Drug Analytical Research
ISSN: 2527-2616
Drug Anal Res, 2019*; v. 3, n.* ***02***, 2-6

Sensitive ultra-fast liquid chromatography method for rosmarinic acid determination in Wistar rat's plasma and brain

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Rosmarinic acid (RA) is a polyphenolic compound recently associated to a neuroprotective potential. Nevertheless, besides the RA poor bioavailability and availability in the central nervous system (CNS) some alternatives to RA delivery, protection, and release been investigated. Recently, our research group optimized chitosan-coated nanoemulsions for RA nasal administration and demonstrated the glioprotective effect against LPS-induced damage in astrocytes. In this context, this study aimed to validate a fast and simplified UFLC method previously reported by our research group for RA determination in Wistar rat's plasma and brain, to be employed in further *in vivo* studies, since the nasal route for RA are not completely understood. The method was validated in terms of specificity, linearity, matrix effect, stability, precision, accuracy and extraction recovery for rat plasma and brain, according to the official guidelines. The method was sensitive, linear (0.1 – 10.0 µg.mL^{-1}), precise and accurate, and showed RA recovery higher than 85% in plasma and brain. Overall results demonstrated that method was successfully validated for determination of RA in rat plasma and brain matrices with high sensibility and with high recovery using simple extraction processes.

Keywords: Rosmarinic acid; UFLC method; plasma; brain; Wistar rats.

Introduction

Rosmarinic acid (RA) is a polyphenolic compound (Fig.1), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. It has numerous biological activities, including a current well-documented anti-inflammatory and antioxidant activities recently associated to a neuroprotective potential [1–6]. Nevertheless, besides the RA poor bioavailability and availability in the central nervous system (CNS), in view of its limited ability to cross the blood-brain barrier (BBB), some alternatives to RA delivery, protection, and release been investigated intended to be used as a new neuroprotective therapy [1,7,8].

Nasal route has been emerged as an alternative strategy for drugs delivery directly to CNS, owing to its ability to by passing BBB, an important obstacle for the delivery of therapeutic agents [9]. In this way, our research group optimized chitosan-coated nanoemulsions for RA nasal administration, since we have recognised that the association of lipid nanotechnology-based delivery systems with mucoadhesive polymers have been a promising approach to improve RA penetration through biological barriers and its residence time in nasal cavity [10]. Additionally, we have recently demonstrated the glioprotective effect against LPS-induced damage in rat astrocyte primary cultures [11].

However, further *in vivo* studies to evaluate this promising neuroprotective approach are still crucial. In this context, this study aimed to validate a fast and simplified ultra-fast liquid chromatography (UFLC) method previously developed by our research group [12] for RA determination in Wistar rat's plasma and brain, to be employed in further *in vivo* studies, since the nasal route for RA are not completely understood.

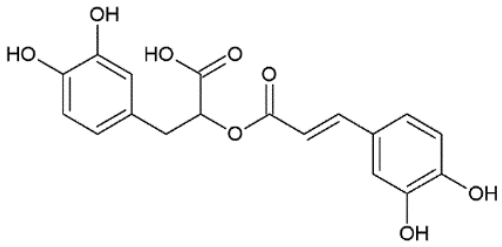

Figure 1. Rosmarinic acid (RA) chemical structure.

Experimental

Chemicals and reagents

RA reference standard with over 98% purity was acquired from Carbosynth Ltd. (Berkshire, GB). Acetonitrile, methanol, and trifluoracetic acid liquid chromatographic grade were purchased from Tedia (Rio de Janeiro, BR). Reverse osmosis using a Milli-Q apparatus (Millipore, Billerica, US) was employed to obtain ultra-pure water.

Instrumentation and chromatographic conditions

UFLC Shimadzu Prominence series system equipped with an automatic injector, a photodiode array (PDA) detector and an LC solutions software (Kyoto, Japan) was employed for RA analyses. Kinetex C18 column (100 x 2.1 mm i.d.; particle size, 2.6 µm; Phenomenex, USA) guarded by a pre-column filter in-line Ultra (Phenomenex, USA) was employed for chromatographic separation at 55 ºC, flow rate of 0.55 mL.min^{-1}, injection volume of 3 µL and detection wavelength of 330 nm. An isocratic eluent composed of water and acetonitrile (83:17, v/v), acidified with 0.1% trifluoracetic acid (v/v), was employed up to 3.5 min [12].

