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Article

Liquid chromatography–mass spectrometry for simultaneous determination of spironolactone and canrenone in plasma samples

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In our study, we aimed to validate a method based on liquid chromatography–mass spectrometry (LC–MS) to quantify spironolactone (SPI) and its active metabolite canrenone (CAN) simultaneously in plasma samples to support *in vivo* experiments. Compounds were separated by using a C_{18} column with the isocratic elution of a mobile phase composed of 0.1% (v/v) formic acid in methanol–water (60:40 v/v) at a flow rate of 0.4 mL min^{-1}. SPI and CAN were detected in an electrospray interface operating in a positive ionization mode and quantified using the selective ion mode monitoring of mass–charge ratios (*m/z*) of 439.0 for SPI and 363.1 for CAN. After calculating the matrix effect using theoretical equations, we observed the strong interference of plasma in the equipment-generated signal, which required creating analytical curves using the matrix as a solvent. The method was nevertheless linear ($r^2 > 0.999$) in a concentration range of 0.4–5.0 μg mL^{-1}, as well as precise, with a coefficient of variation less than 5%. SPI's and CAN's recovery rates from the plasma ranged from 87.4% to 112.1%, while their limits of detection (i.e., 0.07 μg mL^{-1} and 0.03 μg mL^{-1}, respectively) and quantification (i.e., 0.20 μg mL^{-1} and 0.08 μg mL^{-1}, respectively) in the presence of plasma contaminants were low. Therefore, the bioanalytical method seems to be feasible for quantifying SPI and CAN in plasma.

Keywords: Bioanalytical method. High-performance liquid chromatography. Pharmacokinetics. Antiandrogen.

INTRODUCTION

Spironolactone (SPI, 7a-acetylthio-3-oxo-17a-pregn-4-ene-21,17-carbolactone) (Figure 1), an aldosterone antagonist drug discovered in 1957, is used to treat congestive heart failure, hyperaldosteronism, edema, and arterial hypertension (Kosmas *et al*., 2018). It also acts as an inhibitor of the 5-alpha-reductase enzyme in treating acne vulgaris and androgenic alopecia (Afzali *et al*., 2012; Kim, Del Rosso, 2012; Shaw, 2002). After oral administration, SPI metabolizes into canrenone (CAN), as shown in Figure 1, a non-selective aldosterone antagonist that also possesses antiandrogenic, antihypertensive, and cardiovascular effects (Pamnani *et al*., 1990).

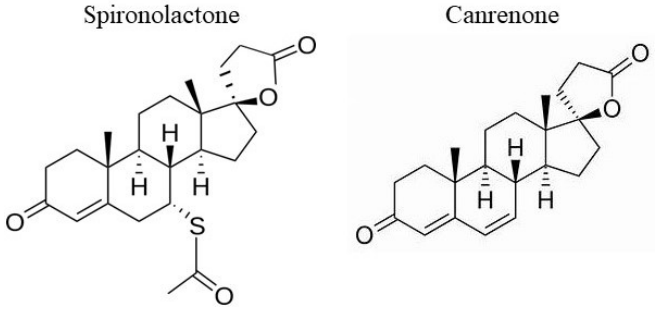

FIGURE 1 – Molecular structures of spironolactone (416.57 g/mol) and canrenone (340.45 g/mol).

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Given its widespread use in clinical practice, SPI features in several pharmaceutical formulations, including capsules, tablets, solutions, suspensions, and syrups (Elkordy, Tan, Essa, 2013; Langguth *et al*., 2005; Rosa *et al*., 2014). However, taking SPI orally can result in side effects such as irregular menstruation, hypotension, cramps, and reduced libido (Sica, 2005). To reduce or eliminate such effects, many drug delivery systems, either for oral or topical administration, have been proposed, including gels and creams (Afzali *et al*., 2012; Ayatollahi, Samadi, 2021), nanoparticles (Akbari *et al*., 2016; Ferreira-Nunes *et al*., 2021), inclusion complexes with cyclodextrins (Kaukonen, Lennernäs, Mannermaa, 1998), liposomes (Laouini *et al*., 2011), and solid lipid nanoparticles (Shamma, Aburahma, 2014).

