linearity was in the range 0.3–150 ng Fingolimod/mL and 1.5–150 ng Fingolimod-P/mL. After protein precipitation, the extraction recoveries of both analytes were always above 60% with minimal matrix effect. The method was accurate and precise, satisfying the criteria set in the European Medicine Agency guidelines for bioanalytical method validation. The method was then applied to measure Fingolimod and Fingolimod-P concentrations in the plasma of 15 RRMS patients under chronic treatment with Fingolimod, administered daily at the dose of 0.5 mg for up to 24 months. No significant differences were observed between samples collected at 6, 12 and 24 months for both analytes, indicating that the drug's bioavailability was unaffected by multiple daily doses up to 24 months. The levels of Fingolimod-P were about two-fold higher than the levels of the parent compound. The availability of this analytical method can allow the monitoring of the impact of plasma levels of the drug and its metabolite on inter-individual variability in clinical responses.

Keywords: Fingolimod; Fingolimod-phosphate; HPLC-MSMS method; human plasma; relapsing–remitting multiple sclerosis

Citation: Fracasso, C.; Passoni, A.; Brambilla, L.; Mantegazza, R.; Rossi, S.; Gobbi, M.; Lucchetti, J. A Validated HPLC-MS/MS Method for Quantification of Fingolimod and Fingolimod-Phosphate in Human Plasma: Application to Patients with Relapsing–Remitting Multiple Sclerosis. *Appl. Sci.* **2022**, *12*, 6102. https://doi.org/10.3390/app12126102

Academic Editor: Burkhard Poeggeler

Received: 12 May 2022
Accepted: 13 June 2022
Published: 15 June 2022

1. Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system characterized by neuroinflammation that lead to extensive astrogliosis, demyelination, and neurons lost [1]; MS represents the leading cause of neurological disability in young

people and is commonly diagnosed between 20 and 40 years old [2]. Relapsing–remitting MS (RRMS) accounts for about 80% of patients with MS and is characterized by recurrent acute exacerbations (relapses) of neurological symptoms, followed by periods of partial or complete recovery (remission) [3].

Disease-modifying therapies (DMTs) represent the standard care for RRMS, aiming to reduce the frequency and severity of relapses, extending the time intervals between relapses and slowing the progression to permanent disability [4]. Among the most prescribed first-line DMTs, interferon beta and glatiramer acetate are administered by injections and associated with injection-site reactions, poor patient adherence and moderate efficacy [5]. Novel oral agents with different mechanisms of action have been approved in recent years for the treatment of MS, both as first- and second-line therapies [6]. Among them, Fingolimod (FTY720, Gilenya; Novartis International AG, Basel, Switzerland) was the first oral therapy approved for the treatment of RRMS; Fingolimod is a sphingosine 1 phosphate-receptor modulator that selectively and reversibly retains naïve and central memory T-lymphocytes within lymph node avoiding penetration in other tissues such as the central nervous system [7,8]. After oral administration, Fingolimod is slowly absorbed (t_{max}: 12–16 h) and is transformed by reversible phosphorylation to Fingolimod-Phosphate (Fingolimod-P), the active metabolite [9,10]. Based on two large clinical trials [11,12], the use of Fingolimod was approved in the USA (September 2010) as a first-line treatment and recommended in the EU (by March 2011) for patients with high disease activity despite previous treatment with at least one other DMT and individuals with rapidly evolving severe RRMS. Efficacy data of Fingolimod have been successively updated by long-term observational studies on some of those patients enrolled in the original trials [8].

Here, we validated a liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) method for simultaneous quantification of Fingolimod and its active metabolite Fingolimod-P in human plasma. The method was set up for its application within a multicentric randomized pragmatic trial whose primary objective was to compare the effectiveness of Fingolimod versus dimethyl-fumarate in RRMS patients (PRAG-MS, EUDRACT number 2017-000559-26, ClinicalTrials.gov Identifier: NCT03345940). In fact, the project also aimed to investigate, as an exploratory endpoint, the association between blood drug concentrations and clinical responses over 6, 12 and 24 months of treatment. Unfortunately, the project was terminated due to the slowness of the recruitment activity. Here, we present the data obtained measuring Fingolimod and Fingolimod-P in a subset of the recruited patients, as a complement of the method development and as a proof of its applicability to clinical samples.

2. Materials and Methods

2.1. Reagents and Chemicals

HPLC-MS grade 2-propanol (2-Prop), methanol (MeOH), acetonitrile (ACN), and formic acid (HCOOH) were from Sigma-Aldrich Co., (Milan, Italy). A milli-Q system (Millipore, Bedford, MA, USA) provided HPLC-MS grade water Fingolimod, Fingolimod-P and the deuterated internal standards (IS) Fingolimod-D4 and Fingolimod-P-D4 were from TLC Pharmaceutical standards (ON, Canada). Blank human plasma samples were from a pool of donors (batch PLA002211C002) and from six individual donors (batches PLA021A020C000-1/6), all of them purchased from Biopredic (Biopredic International, Saint Grégoire, France).

