Received: 7 June 2016 | Revised: 5 September 2016 | Accepted: 24 October 2016

DOI: 10.1002/bmc.3877

WILEY Biomedical Chromatography

RESEARCH ARTICLE

LR12-peptide quantitation in whole blood by RP-HPLC and intrinsic fluorescence detection: Validation and pharmacokinetic study

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Abstract

A simple, sensitive, selective and robust HPLC method based on intrinsic fluorescence detection was developed for the quantitation of a dodecapeptide (designated as LR12), inhibitor of Triggering Receptor Expressed on Myeloid cells-1, in rat whole blood. Sample treatment was optimized using protein precipitation and solid-phase extraction. Chromatographic separation was carried out in a gradient mode using a core–shell C_{18} column (150×4.6mm, 3.6µm) with mobile phases of acetonitrile and water containing trifluoroacetic acid at 1.0mL/min. The method was validated using methodology described by the US Food and Drug Administration guidelines for bioanalytical methods. Linearity was demonstrated within the 50–500ng/mL range and the lower limit of quantitation was 50ng/mL. Finally, a preliminary pharmacokinetic study after intraperitoneal injection of LR12 in rats was conducted to evaluate both LR12 monomer and its corresponding disulfide dimer, the main product of degradation. Beyond the fact that this paper describes the first fully validated method for LR12 analysis in blood samples, the approach followed here to optimize pre-analytical steps could be beneficial to develop HPLC and/or MS methods for other pharmaceutical peptides.

KEYWORDS

bioanalytical validation, intrinsic fluorescence detection, peptide, RP-HPLC, TREM-1

1 | INTRODUCTION

The LR12 dodecapeptide (Figure 1a) is an inhibitor of the Triggering Receptor Expressed on Myeloid cells-1 (TREM-1). Discovered in 2000, TREM-1 is a member of the immunoglobulin superfamily expressed at the surface of neutrophils, mature monocytes, macrophages and hypoxic dendritic cells (Bouchon, Dietrich, & Colonna, 2000). It is generally recognized as an amplifier of innate immunity and it is known to play a major role in the pathophysiology of inflammation-associated disorders.

Several preclinical approaches have been developed for TREM-1 blockade, from genetic invalidation to the use of antibodies or decoy receptors, with the purpose of either identification of TREM-1 involvement in pathologies or treatment. A common drawback of pharmacological inhibition using recombinant proteins or antibodies is that issues regarding the toxicity or the immunogenicity may arise. In this context, small peptides specifically inhibiting TREM-1 were developed, and were shown to inhibit TREM-1-induced leukocyte activation in a dose-dependent manner (Derive et al., 2012). They also displayed beneficial effects when administered in preclinical models of *Pseudomonas aeruginosa*-induced pneumonia, melioidosis, experimental hemorrhagic shock, ischemia–reperfusion, severe acute pancreatitis and myocardial infarction (Boufenzer et al., 2015; Gibot et al., 2006, 2008, 2009; Kamei et al., 2010; Wiersinga et al., 2007). Similar encouraging results have been reported in septic shock in minipigs, as well as in lipopolysaccharide-challenged nonhuman primates (Derive, Boufenzer, & Gibot, 2014; Derive et al., 2013). These peptides reflect a highly conserved region in the TREM-1 family identified to be implicated in ligand binding (Kelker, Debler, & Wilson, 2004b; Kelker et al., 2004a). Among them, LR12 is a dodecapeptide analog specific

Abbreviations used: PD, pharmacodynamic; PK, pharmacokinetic; SPE, solid-phase extraction; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TREM-1, Triggering Receptor Expressed on Myeloid cells-1.