Standard and matrices solutions preparation

RA standard

A stock solution of RA (1.0 mg.mL^{-1}) was prepared in methanol. The stock solution was then diluted in water/acetonitrile mixture (80:20; v:v) to obtain a series of working standard solutions. The stock and standard solution were maintained in darkness at 4 ± 2 °C.

Rat plasma and brain matrices

Plasma matrix from Wistar rats was prepared by proteins precipitation using trichloroacetic acid 5% (w/v). Plasma homogenates were centrifuged (1,000 g, 10 min), the supernatants were collected, diluted 1:1 in water:acetonitrile mixture (80:20; v:v) and filtered through a 0.22 μm nylon syringes filter.
Brain matrix from Wistar rats was obtained by homogenization of tissue with methanol (3 mL per g of tissue) and further sonication in ultrasonic bath for 30 min. Brain homogenates were centrifuged (1,000 g, 10 min), the supernatants were collected and filtered through a 0.22 μm nylon syringes filter.

Method validation

The UFLC method previously reported by our research group [12] was validated in terms of specificity, linearity, matrix effect, stability, precision, accuracy and extraction recovery for rat plasma and brain, according to the official guidelines [13–15].

System suitability

Before method validation, system suitability parameters (peak area, retention time, theoretical plates and tailing factor of RA) were achieved to verify the appropriateness of the chromatographic system for the proposed analysis [16].

Specificity

The specificity was obtained by comparing chromatograms (peak purity and retention time) of pure RA standard solution with chromatograms of matrices (plasma and brain) spiked with RA at the concentration of 10 μg.mL^{-1} and matrices solutions (blank samples).

Linearity, matrix effect and stability

The linearity was determined by regression analysis using the least square method. Three standard curves were obtained in three consecutive days by plotting the measured peak area versus the RA concentration in standard and matrices (0.1, 2.0, 4.0, 6.0, 8.0, and 10.0 μg.mL^{-1}), by six replicates per concentration. Analysis of variance (ANOVA) were used to analyse the results using a significance level of α=0.05.
The matrix effect of plasma and brain was evaluated by the comparison of RA-spiked matrices standard curve slopes obtained during the linearity assay with pure RA standard curve slope.
The stability of RA standard and RA-spiked matrices was assessed by performing the analysis of peak area and detecting any alteration in the chromatographic pattern of the stored samples (room temperature = 25 ± 1 ºC) for 24 h compared with a freshly prepared sample, intending to overestimate the time expended during routine analysis.

Precision and accuracy

The precision was evaluated as both intra-day precision (repeatability) and inter-day precision (intermediate precision) expressed as the relative standard deviations (% RSD) at four RA different levels (0.1, 2.0, 6.0, 10.0 μg.mL^{-1}), in six replicates at each level.
The accuracy was evaluated by adding known amounts of the RA standard at four different levels (0.1, 2.0, 6.0, 10.0 μg.mL^{-1}), in six replicates at each level, and was determined as follows: AC % = (mean experimental concentration x 100/ mean theoretical concentration).

Extraction recovery

The RA extraction recovery was evaluated from Wistar rats plasma and brain spiked with known amounts of RA standard leading to a theoretical concentration of 0.1, 2.0, 6.0 and 10.0 μg.mL^{-1}. To plasma matrices trichloroacetic acid 5% (w/v) in proportion 1:1 (v:v) was added. Then, plasma homogenates were centrifugated (1,000 g, 10 min), the supernatants were collected and diluted in water/acetonitrile mixture (80:20; v:v). Brain tissues (previously washed with saline) were homogenized with 2 mL of methanol per g of tissue in an IKA® Ultra-Turrax T8 mixer (IKA Works Inc., Wilmington, US). All matrices were filtered through a 0.22 μm nylon syringes filter and analyzed by UFLC method. The recovery was expressed as percentage, assessed by comparing the theoretical final concentration based on the spiked amount and the experimental result attained after extraction procedure.