2.2. Stock and Working Solution of Fingolimod and Fingolimod-P

Stock solutions of Fingolimod and Fingolimod-P were prepared in MeOH at 1 mg/mL and diluted in the same solvent to obtain intermediate solutions at 100 µg/mL. These intermediate solutions were combined and serially diluted in MeOH to seven calibration working solutions containing both analytes at 0.03, 0.05, 0.1, 0.2, 0.5, 1 and 3 µg/mL. Four independent quality-control (QC) working solutions containing both analytes at 0.03, 0.08, 0.8 and 2 µg/mL for were used to prepare QC samples. Stock solutions of the internal standards Fingolimod-D4 and Fingolimod-P-D4 were prepared in MeOH at 1 mg/mL and

subsequently diluted in the same solvent to the working solution 0.2 µg/mL containing both internal standards. Stock and working solutions were stored at −20 °C until use.

2.3. Preparation and Processing of Plasma Samples

Seven- (Fingolimod) or eight-point (Fingolimod-P) calibration curves were prepared by adding five µL of Fingolimod/Fingolimod-P calibration working solutions (one µL of the Fingolimod lowest working solution for the LLOQ as an exception) to 100 µL of control plasma: final plasma concentrations range of 0.3–150 ng/mL for Fingolimod and 1.5–150 ng/mL for Fingolimod-P. Similarly, QC plasma samples were prepared adding the QC working solutions to obtain the final plasma concentrations of 0.3 ng Fingolimod/mL or 1.5 ng Fingolimod-P/mL (lower quality control, LLQC), 4 ng/mL (low quality control, LQC), 40 ng/mL (mid quality control, MQC) and 100 ng/mL (high quality control, HQC). After the addition of 5 µL of IS 0.2 µg/mL (final plasma concentration 10 ng/mL), protein precipitation was achieved by mixing plasma samples with 500 µL of cold ACN/HCOOH (99/1, *v*/*v*) followed by 10 min centrifugation at 14,000× *g* (4 °C). A total of 4 µL of the supernatants were injected into the HPLC-MS/MS system.

Plasma samples from treated patients were thawed at room temperature and processed in the same way.

2.4. HPLC-MS/MS System

The Agilent 1200 HPLC system (Santa Clara, CA, USA) coupled with the AB SCIEX triple quadrupole 5500 (AB Sciex, Thornhill, ON, Canada) was used for quantification of Fingolimod and Fingolimod-P in human plasma.

Chromatographic separation was made using a Luna C18 column (150 × 2 mm, 3 µm particle size; Phenomenex Inc., Torrance, CA, USA) with Security Guard C18 4 × 2 mm (Phenomenex Inc.). Mobile phases (MP) were 0.1% formic acid in water (MP-A) and acetonitrile (MP-B), and flow rate was set at 200 µL/min. Autosampler and column oven temperatures were set, respectively, at 8 °C and 35 °C. Elution started with an 8-min linear gradient from 40% to 99% of MP-B held for 3 min and followed by a 1-min linear gradient for returning to the initial condition that was kept for 5 min for column equilibration. The total run time was 17 min. Retention times were 4.68 min (Fingolimod-D4), 4.69 min (Fingolimod) and 5.60 min for both Fingolimod-P and its internal standard Fingolimod-P-D4.

The mass spectrometer was equipped with an electrospray ionization source (ESI) operating in positive ion mode. The mass spectrometer analysis was made in multiple reaction monitoring (MRM) measuring the fragmentation products of the protonated molecular ions of analytes and their internal standards. For each analyte, two ion mass transitions were acquired, one for peak quantification (quantifier ions) and one for analyte confirmation (qualifier ions). The optimized ion transitions and the corresponding collision energy (CE) are show in Table 1.

Table 1. Mass precursor-to-fragment ion transitions (with collision energy, CE) and retention times of analytes.

Analyte	Precursor Ion, *m*/*z*	Quantifier/Qualifier Ions, *m*/*z* [CE, V]
Fingolimod	308.2	143.1 [27]/255.2 [21]
Fingolimod-P	388.2	255.2 [23]/290.2 [18]
Fingolimod-D4	312.2	147.1 [27]/259.2 [21]
Fingolimod-P-D4	392.2	259.1 [23]/294.2 [18]

The optimized mass spectrometric parameters for curtain gas pressure, collision gas pressure ion spray voltage, probe temperature, declustering potential, entrance and collision exit potential were, respectively, 32.0 psi, 7.0 psi, 400 °C, 5500 V, 50 V, 11 V and 16 V. The turbo ion spray source was set at 350 °C. The Analyst Software 1.6.1 (AB Sciex) controlled the HPLC-MS/MS system, and the same software was used for data collection.

2.5. HPLC-MS/MS Method Validation

The analytical method was validated in human plasma according to EMA guidelines [13]. A freshly prepared calibration curve was used in each validation run.