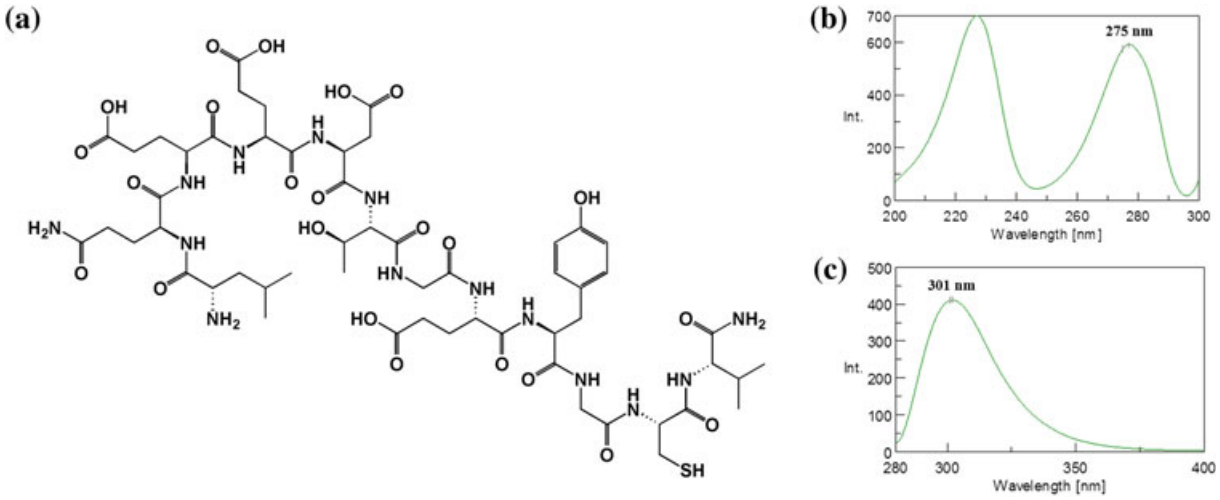


FIGURE 1 Structure of LR12 monomer (H-LQEEDTGEYGCV-NH_2, murine sequence, MW 1341.4, calculated isoelectric point 4.0) (a) and excitation (b) and emission (c) spectra of LR 12 (2.5 µg/mL in acetonitrile–water (14:86, *v*/v) added with trifluoroacetic acid 0.1%, *v*/v)

of murine species. Until now, studies conducted with LR12 (and other TREM-1 inhibitory peptides) were intended to demonstrate their therapeutic activities and therefore focused on histological/biological changes and/or better survival after administration. To the best of author's knowledge, no validated bioanalytical method has been reported so far for such peptides. As a result, for further pharmacokinetic/pharmacodynamic (PK/PD) studies, a simple, reliable, and relatively cheap analytical method to quantify LR12 in simple and complex matrices is needed.

Peptide quantitation in biological samples is commonly obtained after chromatographic separation and several detection modes can be applied, such as immunoassay, electrochemical detection, spectrophotometry, spectrofluorimetry and mass spectrometry (MS) (Rissler, 1995). Immunoassays have become less popular because of cross-reactivity risks, as the antibodies lack sometimes of specificity, especially with peptides corresponding to endogenous sequences (John, Walden, Schäfer, Genz, & Forssmann, 2004). At the same time, hyphenated techniques have been more and more applied owing to their sensitivity, speed and selectivity. Although MS is generally recognized as a method of choice because of its high sensitivity and selectivity, its use for peptide quantitation could be hampered by adsorption phenomenon and instability issues (Ewles & Goodwin, 2011; van den Broek, Sparidans, Schellens, & Beijnen, 2008). Moreover, owing to the high cost of the MS equipment, such a detection mode is not always available. Recently, HPLC in combination with intrinsic fluorescence detection has been explored for the highly selective and highly sensitive quantitation of peptides and proteins. Intrinsic fluorescence detection (based on native fluorescence of tryptophan, tyrosine or phenyl alanine) is a label-free methodology for quantitation of peptides and proteins, which provides similar sensitivity, better linearity and repeatability compared with MS detection (Russell et al., 2011; Saraswat, Snyder, & Isailovic, 2012). When possible, exploitation of tryptophan fluorescence is usually preferred owing to its high quantum yield, but successful use of tyrosine fluorescence has been reported (Saraswat et al., 2012; Zhdanova et al., 2015).

As a result, the aim of this work was to develop a simple chromatographic method with spectrofluorimetric detection and without a derivatization step, taking advantage of the intrinsic fluorescence of the LR12 tyrosine moiety. Pre-analytical steps have been optimized, to avoid the spontaneous oxidation of the monomer form which leads to the formation of the disulfide dimer form. Whole blood samples were used: after protein precipitation, a solid-phase extraction (SPE) was carried out before HPLC analysis. An LC–MS/MS analysis was performed with a qualitative approach to identify both monomer and dimer in the blood samples. Then, the method was validated according to the guidelines of the US Food and Drug Administration (2013). The optimized HPLC-spectrofluorimetric method was then successfully applied to determine LR12 concentration in an *in vivo* pharmacokinetic study in rats after intraperitoneal administration.

2 | EXPERIMENTAL

2.1 | Chemicals

The murine analog peptide LR12 (H-LQEEDTGEYGCV-NH_2) and its dimeric form were provided with >90.0% purity by Pepscan Presto BV (The Netherlands). Acetonitrile and methanol were of HLPC grade (Sigma-Aldrich, USA). High-purity (99%) trifluoroacetic acid (TFA), trichloroacetic acid (TCA) and 1.0M hydrochloric acid (HCl) were obtained from Sigma-Aldrich, Acros Organics (Thermo Fisher Scientific, USA) and Fluka (Germany), respectively. Isoflurane was purchased from Baxter (France) and all products for surgery and animal welfare from a veterinary provider (Centravet, France). Ultrapure water (resistivity 18.2 MΩ.cm) was used for all experiments.