Nevertheless, the principal challenge in evaluating the effectiveness of such new formulations is quantifying SPI and CAN in formulations and biological matrices, including plasma, to support pharmacokinetic studies. In response, some chromatographic methods have been proposed for quantifying SPI and CAN in solid doses (Anderson *et al*., 2017), in skin to support permeation studies (Ferreira-Nunes *et al*., 2019), and in blood for pharmacokinetic studies (Dong *et al*., 2006; Kaukonen, Lennernäs, Mannermaa, 1998; Sandall *et al*., 2006; Takkis *et al*., 2017; Vlase *et al*., 2011). Those methods, however, have either not demonstrated enough selectivity or sensitivity to analyze the drugs in plasma (Anderson *et al*., 2017; Ferreira-Nunes *et al*., 2019; Kaukonen *et al*., 1998) or use a complex mixture of buffer and solvents (Kaukonen, Lennernäs, Mannermaa, 1998; Takkis *et al*., 2017; Vlase *et al*., 2011) that can compromise chromatographic columns. Some of the methods also require time-consuming analysis or uncommon, sophisticated techniques such as atmospheric pressure chemical ionization (Dong *et al*., 2006).

Drug detection with mass spectrometry (MS) boosts the sensitivity and selectivity of chromatographic methods. Even so, when using MS coupled with liquid chromatography (LC)—that is, LC–MS—the matrix effect (ME) can increase or suppress the signal of the analytes, which can consequently compromise the ultimate quality of analyses. Thus, quantifying SPI and CAN in the plasma matrix compared with an organic solvent can be valuable for considering ME in methods used to support pharmacokinetic studies.

Considering all of the above, we set out to validate a straightforward bioanalytical method to quantify SPI and CAN simultaneously in plasma samples of mice to support preclinical studies on formulations containing the drug.

MATERIAL AND METHODS

Chemicals and reagents

Analytical standards for SPI (>99.9%) and CAN (>99.9%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade methanol was obtained from J.T.Baker (Phillipsburg, NJ, USA), while ultrapure water was obtained by using the Milli-Q water purification system (Millipore, Illkirch-Graffenstaden, France). The HPLC-grade formic acid used to prepare the mobile phase was purchased from Sigma-Aldrich (Steinheim, Germany).

Blood plasma

Plasma was collected via cardiac puncturing after mice were euthanized. All animals were anesthetized with isoflurane and left in a CO_2 chamber, after which cardiac puncturing was performed to collect the blood stored in Falcon tubes and centrifuged at 2,150 × *g* for 10 min to separate the plasma. The plasma samples were stored under refrigeration (4 °C) for up to 7 days before use.

The project was approved by the Ethics Committee on Animal Use of the Faculty of Health Sciences, University of Brasília (Protocol number: 028/2020).

Preparation of stock solutions

Stock solutions (100 μg mL^{-1}) were prepared by dissolving 1 mg of the analytes (i.e., SPI and CAN) in 10 mL of a mixture of methanol and ultrapure water acidified with 0.1% formic acid (60:40, v/v). The mixture was subsequently placed in an ultrasound bath for 1 min to facilitate dissolution, and the resulting solution was stored in a refrigerator (4 °C) for up to 7 days before use.

Instrumentation and analytical conditions

Drug quantification was performed using an LC–MS apparatus (2020 model, Shimadzu, Kyoto, Japan), composed of an HPLC system coupled with a mass spectrometer with an electrospray interface. Chromatographic separation was performed on a C_{18} reversed-phase column (4.6 × 150 mm, 5 µm; Sigma Aldrich, Steinheim, Germany) maintained at 35 °C. The mobile phase was composed of methanol with 0.1% (v/v) formic acid and ultrapure water with 0.1% (v/v) formic acid, in a proportion of 60:40 (v/v). The mobile phase flow was 0.4 mL min^{-1}, while the sample injection volume was 3 µL. The duration of analysis for each sample was 23 min. Electrospray interface source operated in positive ionization mode, and, concerning the general settings, the drying gas flow was 15 L min^{-1}, the nebulizer gas flow was 1.5 L min^{-1}, and the capillary voltage was 4,500 V. Scanning and selected ion mode monitoring were performed to observe all sample compositions and quantify the drugs using their specific *m/z* ratios (i.e., 439.0 for SPI and 363.1 for CAN). Last, data acquisition and processing were performed using LC Solution software (version 5.89; Shimadzu, Kyoto, Japan).

Method validation

The bioanalytical method was validated according to the parameters of selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision (i.e., repeatability and intermediate precision), and accuracy, all based on the guidelines of the International Conference on Harmonization (ICH, 2019) and the US Food and Drug Administration (FDA, 2018).

Selectivity

In analyses using LC–MS, the selectivity of the analytical method is evaluated according to the *m/z* ratio of each analyte of interest. During analysis performed with MS, the identification of species corresponding to chromatographic peaks generates a unique pattern for the sample that ensures the technique's selectivity (Wille *et al*., 2012). We chose an *m/z* ratio of the fragments after ionization for each analyte: 439.0 for SPI and 363.1 for CAN. In addition, ion 341.2, which allowed quantifying both analytes, was used to monitor the analysis performed by the equipment (Dong *et al*., 2006).