2.5.1. Linearity, Lower Limit of Quantification and Selectivity

All calibration curves included one blank plasma sample, one blank plasma fortified only with IS, and eight-point calibration standards at 0.3, 1.5, 2.5, 5, 10, 25, 50 and 150 ng/mL for Fingolimod or seven-point at 1.5, 2.5, 5, 10, 25, 50 and 150 ng/mL for Fingolimod-P. Responses, i.e., peak area ratio of the analyte to IS, were plotted against the corresponding drug concentration, fitting the data with a weighted ($1/x^2$) linear regression curve. The determination coefficient (r^2) and the mean accuracy of calibrators were used to assess the quality of calibration curve. The lowest calibration standard point (0.3 and 1.5 ng/mL for Fingolimod and Fingolimod-P, respectively) was defined as the lower limit of quantification (LLOQ) of the method if its signal was at least five times the signal of the blank sample.

Selectivity of the method was verified analyzing plasma matrices from six different donors fortified or not with Fingolimod and Fingolimod-P at the LLOQ and their IS at the concentration used.

2.5.2. Accuracy and Precision

Six replicates of QCs at each plasma concentration (0.3, 4, 40 and 100 ng Fingolimod/mL and 1.5, 4, 40 and 100 ng Fingolimod-P/mL) were analyzed in a single run to define the intra-day accuracy and precision (CV%), and over three separate days for inter-day accuracy and precision.

2.5.3. Dilution Integrity

A plasma sample spiked with both analytes at a concentration of 5-fold of the ULOQ was diluted 10-fold (v/v) with blank plasma matrix (six replicates) and the calculated mean concentration had to be from 85 to 115% of the nominal value.

2.5.4. Carry-Over

Carry-over was examined by injecting Fingolimod and Fingolimod-P at the highest plasma concentration (upper limit of quantification, ULOQ, 150 ng/mL). Carry-over was negligible if the signal of the two analytes measured in the blank plasma injecting after ULOQ was <20% the LLOQ signal.

2.5.5. Matrix Effect and Recovery

Matrix effect (MF) and recovery (Rec%) of Fingolimod, Fingolimod-P and the IS were evaluated in six plasma matrices from six different donors analyzing the LQC (4 ng/mL) and HQC (100 ng/mL).

MF was defined by the ratio of (A) the peak area of the Fingolimod and Fingolimod-P added to the plasma matrix after extraction to (B) the peak area of pure solutions of the analytes at the same concentrations (MF = A/B). For each plasma matrix the IS-normalized MF was obtained by dividing the MF of the analyte by the MF of the IS, and the CV% across the six matrices, for each concentration, has been assessed.

Extraction recovery was determined by comparing (C) the peak area of Fingolimod and Fingolimod-P, and for internal standards, spiked to plasma samples before extraction and (A) the peak area of the analytes spiked to the plasma matrix after extraction. Recovery ($C/A*100$) should be reproducible across QC levels.

2.5.6. Stability Studies

Stability of Fingolimod and Fingolimod-P was investigated as follows:

Stock and working solutions long-term stability. Stability of the stock and working solutions of both analytes were assessed at the storage conditions (−20 °C) analyzing six repli-

cates of (i) Fingolimod and Fingolimod-P stock solutions (diluted 1:1000), (ii) Fingolimod and Fingolimod-P working solutions (at the lowest and highest concentration) and (iii) IS working solutions. The mean peak area of the analytes had to be within 15% of the mean peak area in freshly prepared solutions (t = 0).

Stability in plasma. Six replicates of LQC (4.0 ng/mL) and HQC (100 ng/mL) for both analytes were subjected to two *freeze–thaw cycles*. Briefly, after 24 h at the storage conditions (−20 °C and −80 °C), QC samples were thawed to room temperature and the procedure were repeated twice. *Bench-top stability* was evaluated by analyzing six replicates of LQC (4.0 ng/mL) and HQC (100 ng/mL) for both analytes kept at room temperature for 4 h, i.e., maximum samples preparation time. Several QC samples were stored at −20 °C and −80 °C for monitoring *long-term stability*; specifically, LQC and HQC (six replicates for each) were analyzed after 1 3, 6 and 12 months of storage. QCs were analyzed against a freshly prepared calibration curve and compared to the nominal concentrations.

Autosampler stability. Six replicates of extracted LQC and HQC containing both analytes were kept in autosampler for 48 h at 6 °C and compared to freshly analyzed QC samples.

2.6. Application to Clinical Samples

Plasmatic levels of Fingolimod and Fingolimod-P were measured in 15 patients with RRMS, enrolled at the Foundation IRCCS Neurological Institute Carlo Besta (Milan, Italy), within the PRAG-MS multicenter randomized pragmatic trial.

The trial complied with the 1975 Declaration of Helsinki as revised in 2008 and was approved by the Institutional Ethics Committee and from the National Competent Authorities in March 2017. Written informed consent was obtained from each participant prior to entering the study. Fingolimod was administered orally at a dose of 0.5 mg once a day for 24 months and drug measurement was performed at 6, 12 and 24 months during the treatment period, i.e., the time-points pre-selected for clinical assessments. The included RRMS patients were both males (M) and females (F), with a M:F = 4:11 (M 27%) and a mean age of 36.3 years (±10.3) at the beginning of the trial. Mean disease duration at inclusion was 6.4 years (±10.0) with a median EDSS (Expanded Disability Status Scale) of 1.5 (0–4.5).