Ethical committee approval

All animal procedures were conducted with prior approval from the ethical approval of Federal University of Health Sciences of Porto Alegre, Brazil (Protocol 220/2017) and in accordance with the Brazilian Guidelines for the Care and Use of Animals in Scientific Research Activities (DBCA) and the National Council of Control of Animal Experimentation (CONCEA).

Results and Discussion

Method validation

Different methodologies for RA quantification through liquid chromatography techniques has been reported in literature [17–29]. Nevertheless, most of these methodologies focus on RA analysis in plant extracts and involves time consuming/ gradient eluent systems. In this context, recently our research group develop a fast and simplified UFLC method [12] for RA analysis based on its capability to promote ultra-high-speed analysis with shorter runtimes in comparison with previous literature, high precision and sensibility.
The UFLC method was the first report for RA determination in nanoemulsions, and porcine skin and nasal mucosa retention/permeation assay. In the literature, only few studies around validation of bioanalytical methods for biological matrices were found for RA determination, although, none of

them included rat plasma and brain under the same experimental conditions [22,30–32].

Regarding the system suitability, overall results demonstrated that the UFLC method is suitable for RA analysis in rat plasma and brain [16]. The parameters values and their relative standard deviation (RSD, %) were: retention time of 2.63 (0.05) min, theoretical plates of 5446.82 (0.08), and tailing factor of 1.25 (0.06).

The method was specific for RA determination in rat plasma and brain matrices, once the RA peak purity was demonstrated in RA spiked-matrices and no co-eluting substances in the same RA retention time were detected in matrices (Fig. 2).

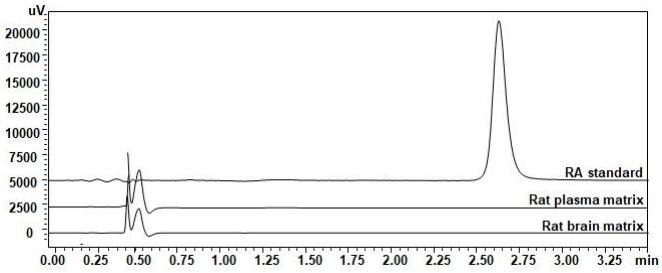

Figure 2. RA chromatographic profile standard at 10 μg.mL^{-1} and specificity in blank samples of rat plasma and brain. RA: rosmarinic acid.

As presented in Table 1, the method was linear for RA determination in rat plasma and brain matrices in range of 0.1–1.0 μg.mL^{-1} according with correlation coefficients. Linear regression and the absence of linearity deviation were also demonstrated by ANOVA evaluation of regression significance and confidence interval on the intercepts. LOQ was fixed at 0.1 μg.mL^{-1} as it was the lowest concentration of RA that could be quantified with acceptable precision and accuracy.

The results for rat plasma and brain matrices effects (Table 1) were lower than 2.7% and indicates a low matrix effect in RA analysis [12,33]. The matrix effect is a very important parameter in biological samples to ensure the RA correct quantification in presence of matrices [34,35].

The results obtained for intra and inter-day precision, accuracy and stability in standard and rat plasma and brain matrices are shown in Table 2. Four different concentrations of RA standard and spiked-matrices were evaluated: 0.1 μg.mL^{-1} (LOQ), 2.0 μg.mL^{-1} (lowest concentration), 6.0 μg.mL^{-1} (medium concentration), and 10.0 μg.mL^{-1} (highest concentration) for each sample. The method was considered precise and accurate for RA determination in rat plasma and brain matrices according to official guidelines [13–15]. The intra-day and inter-day precision results demonstrated a relative standard deviation (RSD, %) lower than 5%. The accuracy ranged within 104.69 to 110.99% range. The stability data also demonstrated that the RA concentration remained constant in matrices even after 24 h of storage (room temperature = 25 ± 1 ºC).

Table 1. Linearity and summary of the output of the ANOVA of RA standard, rat plasma brain matrices.