To confirm that plasma constituents would not interfere with the quantification of the drugs, samples containing the matrices without the analytes (i.e., blank samples) were analyzed. To obtain those interferents, 0.5 mL of plasma was pipetted into a glass tube, and 2 mL of dichloromethane solution–ethyl acetate (20:80 v/v) was added. The resulting mixture was vortexed for 1 min and centrifuged at 2,150 × *g* for 10 min. Afterward, the supernatant was collected with a micropipette and dried in an oven at 45 °C for 12 h. Last, the material was resuspended by adding 1 mL of the mobile phase, filtered through a 0.45 µm filter, and analyzed using LC–MS.

Linearity and matrix effect

Analytical curves were constructed from the SPI and CAN stock solutions at a final concentration of 100 µg mL^{-1}. Six dilutions were performed from each solution to obtain six drug concentrations: 0.4, 0.6, 0.8, 1.0, 2.5, and 5.0 µg mL^{-1}. The analysis was performed in triplicate under the mentioned conditions using the mobile phase for the blank samples. Due to the ME, linearity was verified in the presence of possible interferents in the plasma. Data were statistically analyzed by linear regression; angular coefficient significance and proportionality were calculated with Student's *t* test ($p = .05$), whereas response factors were calculated considering the ratio of peak area to analyte concentration (ICH, 2019).

The ME of the contaminants was evaluated by determining the contaminants' significance using the signals of SPI and CAN in the matrix curve (*Sm*) and both drugs together in the solvent curve (*Ss*), as shown in Eq. 1 (ICH, 2019).

$$ME(\%) = \left(\frac{Sm}{Ss}\right) \times 100 \quad (1)$$

The variation permitted in the linearity test for bioanalytical methods has to be less than 15% (FDA, 2018; Ferreira-Nunes *et al*., 2017).

Limits of detection and quantification

The minimum concentrations of SPI and CAN to be detected and quantified (i.e., LOD and LOQ, respectively) were calculated based on the standard deviation (σ) and slope (*S*) of the three calibration curves according to Eqs. (2) and (3), respectively:

$$LOD = 3.3\ x \frac{\sigma}{S} \quad (2)$$

$$LOQ = 10\ x \frac{\sigma}{S} \quad (3)$$

Precision

Precision was assessed in terms of repeatability (i.e., intra-run precision) and intermediate precision (i.e., inter-run precision). To begin, the method's repeatability was evaluated considering the dispersion of peak area values of the three concentrations analyzed (0.4, 1.0, and 5.0 μg mL^{-1}), which include the linear range of the method, in triplicate for each concentration and analyte.

By contrast, intermediate precision was evaluated considering the dispersion of peak area values of three concentrations (0.4, 1.0, and 5.0 μg mL^{-1}), which include the linear range of the method, in triplicate for each concentration and each analyte, obtained using two different equipment and two different analysts. Results for precision were recorded in terms of the coefficient of variation (CV%) and, for a bioanalytical method, needed to be less than 15% (FDA, 2018; ICH, 2019).

Accuracy

The method's accuracy was assessed by calculating the recovery of SPI and CAN from the mouse plasma. To that end, 0.5 mL of plasma was pipetted into a glass tube, to which different volumes of the stock solutions of SPI and CAN in the mobile phase (i.e., 4.0, 10.0, and 50.0 μL) were added. Next, 2 mL of a dichloromethane solution:ethyl acetate (20:80 v/v) was added and homogenized by vortexing for 1 min. Afterward, samples were centrifuged at 2,150 × *g* for 10 min, and the supernatant was collected with the aid of a micropipette. Last, the solvent was dried in an oven at 50 °C for 12 h and resuspended with 1 mL of the mobile phase.

The drugs' recovery was determined according to the ratio of SPI and CAN extracted from the plasma to the amount initially added. Results of drug recovery were calculated using Eq. (4):

$$Recovery\ (\%) = \left(\frac{measured\ concentration}{theoretical\ concentration}\right) \times 100 \quad (4)$$

The acceptable limits established by regulatory agencies vary between 85.0 and 115.0% because such methods involve drug extraction from a biological matrix (FDA, 2018; ICH, 2019).

RESULTS AND DISCUSSION

Although at least seven published works (Anderson *et al*., 2017; Dong *et al*., 2006; Ferreira-Nunes *et al*., 2019; Kaukonen, Lennernäs, Mannermaa, 1998; Sandall *et al*., 2006; Takkis *et al*., 2017; Vlase *et al*., 2011) show that chromatographic methods can be used to quantify SPI in different chemical and biological matrices, the method proposed herein was designed to analyze SPI and its metabolite CAN simultaneously with enough sensitivity and selectivity considering the interfering samples of blood plasma. The rapid run time of the analysis, at only 20 min, constitutes a relevant analytical advantage given the challenge of simultaneously quantifying SPI and its metabolite, CAN, which have similar chemical structures, in the presence of biological interferents.