2.2 | HPLC apparatus

The HPLC-spectrofluorimetric system consisted of a TSP model P1000XR pump, a TSP SpectraSystem SCM1000 degasser and a TSP model SpectraSeries AS300 autosampler (Thermo Separation Products, USA) set to inject 100 µL. The chromatographic separations

were carried out at 40°C on a core–shell column (Phenomenex Aeris Peptide XB C_{18}, 3.6µm, 150×4.6mm protected by a Phenomenex guard cartridge, Phenomenex, USA). The mobile phase was a linear gradient of a mixture of water (solvent A) and acetonitrile (solvent B), both containing 0.1% (*v*/v) TFA, at a flow rate of 1.0mL/min. The gradient conditions are shown in Table 1. The spectrofluorimetric detector (FP-2020PLUS, Jasco, Japan) was set at λ_{exc}=275nm and λ_{em} =301nm (gain = 100, attenuation = 1). These wavelengths were previously identified as excitation (Figure 1b) and emission (Figure 1c) maxima in LR12 spectra recorded in the initial mobile phase mixture (LR12 at 2.5µg/mL; bandwith, 5nm; response, 0.5s; sensibility, low; FP-8300 spectrofluorimeter, Jasco). Chromatographic data acquisition and analysis were performed using Azur 5.0 software (Datalys, France).

2.3 | Standard solutions and system conformity

Stock peptides solutions (1.0mg/mL) were prepared by dissolving the appropriate amount of LR12 monomer or dimer in 0.1M HCl, then aliquoted and stored at −80°C for maximum 6months. Standard solution (mix of monomer and dimer, 500ng/mL each) was prepared daily by diluting stock solutions in solvent A. After thawing, aliquots of stock solutions were stored for 3days at +4°C before being discarded (recoveries of 100.1±5.7% for monomer and 101.5±3.0% for dimer after 3days, n=3). To stabilize the system (ion pairing with TFA), standard solution was injected five times consecutively, and the RSD values of the monomer and dimer peak areas were calculated on the three last injections.

2.4 | Animals, blood sampling protocol and optimized pre-analytical steps

All experiments were performed in accordance with the European Community guidelines (2010/63/EU) for the use of experimental animals. Protocols and procedures were approved by the regional and national ethical committees on animal experiments (project 'Slow-release', APAFIS#1146–2015071313458604 v3). A total of 33 male Wistar rats (280–350g, Charles River, France) were used in this study.

To obtain arterial blood samples, rats were anesthetized (starting 15min before blood collection time) with isoflurane (4% for induction, then 2% in oxygen 2L/min). After heparin administration (500IU, i.v.), a laparotomy was performed to expose the abdominal aorta. Blood was collected directly from the aorta (PTFE catheter, Intraflon 2, Vygon, France) into Vacutainer (Becton Dickinson, USA) tubes containing heparin lithium. Blood sampling lasted until heart beat had ceased. To avoid any peptide dimerization during sampling process and to stabilize LR12, 40µL of a 1.25mg/mL TCA solution was added into each tube before blood sampling, corresponding to a final TCA solution–blood ratio of approximately 1% *v*/v.

Acidified blood was then mixed 1:1 with 10% (*w*/*v*) TCA and centrifuged for protein removal (10min, 14,000***g***, room temperature). Supernatants were collected and concentrated five times during a SPE step performed on Agilent Plexa Bond Elute 30mg 1mL columns (Agilent Technologies, USA) using a vacuum manifold (vacuum 5 inHg, Macherey-Nagel, Germany). Briefly, after classical cartridge conditioning (2×1.0mL methanol and 2×1.0mL water), 1.5mL of supernatant resulting from centrifugation was placed on the SPE cartridge, washed with 1.5mL of mobile phase A and finally eluted with 300µL of a binary mixture composed of 60:40 (*v*/v) acetonitrile–water both containing 0.1% TFA. Finally, the extracted samples were evaporated for 20min at 40°C under nitrogen (5psi) in a Turbovap® evaporator (Biotage, Sweden). Residual samples were mixed with 200µL of solvent A before injection into the HPLC system. The final volume was insufficient to obtain repeated injections of the same sample after extraction: as a result, in the following text, repeated determinations of a sample mean that several extractions (and injections) were realized from the same blood sample.

TABLE 1 Gradient elution program used in the final chromatographic conditions

Time (min)	A (%, *v*/v)	B (%, *v*/v)
0.0	86	14
5.0	86	14
12.0	80	20
12.5	50	50
18.5	50	50
19.0	86	14
25.0	86	14

2.5 | Method validation

The method was validated for selectivity, precision, accuracy and linearity according to the US Food and Drug Administration (2013) guidelines. The selectivity was checked in regard to six independent rat blood sources (blanks) and a zero sample (mobile phase A, blank gradient). The precision of the assay was studied with respect to repeatability and intermediate precision and it was evaluated using blood samples from three rats spiked at three levels (low, mid and high concentrations) covering the range of expected concentrations. The accuracy was determined using three determinations per concentration with three concentrations (low, middle and high); results were calculated as recoveries vs known amounts of LR12 spiked in blood samples. The linearity was determined on six runs conducted over several days with five concentrations (from 50 to 500ng/mL) of LR12 monomer and dimer spiked in blood samples. The lower limit of quantitation (LLOQ) was established using five samples, according to the guidance for bioanalytical method validation from the US Food and Drug Administration (2013). For all experiments, blood was spiked with LR12 right after sampling in TCA-containing tubes, and the whole protocol of extraction described in above was applied to each sample.

