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Development and Validation of a Liquid Chromatographic Method for CasiopeinaIII-ia® in Rabbit Blood and its Application in Pre-clinical Pharmacokinetic Research

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Abstract. A rapid and simple high-performance liquid chromatography (HPLC) method using extraction with zinc sulfate was developed for determining Casiopeina III-ia (CasIII-ia) levels and then validated for the linear 10-120 µg/mL range in 200 µL of rabbit blood. An HPLC method for quantifying CasIII-ia in plasma has been previously reported; however, studies on its distribution found that CasIII-ia concentrations were higher in whole blood than in plasma. The analysis was performed using the following parameters: methanol-sodium phosphate buffer (pH 6.5; 0.01 M) (40:60 v/v) mobile phase, using a 250 x 4.6 mm I.D Symmetry® C_{18} column and a particle size of 5 µm (Waters Associates, Milford, MA, USA) with a C_{18} 5 µm guard column (Phenomenex®) was kept at a isocratic flow-rate of 0.8 ml/min, room temperature at a wavelength of 262 nm. Acetaminophen was used as the internal standard. The results showed that the assay is sensitive at 10 µg/mL. A linear relationship of r^2=0.9954 for Cass-III-ia was plotted against concentrations ranging from 10 to 120 µg/mL; the analytical method complies with linearity. The maximum intra-day relative standard deviation (RSD) was 5.10 %. An average 84.8 % intra-day (n=15) and 92.5 % inter-day (n=30) recovery % of CasIII-ia was recovered for the whole blood samples. The results demonstrated the applicability of this method for obtaining its *in vitro* distribution and also it´s for use in pharmacokinetic studies at the preclinical level on rabbits. The present study shows an assay rapid, simple, precise, and accurate for quantifying Cas III-ia in rabbit whole blood. The pharmacokinetic study, carried out in rabbits, obtained the following pharmacokinetic parameters: (kel) = 0.0150 min^{-1}; half-life time ($T_{1/2}$) = 53.9 min; with an apparent volume of distribution (Vd) = 202.8 mL; clearance (Cl) = 2.0 mL /mi; and area under the curve (AUC)=23163.8 µg/mL.min. The results contribute to the preclinical characterization of Cas III-ia.
Keywords: Antineoplastic; Casiopeína III-ia; validation HPLC-UV; pharmacokinetics; copper compound.

Resumen. Se desarrolló un método rápido y sencillo por cromatografía líquida de alta resolución (HPLC) utilizando la extracción por precipitación con sulfato de zinc para determinar los niveles de Casiopeína III-ia (CasIII-ia), se validó en un rango lineal de 10-120 mg/mL en 200 µL de sangre de conejo. Un método HPLC para cuantificar Cas III-ia en plasma ha sido reportado previamente; sin embargo, estudios realizados sobre su distribución encontraron que las concentraciones de Cas III-ia eran más altas en sangre total que en plasma. El análisis se realizó utilizando los siguientes parámetros: fase móvil fue metanol y solución amortiguadora de fosfato sódico (pH 6,5; 0,01 M) (40:60 v/v) a un flujo de 0,8 mL/min, temperatura ambiente a una longitud de onda de 262 nm. Se utilizó acetaminofén como estándar interno. Los resultados obtenidos mostraron que el ensayo es sensible a 10 µg/mL. Se encontró una relación lineal de r^2=0.9954 para CasIII-ia contra

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concentraciones que van de 10 a 120 µg/mL; el método analítico cumple con la linealidad. La desviación relativa estándar (RSD) fue de 5.10 %. La media del recobro fue de 84.8 % intra-dia (n=15) y de 92.5 % inter-día (n=30) para Cas III-ia en muestras de sangre total. Los resultados demuestran la aplicabilidad de este método para la obtención de la distribución *in vitro* y para su uso en estudios farmacocinéticos a nivel preclínico en conejos. El presente estudio muestra un ensayo rápido, sencillo, preciso y exacto para cuantificar CasIII-ia en sangre total de conejo. El estudio farmacocinético, realizado en conejos se obtuvieron los siguientes parámetros farmacocinéticos: (kel) = 0,0150 min-1; tiempo de semivida ($T_{1/2}$) = 53,92 min; volumen aparente de distribución (Vd) = 202,8 mL; aclaramiento (Cl) = 2,0 mL /mi; y área bajo la curva (AUC)=23163,8 µg/mL.min. Los resultados contribuyen a la caracterización preclínica de Cas III-ia.
Palabras clave: Antineoplásico; Casiopeína III-ia; validación HPLC-UV; farmacocinética; compuestos de cobre.