Our method was validated in terms of selectivity, linearity, LOD, LOQ, precision, and accuracy, and a study to assess the ME of the plasma interferents was performed as well, as described below.

Selectivity

LC–MS can be used to identify molecular species according to their *m/z* ratio and the form of ionization (i.e., positive or negative) in the electrospray (Wille

et al., 2012; Wong, MacLeod, 2009). Thus, during analysis, chromatographic peaks either known or unknown can be monitored according to the *m/z* ratio. Furthermore, during the ionization of drugs, fragments with different *m/z* ratios can be generated and ionized as well. The technique is sensitive and specific (Oliveira *et al*., 2020), because the likelihood of detecting two molecules showing the same fragmentation pattern is minimal.

Figure 2 depicts the chromatograms obtained for SPI and CAN in a run following the proposed method, and in Figure 3, their MS, the latter correlating to the *m/z* ratios of the generated ions.

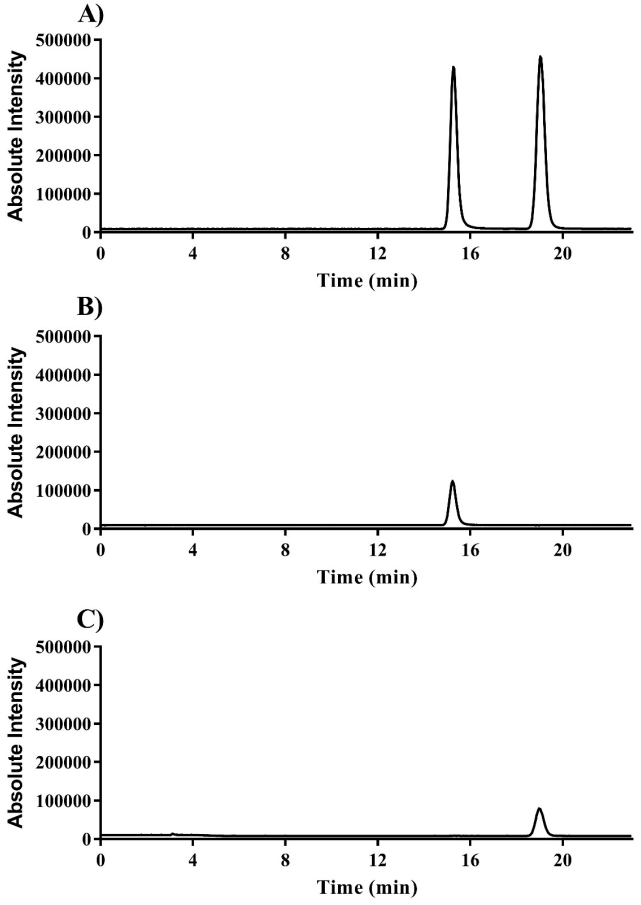

FIGURE 2 – (A) Chromatogram obtained after analyzing spironolactone and canrenone (5.0 μg mL^{-1}) in the ion mode selected for the ion in common (*m/z* = 341.1); (B) chromatogram of the spironolactone fragment (*m/z* = 439.0) with a retention time of 15.8 min; and (C) chromatogram of the canrenone fragment (*m/z* 363.1) with a retention time of 19.8 min.