2.6 | Mass spectrometry

LC–MS/MS experiments were carried out on a UFLC-XR device (Shimadzu) coupled to a QTRAP® 5500 MS/MS hybrid system triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Foster City,

USA) equipped with a turbo VTM ion source. Instrument control, data acquisition and processing were performed using the associated Analyst 1.5.2 software. The RP-LC separation was carried out with the same chromatographic conditions described above. MS analysis was carried out in positive ionization mode using an ion spray voltage of 5500V. The curtain gas flow was set at 25psi using nitrogen. The turbo VTM ion source was set at 550°C. Nebulizer gas and auxiliary gas flow (air) were set at 50psi. Finally, the multiple reaction monitoring (MRM) transitions were monitored with a dwell time of 150ms using 30eV collision energy for all the transitions.

2.7 | Application of the method in a pharmacokinetic study

Male Wistar rats received a 5mg/kg intraperitoneal injection of LR12 (extemporaneous solution at 0.6mg/mL in NaCl 0.9%). All injections were done between 9 and 12a.m. At predetermined times after LR12 administration (15, 30min, 2, 5 or 24h), arterial blood was collected according to the previously described protocol. Results are presented as means ± SD (n=3 animals per timepoint).

In a second set of experiments, in order to determine pharmacokinetics during the first 15min following LR12 administration, the protocol was modified as follows: instead of a laparotomy, the left femoral artery was cannulated under anesthesia. Heparin (i.v.) and LR12 (i.p.) were administered immediately after surgery then 1.5mL of blood was sampled (at 2.5, 5, 7.5, 10 and 12.5min after LR12 injection) in heparin-lithium tubes containing 20µL of a 1.25mg/mL TCA solution. After the last blood collection, animals were immediately sacrificed (pentobarbitone 120mg/kg, i.v.). Three rats were used in this protocol. Thereafter, pre-analytic treatment of samples was similar to that previously described.

3 | RESULTS AND DISCUSSION

3.1 | Method development and sample treatment optimization

3.1.1 | Sample collection and storage

At the beginning of this study, as in many quantitation bioanalytical methodologies, the sample treatment was identified as the key point. The LR12 monomer is indeed a peptide with a short half-life in whole blood, reported for example equal to 2.25min in monkey's blood by Derive *et al.* (2014). A classical first step of centrifugation (10,000***g***, 10min at 4°C) to perform the protein precipitation and to obtain plasma was tested first, but this option was quickly discarded owing to the loss of 40% of the LR12 concentration. The second tested approach was direct deproteinization of whole blood (freshly sampled blood mixed 1:1*v*/v with a 10% *w*/*v* TCA solution). However, with this saving-time protocol, a dramatic decrease in the LR12 concentration was also observed (loss of 40% after 10min and 60% after 20min). Then an acidification step of the whole blood before protein precipitation was found to stabilize the peptide and gave satisfactory recoveries. Thus, 40µL of a 1.25mg/mL TCA solution was added into each blood collection tube before sampling, leading to a final TCA solution–blood ratio of ~1% *v*/v (4mL of blood). After centrifugation (10min, 14,000***g***, room temperature), LR12 peptides were stable in the supernatant after a storage period of 15days at −80°C, as shown by recoveries of 103.7±5.6% for monomer and 97.4±12.3% for dimer at day 15 (initial concentration: 150ng/mL, n=3).

3.1.2 | Sample treatment

Diluted standard solutions were submitted to solid phase extraction realized as described above, testing several mobile phases A/B elution mixtures (from 0 to 40% *v*/v of the aqueous mobile phase A). The obtained samples were noncompatible with the HPLC system owing to an excessive amount of acetonitrile leading to great distortion of each chromatographic peak under study. A final evaporation step was therefore added to eliminate the organic part of the eluting SPE solvent. However, whatever the composition of the elution mixture, samples evaporation to dryness led to the disappearance of the LR12 monomer to the benefit of the dimer [Figure 2a, elution mixture 40:60 phases A/B, only 10% of monomer