Blood samples were collected and analyzed from all 15 patients at month 6, from 11/15 at month 12 and from 6/15 at month 24. Blood was collected shortly after the treatment intake (2–5 h) in K3-EDTA tubes, and plasma was separated by centrifugation at 3000 r.c.f (4 °C) for 15 min and stored at −20 °C or −80 °C, until used for the analysis. The researchers involved in the drug measurements had no access to the patient data.

2.7. Statistical Analysis

The GraphPad Prism® program v. 7.05 (GraphPad Software, Inc., La Jolla, CA, USA) was used for processing raw data and for statistical analysis. A 1-sample Kolmogorov–Smirnov Z normality test was used to check data distribution. Plasma levels of Fingolimod and Fingolimod-P were then analyzed by one-way ANOVA followed by Tukey multiple comparison post-hoc test.

3. Results

3.1. HPLC-MS/MS Method Validation

3.1.1. Linearity, LLOQ and Selectivity

All the three calibration curves were linear in the selected concentrations range 0.3–150 ng/mL for Fingolimod and 1.5–150 ng/mL for Fingolimod-P; the determination coefficients (r^2) were always >0.9955 for Fingolimod and >0.9943 for Fingolimod-P and the slopes were highly reproducible with a precision (CV%) less than 15% for both Fingolimod and Fingolimod-P calibration curves (Table 2). The accuracy and precision of the back-calculated concentrations were within the acceptance limits (Table 2).

Table 2. Linearity of calibration curves and LLOQ for Fingolimod and Fingolimod-P (0.3 ng/mL and 1.5 ng/mL, respectively) in human plasma.

Fingolimod											
Three Runs	**Nominal Concentrations (ng/mL)**								**Calibration Curve**		
	0.3	**1.5**	**2.5**	**5.0**	**10.0**	**25.0**	**50.0**	**150.0**			
	Calculated Concentrations (ng/mL)								**Slope**	**Intercept**	**r^2**
Mean	0.302	1.47	2.37	5.30	10.24	25.40	49.50	149.00	0.1225	0.0049	0.9954
±SD	0.003	0.09	0.25	0.35	0.50	2.27	1.76	3.46	0.0058	0.0012	0.0002
Accuracy	100.8%	97.8%	94.8%	106.0%	102.4%	101.6%	99.0%	99.3%			
Precision (CV)	0.8%	5.9%	10.3%	6.5%	4.9%	8.9%	3.6%	2.3%	4.8%		
Fingolimod-P											
Three Runs	**Nominal Concentrations (ng/mL)**								**Calibration Curve**		
	1.5		**2.5**	**5.0**	**10.0**	**25.0**	**50.0**	**150.0**			
	Calculated Concentrations (ng/mL)								**Slope**	**Intercept**	**r^2**
Mean	1.65		2.51	4.69	9.41	26.33	50.07	150.67	0.0963	−0.0597	0.9951
±SD	0.08		0.12	0.17	0.22	1.30	1.22	2.08	0.0116	0.0234	0.0007
Accuracy	109.8%		100.5%	93.8%	94.1%	105.3%	100.1%	100.4%			
Precision (CV)	4.6%		4.7%	3.7%	2.3%	4.9%	2.4%	1.4%	12.0%		

The peak area measured with the lowest Fingolimod and Fingolimod-P concentration (0.3 and 1.5 ng/mL, respectively) was 5-fold the signal measured in the blank human plasma (data not shown), prompting us to consider this concentration as the LLOQ for both analytes.

Selectivity of the method was evaluated confirming that the interfering peaks in six independent plasma matrices, which co-eluted at the same retention time of Fingolimod and Fingolimod-P, were always ≤20% of the peak area of Fingolimod and Fingolimod-P at LLOQ level (0.3 and 1.5 ng/mL, respectively). Absence of interfering peaks in mobile phase solution, in blank plasma and in blank plasma spiked with IS was also assessed qualitatively (Figure 1). Similarly, interfering peaks which co-eluted at the same retention time of IS were always ≤5% of the peak area of IS at the concentration used (10 ng/mL) (data not shown).

3.1.2. Accuracy and Precision

Six replicates of QC samples at 0.3 ng/mL (Fingolimod) or 1.5 ng/mL (Fingolimod-P) and at 4.0, 40 and 100 ng/mL were analyzed within a single-run analysis (intra-day) and over different days (inter-day). Accuracy and precision were consistent with the requirements indicated in EMA guidelines (Table 3).

3.1.3. Carry-Over

Fingolimod and Fingolimod-P were injected at the highest concentration (ULOQ, 150 ng/mL) followed by injection of blank matrix: carry-over was negligible (<20% the LLOQ signal, data not shown) using the needle-wash solution H_2O:MeOH (1:1 v/v) with 1% HCOOH.