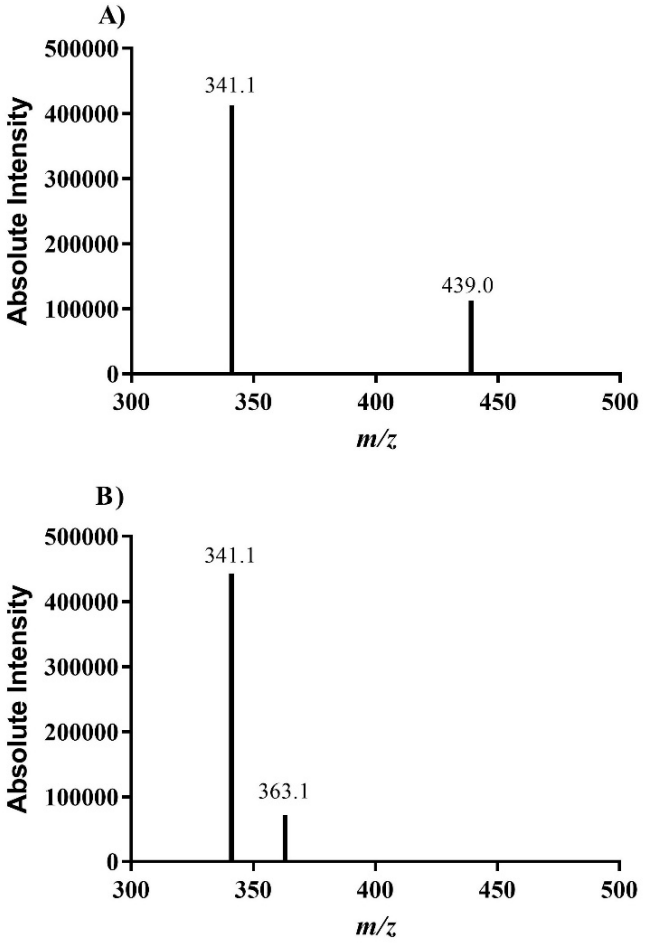

FIGURE 3 – Mass spectrum of (A) spironolactone and (B) canrenone. Mass spectra were obtained after ionizing the samples in positive mode by electrospray. The figure shows the prominent peaks obtained after analyzing the total ion chromatogram of the drugs to verify the primary signals.

As shown in Figures 2 and 3, the primary ions observed for SPI were $[M + Na]^+$ (i.e., *m/z* = 439.0) and $[M – SCOCH_3]^+$ (i.e., *m/z* = 341.1) and for CAN were $[M + Na]^+$ (i.e., *m/z* = 363.1) and $[M + H]^+$ (i.e., *m/z* = 341.1), which corroborates the results of Dong *et al*. (2006). Therefore, the common ion (i.e., *m/z* = 341.1) was chosen to monitor the analyses, and the specific ions for each drug were used to quantify the analytes.

The selectivity of the analytical method was also evaluated by comparing the chromatograms of the blank sample (i.e., dilution solvent), the drugs, and the analytical interferents in the plasma (Figure 4). Data collected along those lines indicate that the method is selective for quantifying SPI and CAN in plasma.