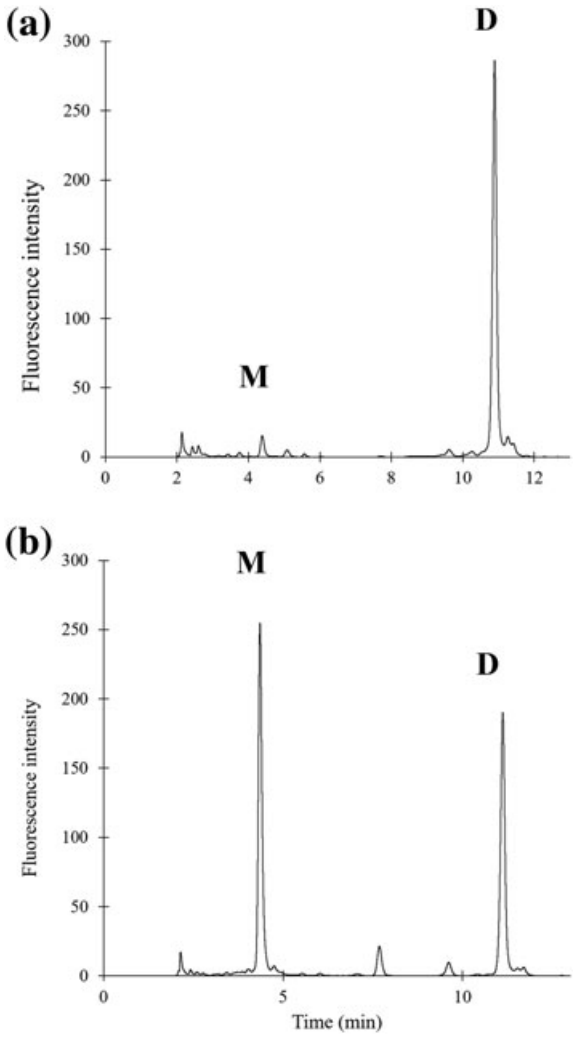


FIGURE 2 LR12 monomer instability when evaporated to dryness: typical chromatograms of samples containing initially a mix of LR12 monomer (M) and dimer (D) at 250.0ng/mL, evaporated to dryness (a) or after partial evaporation (b)

(M) was recovered while recovery of the dimer (D) increased to reach 145%]. Interestingly, dimerization was not observed when samples were only partially evaporated (Figure 2b, elution mixture 40/60 phases A/B, full recoveries of monomer and dimer, i.e. 101.2±3.5 and 99.2±4.1%, respectively). Use of a pure organic solvent for elution was assumed to be the better option to allow gentle evaporating conditions, but the 40:60 A/B mixture was the option finally chosen, as the only one leading to full recoveries. Reproducibility of the partial evaporation was checked on the first 30 samples by following the weight loss of the eppendorf tube content (RSD 5.7%).

Optimized parameters were 40°C, 5psi of nitrogen and 20min: this protocol led to a partial evaporation, with a reproducible residual sample volume of 50µL. As temperature, nitrogen pressure and time are crucial parameters during the evaporation step, they were always strictly controlled in the study. Residual sample was mixed with 200µL of mobile phase A before injection.

In conclusion of this sample treatment part, a partial evaporation (not to dryness) was compatible with high recoveries for both monomer and dimer. The optimized final sample treatment protocol is illustrated in Figure 3. The high and reproducible recoveries obtained at all QC levels with this protocol obviated the need for using an internal standard (results detailed below and in Table 2).

3.1.3 | LC conditions

Mobile phases were selected to be compatible with MS detection, bearing in mind that a volatile acidic component has to be added to maintain an acidic environment (pH2.0) to get the best stability of the analytes. Experiments made with formic acid have not been very successful because many blood matrix interferences were observed (data not shown). Finally, TFA was added to mobile phases, because it acts as a good pH regulator and also as an ion pairing agent. Selectivity was obtained thanks to displacement of the analyte chromatographic peaks enabled by this method change. During this development, the HPLC system was the same as described previously, except for the column (Nucleosil C_{18}, 5µm, 250×4.6mm, Macherey-Nagel, Germany), and elution was performed in isocratic mode with 86:14 (v/v) water–acetonitrile containing 0.1% TFA. Nevertheless, after only a few injections of real blood samples, a significant increase in the system pressure was observed indicating clogging of the column. Two modifications were then applied: first, a switch of stationary phase from porous to semi-porous core–shell microparticles and, second, elution in gradient mode according to the program described in Table 1. Chromatographic columns packed with core–shell particles appeared a decade ago and have become popular for peptide and protein analysis owing to their good efficiency (Gritti, Cavazzini, Marchetti, & Guiochon, 2007; Gritti et al., 2010; Larose, Julien, & Bilodeau, 2013; Staub, Zurlino, Rudaz, Veuthey, & Guillaurme, 2011). In this case also, a slight improvement in selectivity was achieved with the core–shell column. Moreover, as the porosity of the stationary phase is decreased, one can expect less clogging of the column by the blood samples. Thanks to this and mainly the gradient elution, column lifetime was drastically improved, as shown by the stability of the number of theoretical plates for the analytes, unchanged after more than 300 injections.