Introduction

Several metal complexes have shown promising antineoplastic activity against cancer cells and tumors, both *in vitro* and *in vivo* [1]. One group of such complexes, which resulted from the search for new anticancer drugs based on endogenous (essential) metals that could have presented lower toxicity [2-4], Casiopeinas® have proven to be cytotoxic to cancer cells that are sensitive or resistant to cisplatin and to xenograft tumors in mice. [3]

Some Casiopeinas® have exhibited greater antineoplastic potency than cisplatin in both *in vitro* and *in vivo* studies on a variety of tumor cell lines [3,4] while also showing superoxide genomic instability due to intrachromosomal recombination [5] and a low potency in inducing genomic instability via intrachromosomal recombination [3]. These features suggest that these drugs are able to can diminish undesirable side effects [3], while stability constants and structural data have also been reported [6] CasIII-ia (Fig. 1) has shown, *in vitro*, both a pharmacological effect and selectivity towards tumor lines (MCF- 7, HCT-15, SK-N-SH neuroblastoma, HeLa, and SiHa) and healthy cells such as T lymphocytes and macrophages [3,24] In addition, pharmacokinetic studies on Cas III-ia have also been conducted on various species, including rats [3] and dogs [20]. It should be noted that Cas III-ia is the first drug with anticancer activity and developed at a Mexican university to reach Phase 1 for clinical studies in Mexico.

Some of the mechanisms reported for Casiopeinas® include DNA fragmentation and oxidation, which generate reactive oxygen species (ROS) and, thereby, cause copper reduction [3]. Reactive oxygen species also depolarize the mitochondrial membrane and cause mDNA damage by decreasing the levels of proteins involved in the respiratory chain, causing cell apoptosis via the caspase pathway. Casiopeinas® have been found to interact with the cytochrome p450 isoform CYP1A1 enzyme and present an affinity for adenine [15]. Additionally, it has been reported that Casiopeinas® interact with tubulins, integrins, and proteins such as fibronectin, there by producing changes in the cytoskeleton and, ultimately, cell death. [3]

The hemotoxicity observed in rats, points to a more complex *in vivo* cytotoxicity in the case of Casiopeinas®, as the administration of a single C_I (5 mg/kg) dose was found not to generate serious damage and was within the functional range [3,7]. Acute toxicological studies conducted for Casiopeinas® in different species at a preclinical level have reported the following results: for an LD_{50} dose of Cas III-Ea in NIH mice 12.47 mg/kg (females) and 6.67 mg/kg (males) via intraperitoneal administration and 7.12 mg/kg (females) and 10.15 mg/kg (males) via intravenous administration; and, for an LD_{50} dose of Cas III-Ea in Wistar rats 4.63 mg/kg (females) and 5.26 mg/kg (males) via intraperitoneal administration and 8.48 mg/kg for both (males and females) via intravenous administration. These findings show clear differences between the species, with a lower LD_{50} dose observed for the Wistar rats, indicating that they are more affected. Respective LD_{99} doses of 200 mg/m^2 for Cas III-ia and 160 mg/m^2 for Cas IIgly have been reported for their administration in dogs. The toxicity data found for the different Casiopeinas® can serve as a scientific basis for selecting, by extrapolating from allometric studies, a dose safe for use in future clinical studies. [3]

Studies on the pharmacokinetics of Cas III-ia in different animal species, such as rats and dogs, have the reported on its viability in terms of the corresponding pharmacokinetic parameters, as related to body weight and the physiological processes of each animal species. [3,14]

Cas III-ia (Fig. 1) is a potentially useful antineoplastic agent [13]. It is very active against L1210 leukemia cells, kills cells by inducing apoptosis [11], induces a weak recombinogenic action, and is able to degrade DNA

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in vitro under a range of cultures. The use of high-performance liquid chromatography (HPLC) methods for the quantification of Cas III-ia® and Cas IIgly® in rat plasma has also been reported [8,9]. However, to determine both their preclinical pharmacokinetic parameters and *in vitro* distribution in rabbit blood, a sensitive and specific assay method is needed to measure the levels of these drugs in the blood. Therefore, the present study developed and validated a simple, inexpensive gradient reversed-phase HPLC. The method was validated according to procedures and acceptance criteria based on national (NOM-177-SSA1-2013) [18] and international (FDA,2001) [19] guidelines, as well as the recommendations made by other guides and authors related to the study of casiopeinas [10-12].

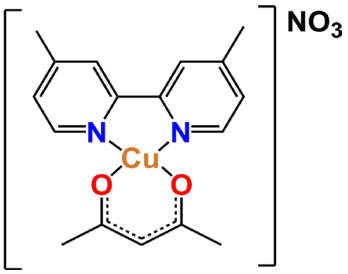

Fig. 1. Chemical structure of Cas III-ia,[Cu(4,4'-dimethyl-1,10-phenanthroline)(acetylacetonate)]NO_3.