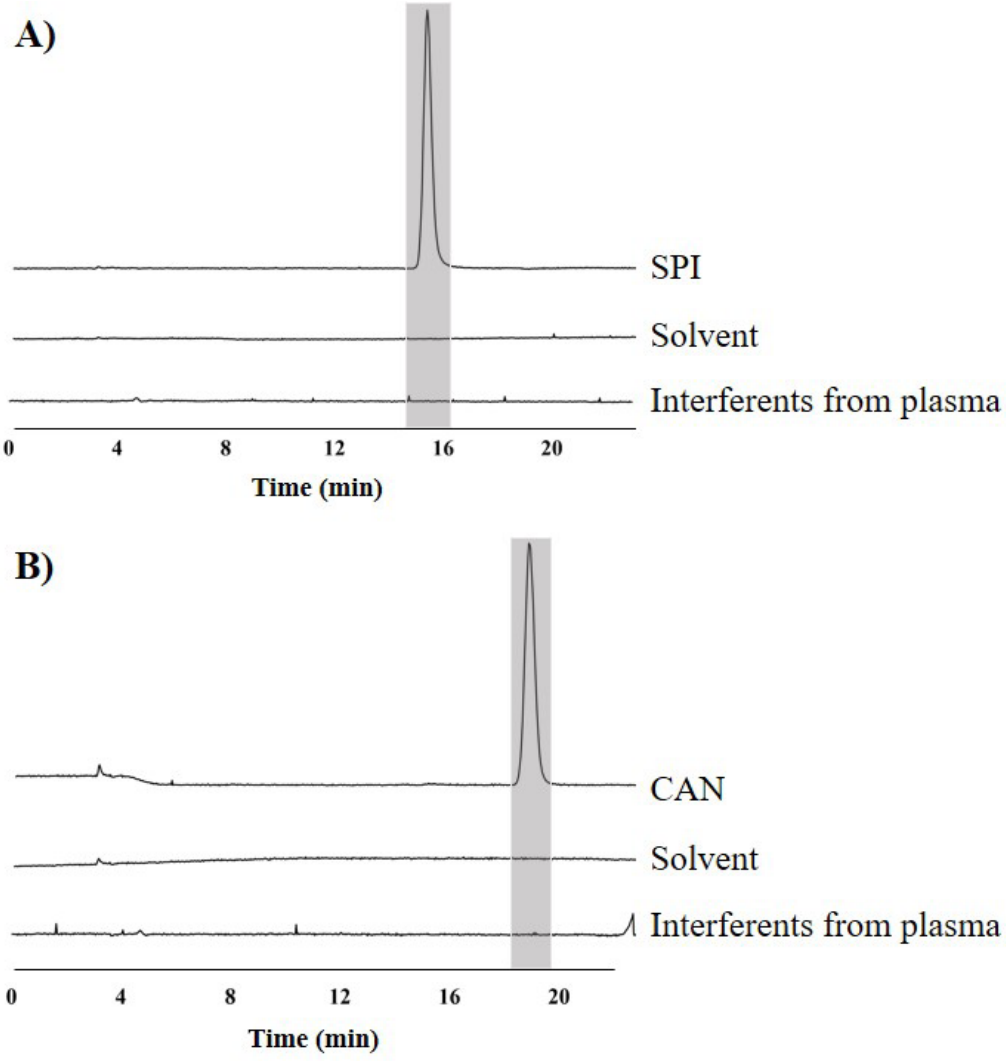

FIGURE 4 – Representative chromatograms of the blank injection of interferents from plasma and solvent (i.e., mobile phase) for (A) spironolactone (SPI) at 5.0 µg mL^{-1} (i.e., *m/z* = 439.0) and (B) canrenone (CAN) at 5.0 µg mL^{-1} (i.e., *m/z* = 363.1).

Linearity

The analytical curves obtained for the quantification of SPI and CAN are shown in Figure 5.

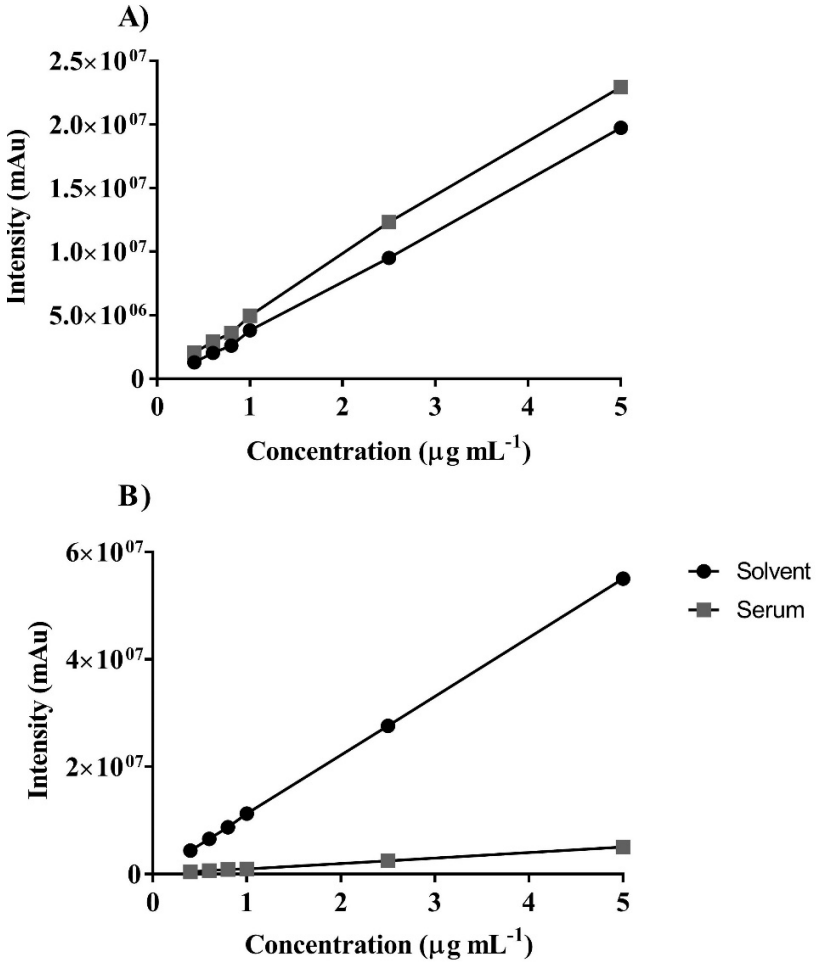

FIGURE 5 – Curves obtained by analyzing samples prepared with solvent and plasma for both drugs; (A) spironolactone's and (B) canrenone's concentrations ranged from 0.4 to 5.0 µg mL^{-1}. Calibration curve equations were (A) y = 2,669,850.49x + 270,168.3 (r^2 = .9996) for the solvent and y = 4,718,556.1x + 139,019.15, with r^2 = .9998 for the plasma matrix and (B) y = 1711,581.50x + 52,883.47 (r^2 = .9997) for the solvent and y = 997,542.0x + 2,183.1, with r^2 = .9995 for the plasma matrix. Data show the average of 3 replicates ± *SD*.

By calculating the linear regression for the curves in which the drugs were dissolved in methanol, we found linear correlation coefficients (r^2) of .9996 and .9997 for SPI and CAN, respectively, both of which were within established limits (ICH, 2019). The high numerical values found for the slopes—2,669,850 ± 27,498 for SPI and 1,711,582 ± 13,851 for CAN—indicate adequate responses of the method in relation to different concentrations. Student's *t* test revealed that the slope was different from 0, as recommended (ICH, 2019). The CV% of the curve areas ranged between 1.03% and 4.88%, which were thus also within the established limit (FDA, 2018).