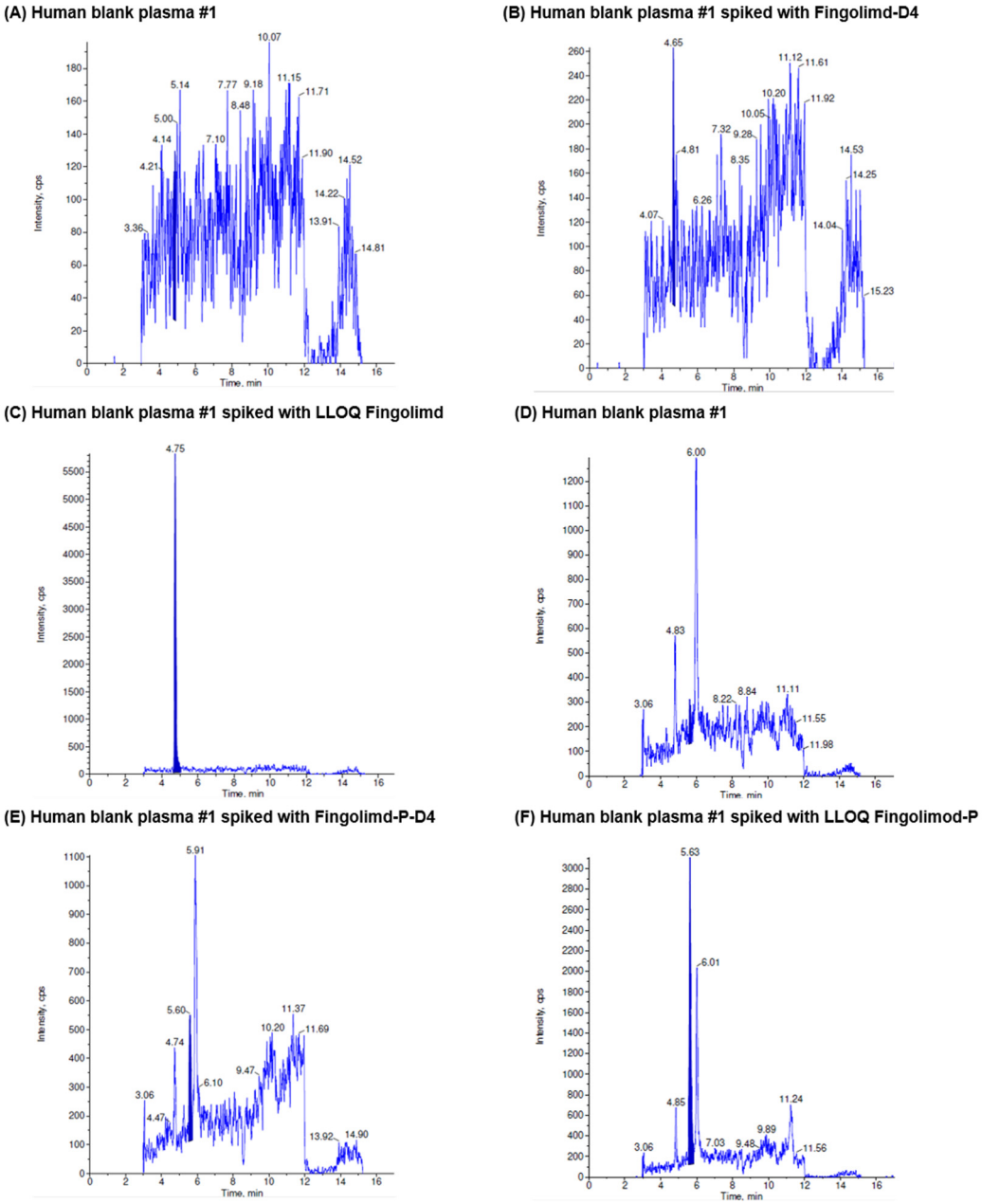

Figure 1. (i) Representative HPLC-MS/MS chromatograms (extracted for Fingolimod, FTY720, quantitative ion transition) in plasma of (**A**) indicative blank matrix (human plasma source #1), (**B**) IS spiked in the blank matrix (human plasma source #1) at the concentration of use (10 ng/mL) and (**C**) Fingolimod spiked in the blank matrix (human plasma source #1) at the LLOQ (1.5 ng/mL, retention time 4.75 min). (ii) Representative HPLC-MS/MS chromatograms (extracted for Fingolimod-P, FTY720-P, quantitative ion transition) in plasma of (**D**) indicative blank matrix (human plasma source #1), (**E**) IS spiked in the blank matrix (human plasma source #1) at the concentration of use (10 ng/mL) and (**F**) Fingolimod-P spiked in the blank matrix (human plasma source #1) at the LLOQ (3 ng/mL, retention time 5.63 min).

Table 3. Intra-day and inter-day accuracy and precision of Fingolimod and Fingolimod-P in human plasma.