3.2 | Mass spectrometry

An ESI-MS/MS strategy was performed to identify univocally both monomer and dimer peptides. First, using an MS infusion experiment, the full-scan electrospray mass spectrum showed the formation of characteristic $[M+nH]^{n+}$ pseudo-molecular ions with the doubly charged species being the most abundant for LR12 monomer (m/z=671.3) whereas the triply charged species were observed for LR12 dimer (m/z=894.3). Second, the product ion scan, performed

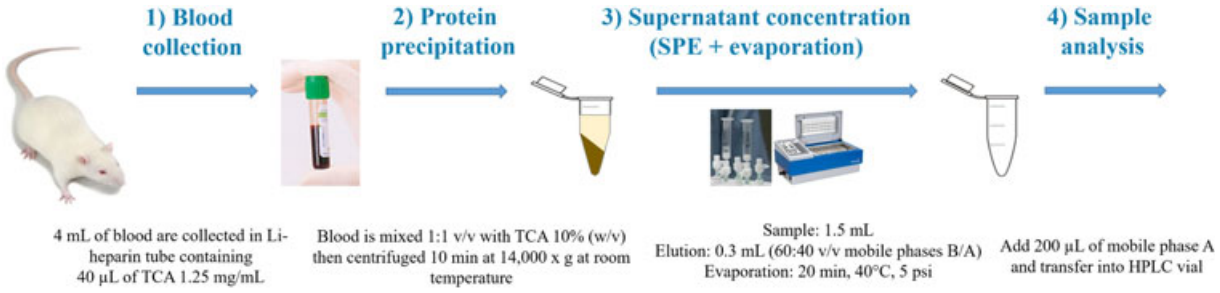


FIGURE 3 Optimized pre-analytical steps for LR12 quantitation in blood samples

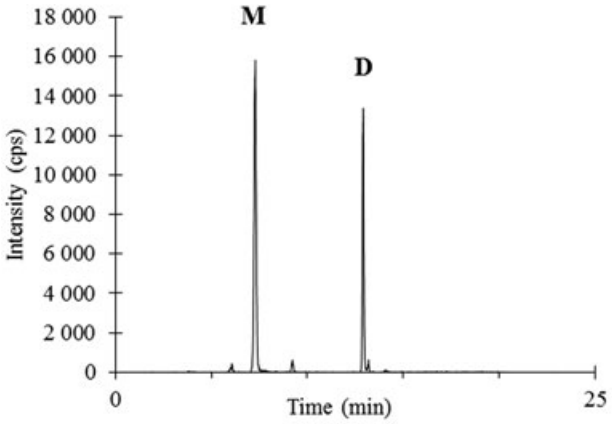
TABLE 2 Precision and accuracy for LR12-spiked blood samples

LR12 form	Nominal blood concentration (ng/mL)	Accuracy (%)		Precision RSD (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Monomer	100	107.34±3.16	108.91±3.90	5.30	6.38
	200	103.71±1.81	103.44±1.85	2.26	2.33
	400	103.66±4.22	104.03±2.72	0.61	2.96
Dimer	100	98.15±3.37	95.76±3.70	2.57	3.87
	200	99.31±5.52	96.01±5.04	3.06	5.25
	400	97.10±2.93	96.33±3.31	3.02	3.44

at 30eV collision energy, showed multiple ions fragmentation and the best MS/MS transitions were reported in Figure 4. Finally, the LC–MS/MS was carried out using the same chromatographic conditions as described previously for fluorescence detection. The chromatographic peak assignment was thus confirmed for both LR12 monomer and dimer (Figure 4).

3.3 | Method validation

The presently developed method (sample treatment and HPLC quantitation) was validated using guidelines described by the US Food and Drug Administration (2013). Selectivity, precision (repeatability and intermediate precision), accuracy, linearity and LLOQ were evaluated.



	m/z (amu)	Identified ion	MS/MS transitions
Monomer (M)	671.3	$(M+2H)^{2+}$	671.3 / 902.5 671.3 / 561.9
Dimer (D)	894.3	$(M+3H)^{3+}$	894.3 / 902.5 894.3 / 808.6

FIGURE 4 Total ion current (TIC) chromatogram obtained by HPLC-MS/MS for LR12 monomer (M) and dimer (D) forms in spiked blood sample (50ng/mL) and HPLC-multiple reaction monitoring conditions

3.3.1 | Selectivity

Selectivity of the method was evaluated both in the mobile phase (Figure 5a) and in blood (Figure 5b) by comparing chromatograms corresponding to LR12 spiked blood samples (mix of monomer and dimer, 50ng/mL each) and those for the initial blank samples. An experiment was performed with blood from six different rats. Representative chromatograms (Figure 5) showed that analyte peaks were well resolved and free of interferences from any endogenous compounds of the blood.

3.3.2 | Repeatability, intermediate precision and accuracy

Blood was collected from three rats and spiked with LR12 at different concentrations (100, 200 and 400ng/mL, respectively). After protein precipitation and centrifugation (see above), supernatants were used immediately (for repeatability and intermediate precision, Day 1) or frozen at −80°C (for intermediate precision, Day 2).