Because our method involves a biological matrix, the ME that can occur by using MS was also evaluated. The ME can increase or suppress the signal detected by MS because the interferents present in the matrix elute together with the analytes of interest, thereby generating different responses from the equipment (Rudzki, Gniazdowska, Buś-

kwaśnik, 2018). Thus, a calibration curve was constructed in the presence of interferents contained in plasma. The analytical curve appears in Figure 5B.

The ME was calculated using theoretical equations considering that plasma is a biological material rich in metabolites, inorganic salts, and lipoproteins. In our work, the signals generated by the equipment from samples diluted in the solvent and samples contaminated with plasma were considered. The results obtained for SPI and CAN were 164.3 ± 11.7 and −127.6 ± 11.6, respectively, which indicate an increase and decrease in the signal for SPI and CAN, also respectively. Thus, the plasma seemed to strongly interfere with the signals generated by the equipment.

By calculating the linear regression for the curves of plasma, we found that the linear correlation coefficients (r^2) obtained were .9998 and .9995 for SPI and CAN, respectively. The high numerical values found for the slopes—4,718,557 ± 85,588 for SPI and 997,542 ± 11,171 for CAN—indicate the method's adequate responses to different concentrations, even in the presence of interferents from the matrix. Student's *t* test showed that the slope was different from 0, as recommended (ICH, 2019). The CV% of the mean of the curve areas ranged between 0.31% and 6.33%, which upholds the variation allowed for bioanalytical methods (FDA, 2018; Ferreira-Nunes *et al*., 2017).

Our method was shown to be linear in quantifying SPI and CAN in the concentration range of 0.4 to 5.0 μg mL^{-1}, in both the solvent and the biological matrix. Furthermore, the analytical curves were prepared in a matrix because the signal of the drugs was interfered with by agents in the plasma.

Limits of detection and quantification

LOD and LOQ values obtained for the method are listed in Table I. The ME has to be considered, because the CAN metabolite extracted from the matrix may have a decreased signal, which may complicate its correct detection and quantification with the equipment.

Considering that the usual dose of SPI is 10 mg and has 90% bioavailability after oral administration (Li *et al*., 2016), for a 70 kg adult with a blood volume of 5 L, the method has to be able to unambiguously quantify more than 2 μg/mL of the drug in plasma in order to support pharmacokinetic studies. Based on the LOQ values shown in Table I (i.e., 0.20 and 0.08 μg/mL for SPI and CAN, respectively), the method can accurately quantify a 10-fold lower concentration of SPI and 25-fold lower concentration of CAN than what is required.

TABLE I – Limits of detection (LOD) and quantification (LOQ) for spironolactone and canrenone in solvent and plasma

Sample	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Spironolactone		
Solvent	0.049	0.149
Plasma	0.068	0.202
Canrenone		
Solvent	0.050	0.170
Plasma	0.030	0.079

Precision

The precision of the analytical method was first evaluated in terms of repeatability. Intermediate precision was calculated considering different analysts and days; such a set of tests helped to detect random errors that cannot be controlled but that are inherent to the method, including those caused by glassware, solvents, reagents, weighing scale, and sample preparation (Ferreira-Nunes et al., 2017).

The method demonstrated repeatability between samples covering the range of the analytical method, with CV% values less than 4.74%, as shown in Table II.

TABLE II – Results of the repeatability of the analytical method for the quantification of spironolactone and canrenone

Sample	Theoretical concentration (μg mL^{-1})	Experimental concentration (μg mL^{-1})	Coefficient of variation (%)
Spironolactone			
Solvent	0.40	0.42	1.89
	1.00	1.04	2.82
	5.00	5.22	0.86
Plasma	0.40	0.42	4.74
	1.00	0.99	3.19
	5.00	4.91	4.09
Canrenone			
Solvent	0.40	0.42	3.51
	1.00	1.03	1.30
	5.00	5.17	0.85
Plasma	0.40	0.39	3.38
	1.00	1.00	4.51
	5.00	4.81	1.33

According to the CV% values shown in Table III, the method demonstrated accuracy within the variation of analysts and days. The highest coefficient of variation for SPI was 4.78%, while the lowest was 0.27%. As for CAN, the most considerable variation was 4.86% and the least was 0.20%. Regarding general CV% (Table III), all values remained less than 15.0% for both analytes (FDA, 2018). Those results imply that the values have low data dispersion and that the method is suitable for its intended purpose.