QCs Nominal Concentrations (ng/mL)		QCs Calculated Concentrations (ng/mL) in Six Replicates							
		Intra-Day				Inter-Day			
		Mean (ng/mL)	±SD (ng/mL)	Accuracy	Precision (CV)	Mean (ng/mL)	±SD (ng/mL)	Accuracy	Precision (CV)
Fingolimod	0.3 (6)	0.30	0.02	99.5%	8.3%	0.30	0.01	101.6%	3.4%
	4.0 (6)	4.24	0.20	105.9%	4.7%	4.16	0.23	103.9%	5.4%
	40.0 (6)	39.52	1.45	98.8%	3.7%	36.93	1.50	92.3%	4.1%
	100.0 (6)	94.52	4.64	94.5%	4.9%	10.28	7.43	100.3%	7.4%
Fingolimod-P	1.5 (6)	1.74	0.09	116.0%	8.3%	1.67	0.10	111.6%	5.8%
	4.0 (6)	4.15	0.14	103.6%	3.4%	4.56	0.30	114.0%	6.5%
	40.0 (6)	40.00	1.71	100.0%	4.3%	38.97	1.45	97.4%	3.7%
	100.0 (6)	101.62	4.37	101.6%	4.3%	104.48	6.22	104.5%	5.9%

3.1.4. Matrix Effect and Recovery

Matrix effect was assessed for Fingolimod and Fingolimod-P (at LQC and HQC) and IS (at the used concentration) in six different plasma matrices (Table 4). Negligible ion suppression/enhancement has been found with an IS-normalized MF reliable through the concentrations tested: CV% was in the range 2.9–4.2% (Fingolimod) and 4.8–4.9% (Fingolimod-P).

Table 4. Fingolimod and Fingolimod-P recovery and matrix effect in human plasma calculated on six different matrices.

Fingolimod												
QC Conc. (ng/mL)	Fingolimod Recovery			Fingolimod-D4 (IS) Recovery			Fingolimod MF			IS-Normalized MF		
	Mean	±SD	CV	Mean	±SD	CV	Mean	±SD	CV	Mean	±SD	CV
4.0 (6)	88.0%	2.3%	2.7%	87.0%	10.7%	12.3%	1.281	0.178	13.9%	1.123	0.033	2.9%
100.0 (6)	90.6%	9.7%	10.7%				1.037	0.077	7.4%	0.956	0.040	4.2%
Fingolimod-P												
QC Conc. (ng/mL)	Fingolimod-P Recovery			Fingolimod-P-D4 (IS) Recovery			Fingolimod-P MF			IS-Normalized MF		
	Mean	±SD	CV	Mean	±SD	CV	Mean	±SD	CV	Mean	±SD	CV
4.0 (6)	73.4%	4.3%	5.9%	71.7%	9.3%	13.0%	1.331	0.195	14.7%	1.109	0.054	4.9%
100.0 (6)	75.5%	9.4%	12.5%				1.021	0.073	7.2%	0.945	0.045	4.8%

Recovery of Fingolimod and Fingolimod-P was calculated at two concentrations (4.0 and 100 ng/mL) for each of the six different matrix considered and gave reproducible values (CV% always <15%) within the range 84.9–101.6% (Fingolimod) and 62.3–86.1% (Fingolimod-P) (Table 4).

3.1.5. Analyte Stability

In the storage conditions tested, Fingolimod and Fingolimod-P stock and working solutions were stable for, respectively, 7 and 2 months at −20 °C, since the absolute peak areas of the working solutions confirmed the stability of both analytes in these storage conditions, with differences always ≤15% compared to the mean peak area measured a t = 0 (Table 5).

Table 5. Stability of stock and working solutions of Fingolimod and Fingolimod-P.

Tested Solutions (Six Replicates for Each)	t = 0			t = 7 Months (s.s.) or 2 Months (w.s.) at −20 °C			
	Mean Peak Area	±SD	Precision (CV)	Mean Peak Area	±SD	Deviation from t = 0	Precision (CV)
Fingolimod s.s. 1 mg/mL diluted 1:1000 (6)	899,167	14,919	1.7%	781,000	18,133	−13.1%	2.3%
Fingolimod-P s.s. 1 mg/mL diluted 1:1000 (6)	1,393,333	13,663	1.0%	1,346,667	12,111	−3.3%	0.9%
Fingolimod w.s. 0.03 µg/mL (6)	26,060	1521	5.8%	28,717	1269	10.2%	4.4%
Fingolimod w.s. 3.0 µg/mL (6)	2,525,000	52,058	2.1%	2,675,000	41,833	5.9%	1.6%
Fingolimod-P w.s. 0.03 µg/mL (6)	19,100	1931	10.1%	17,067	2364	−10.6%	13.9%
Fingolimod-P w.s. 3.0 µg/mL (6)	2,346,667	35,024	1.5%	234,333	80,911	−0.1%	3.5%

s.s., stock solutions; w.s., working solutions.

Stability of Fingolimod and Fingolimod-P in plasma was evaluated at two concentrations (4.0 and 100 ng/mL) under four conditions (Table 6): (i) bench-top stability (4 h at room temperature); (ii) stability after two freeze–thaw cycles; (iii) autosampler stability (48 h at 6 °C); (iv) long-term storage stability (1, 3, 6 and 12 months at −20 °C and at −80 °C).