TABLE 3 Statistical evaluation of linearity (50.0–500.0ng/mL of monomer M or dimer D, spiked in blood samples) with a linear regression model $Y=A \times [M \text{ or } D]+B$ (five concentrations on six days, $n=30$, $\alpha=0.05$)

	Theoretical value	Monomer (M)	Dimer (D)
Slope *A*		1.846	1.531
Standard deviation on *A*		0.050	0.110
Intercept *B*		−83.06	9.58
Standard deviation on *B*		16.15	31.93
Correlation coefficient		0.989	0.940
F regression	4.20	1198.71	214.03
F lack of fit	2.99	2.66	0.08

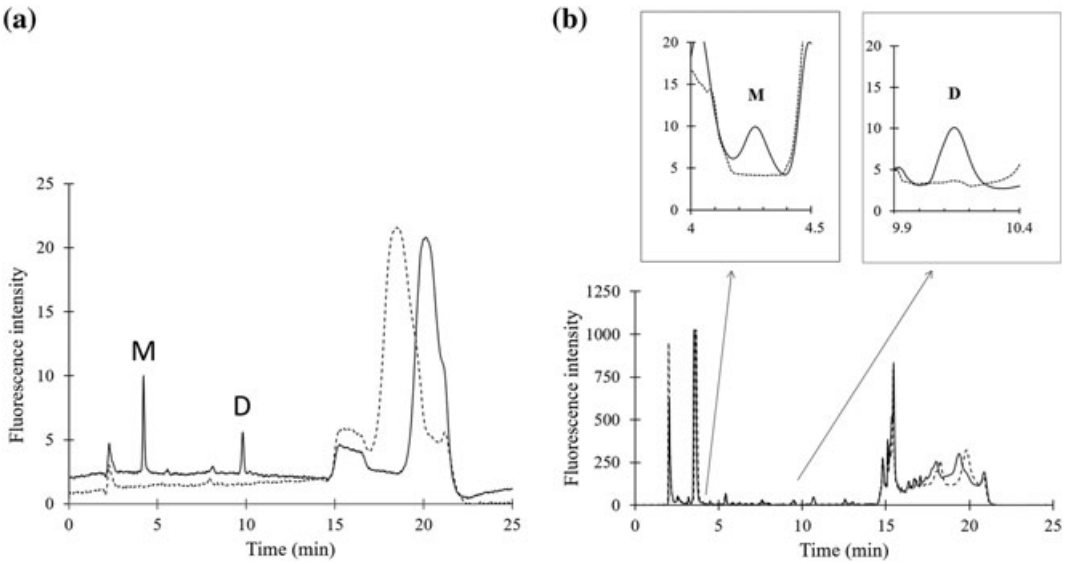


FIGURE 5 Chromatograms illustrating the selectivity of the HPLC-spectrofluorimetric method for LR12 monomer (M) and dimer (D) forms (50ng/mL): (a) in mobile phase and (b) in spiked blood samples

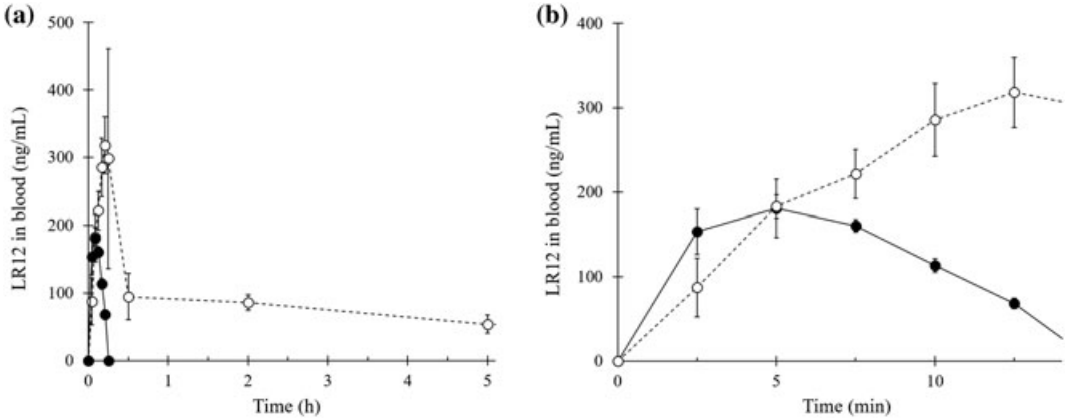


FIGURE 6 Blood concentrations of LR12 monomer and dimer forms after i.p. administration of LR12 (5 mg/kg, monomer form) to Wistar rats ($n = 3$ for each time): in the hours following administration (a) and in the first 15 min (b)

Repeatability (intra-day) was determined by performing three extractions of supernatant from each blood sample: results are shown in Table 2. Relative standard deviation (RSD) values are given for each form of LR12 and were <6.0%.

Intermediate precision (inter-day) was calculated after 3 new extractions of supernatants (from the same blood) and analysis, performed on another day. Results are given in Table 2 and are satisfactory (<7.0%).