TABLE III – Results of the intra- and interassay precision of the analytical method for quantifying spironolactone and canrenone in methanol and plasma

Sample		Theoretical concentration (μg mL^{-1})	Condition		Experimental concentration (μg mL^{-1})	CV (%)	Overall CV (%)
Spironolactone	Solvent	0.40	Analyst 1	Day 1	0.39	4.29	6.69
			Analyst 2	Day 1	0.40	2.67	
			Analyst 1	Day 2	0.38	2.25	
			Analyst 2	Day 2	0.40	4.78	
		1.00	Analyst 1	Day 1	1.04	2.82	3.29
			Analyst 2	Day 1	1.01	1.51	
			Analyst 1	Day 2	1.03	3.01	
			Analyst 2	Day 2	1.03	2.53	
		5.00	Analyst 1	Day 1	5.11	2.57	2.88
			Analyst 2	Day 1	5.02	2.26	
			Analyst 1	Day 2	5.16	2.17	
			Analyst 2	Day 2	5.24	2.47	

TABLE III – Results of the intra- and interassay precision of the analytical method for quantifying spironolactone and canrenone in methanol and plasma

Sample		Theoretical concentration (μg mL^{-1})	Condition		Experimental concentration (μg mL^{-1})	CV (%)	Overall CV (%)
Spironolactone	Plasma	0.40	Analyst 1	Day 1	0.43	3.08	6.04
			Analyst 2	Day 1	0.41	4.26	
			Analyst 1	Day 2	0.42	4.06	
			Analyst 2	Day 2	0.40	4.07	
		1.00	Analyst 1	Day 1	0.99	2.12	3.65
			Analyst 2	Day 1	0.97	2.68	
			Analyst 1	Day 2	1.02	1.85	
			Analyst 2	Day 2	1.02	1.87	
		5.00	Analyst 1	Day 1	4.81	1.58	5.11
			Analyst 2	Day 1	4.81	1.47	
			Analyst 1	Day 2	5.31	0.27	
			Analyst 2	Day 2	5.32	0.84	
Canrenone	Solvent	0.40	Analyst 1	Day 1	0.41	3.51	4.93
			Analyst 2	Day 1	0.40	0.65	
			Analyst 1	Day 2	0.41	3.78	
			Analyst 2	Day 2	0.40	3.03	
		1.00	Analyst 1	Day 1	1.03	3.08	2.10
			Analyst 2	Day 1	1.04	0.20	
			Analyst 1	Day 2	1.05	1.80	
			Analyst 2	Day 2	1.03	0.90	
		5.00	Analyst 1	Day 1	5.21	4.03	2.91
			Analyst 2	Day 1	5.12	2.26	
			Analyst 1	Day 2	5.21	2.42	
			Analyst 2	Day 2	5.20	1.29	
	Plasma	0.40	Analyst 1	Day 1	0.40	4.44	7.37
			Analyst 2	Day 1	0.41	4.86	
			Analyst 1	Day 2	0.38	3.05	
			Analyst 2	Day 2	0.41	2.47	
		1.00	Analyst 1	Day 1	1.00	1.65	5.51
			Analyst 2	Day 1	0.98	2.78	
			Analyst 1	Day 2	1.06	2.64	
			Analyst 2	Day 2	0.95	2.36	
		5.00	Analyst 1	Day 1	4.81	1.33	1.20
			Analyst 2	Day 1	4.79	1.10	
			Analyst 1	Day 2	4.85	0.42	
			Analyst 2	Day 2	4.85	1.03	

Note: CV = coefficient of variation.

Accuracy

The recovery of SPI and CAN was evaluated with reference to drug-contaminated samples of plasma. The high values of accuracy, ranging from 87.36 to 112.09% (Table IV), may relate to the solubilization of SPI and CAN in organic solvents such as methanol, ethyl acetate, dichloromethane, and chloroform (Dong *et al*., 2006). Therefore, the values were satisfactory for analyte extraction from the samples of plasma, with an average of 100.8%.

TABLE IV – Accuracy of the analytical method used for quantifying spironolactone and canrenone

Sample	Theoretical concentration (μg mL^{-1})	Experimental concentration (μg mL^{-1})	Coefficient of variation (%)	Recovery (%)
Spironolactone	0.40	0.43	5.21	108.34
	1.00	1.12	1.76	112.09
	5.00	5.17	5.69	103.56
Canrenone	0.40	0.39	7.75	96.46
	1.00	0.97	6.25	97.03
	5.00	4.36	4.17	87.36

CONCLUSION

We have validated a rapid, straightforward chromatographic method to quantify SPI and its active metabolite CAN simultaneously in plasma samples without using salts in the mobile phase. Even considering the ME when the drugs were placed in contact with the interfering plasma, the method proved to be selective, linear, sensitive, and accurate in all analyses and presented high rates of recovery for drugs extracted from the plasma. Thus, the method seems to be feasible for use in pharmacokinetic studies of medicines containing SPI.

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