Table 6. Stability of Fingolimod and Fingolimod-P in human plasma.

Fingolimod								
Stability Conditions, Six Replicates for Each	LQC—4.0 ng/mL				HQC—100 ng/mL			
	Mean (ng/mL)	±SD (ng/mL)	Accuracy	Precision (CV)	Mean (ng/mL)	±SD (ng/mL)	Accuracy	Precision (CV)
Bench-top (4 h RT) (6)	4.22	0.22	105.6%	5.3%	101.5	5.1	101.5%	5.1%
1 month (storage −80 °C) (6)	4.06	0.23	101.5%	5.7%	88.6	4.7	88.6%	5.3%
3 months (storage −80 °C) (6)	4.00	0.24	99.9%	6.1%	101.8	8.1	101.8%	8.0%
6 months (storage −80 °C) (6)	3.97	0.17	99.3%	4.3%	99.4	7.7	99.4%	7.7%
12 months (storage −80 °C) (6)	3.67	0.16	91.6%	4.4%	92.4	3.6	92.4%	3.9%
1 month (storage −20 °C) (6)	3.59	0.17	89.7%	4.7%	86.8	4.3	86.8%	5.0%
3 months (storage −20 °C) (6)	3.46	0.09	86.5%	6.1%	91.4	5.2	91.4%	5.7%
6 months (storage −20 °C) (6)	3.34	0.17	*83.4%*	5.0%	86.8	2.1	86.8%	2.5%
Freeze and thaw 2nd cycle (6)	3.45	0.17	86.2%	5.1%	85.5	3.8	85.5%	4.4%
Autosampler (6 °C) 48 h	3.46	0.16	100.2%	4.7%	84.3	2.4	98.6%	2.8%
Fingolimod-P								
Stability Conditions, Six Replicates for Each	LQC—4.0 ng/mL				HQC—100 ng/mL			
	Mean (ng/mL)	±SD (ng/mL)	Accuracy	Precision (CV)	Mean (ng/mL)	±SD (ng/mL)	Accuracy	Precision (CV)
Bench-top (4 h RT) (6)	4.09	0.20	102.2%	5.0%	105.4	2.8	105.4%	2.6%
1 month (storage −80 °C) (6)	4.16	0.42	104.1%	10.0%	100.3	5.6	100.3%	5.6%
3 months (storage −80 °C) (6)	4.29	0.31	107.2%	7.1%	99.1	3.3	99.1%	3.3%
6 months (storage −80 °C) (6)	4.05	0.16	101.2%	3.8%	109.0	6.0	109.0%	5.5%
12 months (storage −80 °C) (6)	3.98	0.20	99.5%	4.9%	92.7	9.9	92.7%	10.7%
1 month (storage −20 °C) (6)	3.49	0.19	87.25	5.6%	103.7	3.6	103.7%	3.4%
3 months (storage −20 °C) (6)	3.56	0.14	88.9%	4.1%	98.2	7.9	98.2%	8.0%
6 months (storage −20 °C) (6)	2.79	0.30	*69.8%*	10.8%	88.4	3.4	88.4%	3.8%
Freeze and thaw 2nd cycle (6)	3.82	0.21	95.4%	5.4%	87.9	3.8	87.9%	4.3%
Autosampler (6 °C) 48 h	4.00	0.35	104.8%	8.7%	87.6	3.3	99.6%	3.8%

Six replicates of LQC (4.0 µg/mL) and HQC (100 ng/mL) were analyzed under the tested conditions (Table 6). Stability data met EMA requirements, since the accuracy and precision of tested QCs (at least five replicates) were always within the range 85–115%, except for LQC (for both analytes), stored at −20 °C for 6 months (highlighted in italics inside Table 6). Given these results, the 12-month stability at −20 °C has not been assessed.

3.2. Application to Clinical Samples

Plasma levels of Fingolimod and Fingolimod-P have been measured in 15 patients, of which the 33% (5/15) were naïve and 10/15 switched from a first-line therapy due to inefficacy. No safety issues were recorded, and all MS patient completed the 24-month period of treatment and follow-up. At month 6, one patient experienced mild radiological activity and another one experienced an increase of the EDSS score (diagnosed as a secondary progressive, SP, patient after 24 months of treatment); all the other patients were classified as NEDA (No Evidence of Disease Activity—no relapse, no radiological activity, no increase in EDSS). After 12 and 24 months of treatment, all patients achieved the NEDA status, except for the SP one at month 24.

Figure 2 shows the distribution of Fingolimod and Fingolimod-P concentrations in the plasma of 15 patients given the daily oral dose of Fingolimod (0.5 mg). Fingolimod levels measured after 6, 12, 24 months of treatment were comparable ($p > 0.05$): median with [Q1–Q3] of the plasma levels were, respectively, 0.547 ng/mL [0.316–0.722], 0.400 ng/mL [0.306–0.511] and 0.720 ng/mL [0.391–1.031]. Similarly, comparable levels ($p > 0.05$) of Fingolimod-P were found, showing a median with [Q1–Q3] of 1.230 ng/mL [1.040–1.460], 0.960 ng/mL [0.730–1.350] and 1.390 ng/mL [0.780–1.718] after 6, 12 and 24 months, respectively. Fingolimod-P-to-Fingolimod ratio ranged from 1.281 to 4.244 (mean ± SD 2.2 ± 0.7, $n = 31$) with no differences between the three time-points ($p > 0.05$).