The accuracy of the method was evaluated by injection of blood samples spiked with both LR12 forms (three concentrations covering the linearity range, three determinations per concentrations corresponding to three independent extractions of supernatants from the same blood sample). Accuracy was demonstrated by the high recovery values obtained at the three different concentration levels for both monomer and dimer forms, as shown in Table 2. Although US Food and Drug Administration guidelines recommend at least five determinations per concentration, it was not possible in this case as the volume of blood collected from a rat is not sufficient to perform all the analyses. The number of determinations was therefore reduced to three per concentration, in order to obtain both intra-day and inter-day precision and accuracy from the same blood sample.

Therefore, all of the results indicated that the HPLC-spectrofluorimetric method was reproducible and accurate for the analysis of LR12 (monomer and dimer) in rat blood samples.

3.3.3 | Linearity

The linearity was determined on six runs with five concentrations (from 50 to 500 ng/mL) in blood samples spiked with LR12 monomer and dimer. The linearity was evaluated by linear regression analysis calculated by the least squares regression method. Results are given in Table 3. As the experimental values for F regression are higher than the theoretical ones, the hypothesis of an existing slope for each species can be considered as true (at the risk $\alpha = 0.05$). Moreover, as the experimental values for F lack of fit are lower than the theoretical values, a good correlation of the results with a linear model can be assumed (again at the risk $\alpha = 0.05$).

3.3.4 | Lower limit of quantitation

For both the monomer and the dimer forms, the LLOQ was established at 50 ng/mL (with RSD values less than 20%). This value is in the range of LLOQ (2–300 ng/mL) published in other studies for the determination of various pharmaceutical peptides in biological matrices using HPLC with spectrofluorimetric detection (either intrinsic fluorescence or after a derivatization step; Allen, Stafford, & Nocerini, 1997; Boppana & Miller-Stein, 1994; Chepyala, Tsai, Sun, Lin, & Kuo, 2015; Hinton et al., 1996; Lawless, Hopkins, & Anwer, 1998; Mendoza et al., 1997). Comparable values of LLOQ (from 5 to 40 ng/mL) have also been reported for peptides quantified in biological fluids with HPLC/MS methods (Gil et al., 2012; Jiang et al., 2013; Saraswat et al., 2012).

3.4 | Pharmacokinetic study

The validated method for LR12 quantitation by HPLC-spectrofluorimetric detection was successfully applied to study the pharmacokinetics of LR12 in rats after 5 mg/kg i.p. administration of the monomeric form. As shown in Figure 6, LR12 quickly reached the bloodstream, with both monomer and dimer forms detectable at the first timepoint. While the monomer quickly reached a maximum value [time to peak concentration (t_{max}) = 5 min, peak concentration (C_{max}) = 181 ± 35 ng/mL] before disappearing during a 15 min period, the dimer form (t_{max} = 15 min, C_{max} = 300 ± 162 ng/mL) lasted much longer in the blood but was not detectable 24 h after LR12 administration. The t_{max} values (5–15 min) observed here were comparable with published results of pharmacokinetics of various pharmaceutical peptides in rodents after i.p. injection (Cao, Gao, & Jusko, 2012; Novakovic, Anderson, & Grasso, 2014; Yamamoto *et al*., 2015).

4 | CONCLUSION

The dodecapeptide LR12 has revealed promising therapeutic effects in various preclinical models of inflammatory diseases. In this work, a simple, sensitive, selective and robust HPLC method with intrinsic fluorescence detection is described for LR12 quantitation in rat blood

samples. Instability of LR12 during blood sampling, sample preparation and storage is highlighted. The optimized protocol for blood samples treatment consists of (a) maintenance of an acidic environment during blood sampling, (b) immediate acidic protein precipitation and (c) samples concentration by SPE followed by partial evaporation. Then, an RP-HPLC with an intrinsic fluorescence detection and chromatographic conditions compatible with MS was applied. To our knowledge, it is the first report in the literature of a method capable of determining murine LR12 concentrations in rat whole blood, which was validated with respect to linearity, precision and accuracy. Finally, LR12 monomer and dimer were successfully monitored *in vivo* in rats in a pharmacokinetic study. PK/PD preclinical studies are essential for drug development and for the design of clinical trials, especially dose schedule and identification of surrogate markers. The method developed in this work can be used in preclinical models to evaluate the relation between dose, bioavailability and therapeutic effect of this peptide. Finally, it is potentially helpful for the development of new therapeutic modalities (e.g. sustained release formulation) as well as for the development of a PK method for similar small peptides. A challenging perspective of this work would be to adapt this method to microsampling that would allow the collection of serial data in small animals.

ACKNOWLEDGEMENTS

This work was supported by the 'Fondation pour la Recherche Médicale', grant number DBS20131128445, to Philippe Maincent. The authors thank F. Dupuis and M. L. Bouressam for their kind help during blood sampling. They also acknowledge S. Aury for her technical assistance, as well as S. Boukhenouna for upstream preliminary experiments.

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How to cite this article: Parent M, Boudier A, Maincent P, et al. LR12-peptide quantitation in whole blood by RP-HPLC and intrinsic fluorescence detection: validation and pharmacokinetic study. *Biomedical Chromatography*. 2017;31:e3877. https://doi.org/10.1002/bmc.3877