		RA standard	Rat plasma matrix	Rat brain matrix
	Range (μg.mL^{-1})	0.1 – 10.0	0.1 – 10.0	0.1 – 10.0
	Regression equation	y = 8592x – 183.18	y = 8777.8x – 238.3	y = 8829.7x – 40.559
	R	0.9996	0.9994	0.9996
	Matrix effect (%)	-	2.12	2.69
Regression	Significance F	2.239E-94[a]	6.376E-90 [a]	1.851E-97 [a]
Intercept	p-value	0.32600[b]	0.2865 [b]	0.8226 [b]
	Lower 95%	-556.1767	-693.4420	-377.8245
	Upper 95%	187.8070	208.5224	301.3503

[a] 95% confidence level= significant linear regression; [b] 95% confidence level= no significant linearity deviation; RA: rosmarinic acid; R: correlation coefficient.

Table 2. Intraday and intra-day precision, accuracy and stability evaluation of RA standard, rat plasma brain matrices.

	Level	Precision (RSD)				Accuracy	SE
	(μg/mL)	First day [a]	Second day [a]	Third day [a]	Inter-day	(%)	(%)
RA	0.1	3.99	3.99	3.99	3.51	105.53	104.69
	2.0	4.11	1.38	2.68	3.31	97.01	95.58
	6.0	1.38	0.21	0.33	1.06	101.24	102.37
	10.0	0.72	0.67	0.44	0.77	98.76	99.41
Rat plasma matrix	0.1	1.34	2.54	2.18	4.36	108.94	110.99
	2.0	0.82	2.24	2.16	2.40	96.63	95.68
	6.0	1.37	1.03	1.26	1.56	101.34	99.94
	10.0	0.47	0.45	0.39	0.53	99.01	100.25
Rat brain matrix	0.1	4.88	1.10	2.44	3.81	91.97	90.44
	2.0	1.80	2.99	1.55	2.90	98.86	96.85
	6.0	1.25	2.38	1.72	2.01	100.38	99.91
	10.0	0.26	0.88	0.55	0.68	99.24	99.21

[a] six replicates per day; SE: stability evaluation in 24 hours; RSD= relative standard deviation (%); RA: rosmarinic acid.

The results for RA extraction recovery from rat plasma and brain recovery are presented in Table 3 and were established after preliminary studies. The recovery was higher than 84.9% and lower than 105.1%, without interference of matrix components and with adequate precision RSD lower than 11.5% for both matrices in accordance with FDA recommendations for bioanalytical procedures validation [15].

Table 3. RA extraction recovery from rat plasma and brain matrices.

	Level (μg.mL^{-1})	Recovery (RSD, %)
Rat plasma matrix	0.1	84.91 (11.5)
	2.0	89.39 (4.9)
	6.0	88.18 (1.3)
	10.0	90.49 (14.9)
Rat brain matrix	0.1	93.72 (5.5)
	2.0	97.82 (11.5)
	6.0	105.07 (2.8)
	10.0	98.55 (3.5)

RSD: relative standard deviation (%); RA: rosmarinic acid.

Conclusions

In present study, a fast and simple UFLC method previously reported by our research group was successfully validated for determination of RA in rat plasma and brain matrices with high sensibility and with high recovery using simple extraction processes. This is the first high-throughput liquid chromatography method validated for RA determination in different complex matrices, allowing its use in further *in vivo* studies.

Acknowledgments

The authors are gratefully acknowledged to Conselho Nacional de Desenvolvimento científico e Tecnológico (CNPq - processos 459619/2014-4; 310846/2014-5; 422298/2016-6), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS - processos 16/2551-0000265-7; 16/2551-0000473-0; 17/2551–0001273-9), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES - Finance Code 001) for the financial grants. F.N.S.F., J.H.A. and L.R.M. would like to thank CAPES and CNPq (GD#142202/2018-5) for the doctoral grant. V.L.B., L.S.K., A.T.H., E.B., and H.F.T. are recipients of CNPq research fellowship.

Conflict of interest

The authors declare no conflicts of interest.

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