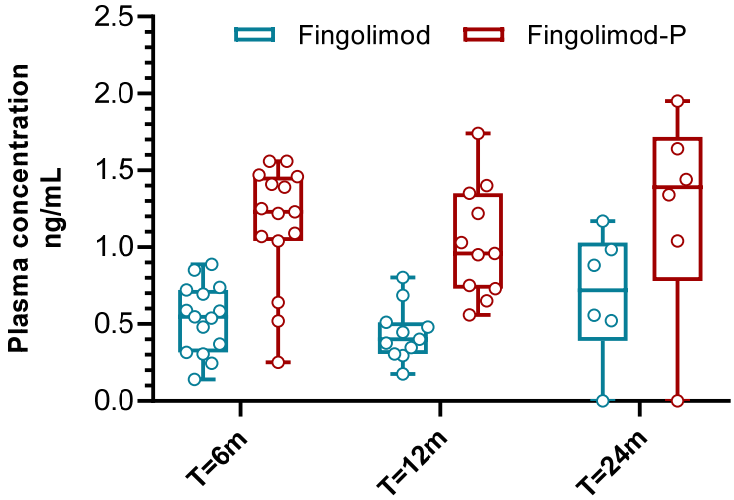

Figure 2. Plasma levels of Fingolimod (light blue) and Fingolimod-P (red) measured in 15 patients after 6 ($n = 15$), 12 ($n = 11$) and 24 ($n = 5$) months of treatment (Fingolimod 0.5 mg/day orally). Box plots show median, quartile range and min-max range. Absolute levels of Fingolimod and Fingolimod-P were analyzed by one-way ANOVA followed by Tukey multiple comparison post-hoc test.

4. Discussion

In this study, we developed and validated a rapid HPLC-MS/MS to quantify Fingolimod and its active metabolite Fingolimod-P in human plasma. The method involved the use of Fingolimod-D4 and Fingolimod-P-D4 as internal standards, plasma protein precipitation with acetonitrile and chromatographic separation with tandem mass spectrometry detection. The method was validated following the criteria set in the "EMA guidelines on bioanalytical method validation" [13]. This method was successfully applied to plasma samples obtained from 15 RRMS patients under chronic treatment with Fingolimod ad-

ministered orally at a dose of 0.5 mg once daily for up to 24 months. To our knowledge, this is the first analysis reporting plasmatic levels of Fingolimod and Fingolimod-P after such long-term treatments with the dosage recommended in the clinical practice.

No significant differences were observed in the levels of Fingolimod between samples collected after 6, 12 and 24 months of treatment, in agreement with literature data showing that steady-state concentrations are reached in less than 2 months [14]. Importantly, the concentration profile at steady state is remarkably flat, with peak-to-trough fluctuations after daily administrations ranging approximately 20% [9] and thus, the time of blood collection is likely not so critical. The similar plasmatic levels for Fingolimod at the different time-points suggests that 24 months of daily treatment does not affect the drug's bioavailability.

Our data also indicate similar plasmatic levels of the active metabolite Fingolimod-P along the 24-month treatment, suggesting that Fingolimod metabolism is unaffected by multiple daily doses. In addition, the levels of the active metabolite Fingolimod-P were consistently higher than the levels of the parent compound, by 2.2-fold on average. Contradictory results are reported in the literature regarding this latter observation. Thus, data obtained in single healthy subject receiving 5 mg/day of Fingolimod for 7 days showed higher levels of the metabolite after the first dose and similar levels of drug and metabolite after the 7th dose [15]. On the contrary, higher levels of the drug in comparison to the metabolite were found in three other studies [9,16] in which Fingolimod was administered at daily doses ranging 0.5–5 mg for 7–28 days. No data are available in the literature at the longer time-points ($\geq$6 months) considered in the present study.

The analytical method described in this work can be useful to evaluate if the inter-individual variability in clinical responses might be explained by inter-individual differences in drug bioavailability, which can underlie either no or poor efficacy (insufficient blood concentrations) or adverse events (too high blood concentrations).

Author Contributions: Conceptualization, C.F., L.B., R.M., S.R., M.G. and J.L.; methodology, C.F., A.P. and J.L.; formal analysis, C.F., L.B., M.G. and J.L.; investigations, C.F., L.B., S.R., R.M., M.G. and J.L.; writing—original draft preparation, C.F., M.G. and J.L.; writing—review and editing, C.F., L.B., R.M., M.G. and J.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Patient-Centered Outcomes Research Institute, grant number MS 1511-33406.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Foundation IRCCS Neurological Institute Carlo Besta (protocol code N. 43, 10 August 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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