Materials and reagents

The Cas III-ia was obtained by the present authors at their laboratory facilities, in accordance with the procedure reports set out in the corresponding patents [2,3]. The whole blood pool of rabbit samples was used for the validation method, while acetaminophen (2.5 µg/ml, USP reference standard) was used as an internal standard. It was added to the corresponding academic solutions (calibration samples stored in methanol and whole blood rabbit control samples), with the relative peak area (drug peak/internal standard peak) also analyzed.

The methanol used in the present study was HPLC grade. The water was produced by the Milli-Q water system (Millipore, Bedford, MA, USA), while the sodium phosphate and other reagents were commercially available and were of analytical grade.

Animals

Male New Zealand rabbits, weighing between 2.0 and 3.0 kg, were used in the present study. The animals were kept under clean conventional conditions and had access to food and water *at libitum*.

Methodology

Chromatographic conditions

The assay was performed using a high performance liquid chromatograph system with a Shimadzu pump (model LC10ADVP; Kyoto, Japan), a Shimadzu variable wave length UV absorbance detector (model SPD10ADVP), a Shimadzu automatic injector (model SIL10ADVP) fitted with a 50 µL sample loop (Cotati, CA, USA), a Shimadzu system controller (model SCL10AVP; Kyoto, Japan), and a chromatography data station, (Shidmadzu, Class VP, Version 5.0, Shimadzu, 1999). Chromatographic separation was carried out under the following conditions: 250 x 4.6 mm I.D Symmetry® C_{18} column and a particle size of 5 µm (Waters Associates, Milford, MA, USA) and a C_{18} 5 µm guard column (Phenomenex®). A system of methanol-sodium phosphate buffer (pH 6.5; 0.01 M) (40:60 v/v) as mobile phase and with an isocratic flow-rate of 0.8 ml/min. The analyses were performed at room temperature. The absorbance at 262 nm was recorded at a sensitivity of the detector expressed in mV (millivolts). The duration of the analytical run was 15 minutes.

Sample preparation

Two-hundred microliters of blood (Samples are collected in tubes containing heparin equivalent to 2 I.U. used as an anticoagulant.) was added to 0.6 µL of methanol and then shaken for 30 s in a vortex, with 50 µL of

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zinc sulfate (10 % *w/w*) and 150 µL of acetaminophen (2.5 µg/mL concentration) added to the mixture, which was vigorously stirred for 30 s and centrifuged for 5 min at 5000 *g*. The supernatant was transferred to vials and a 50 µL then the aliquot was injected into the HPLC system.

Calibration curves in methanol

The stock Cas III-ia solution was prepared by dissolving 10 mg of Cas III-ia in methanol, which was then diluted to 10 mL with the same solvent (1000 µg/mL). Table 1 presents the preparation of the calibration curve in the system in a range of concentrations of 10–120 µg/mL were prepared in mobile phase and analyzed by chromatography.

Table 1. Preparation of calibration curve in system.

Aliquot of stock solution(mL)	Bring to capacity with mobile phase (mL)	Final concentration (µg/mL)
0.1	10	10
0.2	10	20
0.4	10	40
0.6	10	60
0.8	10	80
1.2	10	120

Calibration curves in whole rabbit blood

Four milligrams of Cas III-ia were diluted in 10 ml whole rabbit blood (400 µg/mL, stock solution), the Table 2 shows the preparation of the calibration curve in whole rabbit blood in a concentration range of 10 to 120 µg/mL. Three calibration curves were prepared, and each calibration curve was prepared from a stock solution of 400 µg/mL of CasIII-ia from an independent weighing. The calibration curves were subjected to the pretreatment of the samples indicated in section sample preparation, only the step of collecting the aliquot in a tube previously loaded with heparin was eliminated, since the collection of the aliquot (200 µL) was performed in a microtube without heparin because the pool of whole blood that was used to prepare the calibration curves was collected in falcon tubes of 50 mL previously loaded with heparin (containing heparin equivalent to 20 I.U. used as an anticoagulant) to avoid the formation of clots. The treated samples were injected and analyzed by chromatography.

Table 2. Preparation of calibration curve in whole rabbit blood.

Aliquot of stock solution (mL)	Bring to capacity with whole rabbit blood (mL)	Final concentration (µg/mL)
0.25	10	10
0.5	10	20
1.0	10	40
1.5	10	60
2	10	80
3	10	120

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Limit of quantification and detection based on signal to noise

Limit of quantification (LOQ): the response of blanks (rabbit whole blood) and the response of analytical samples (rabbit whole blood spiked with Cas III-ia) were determined. The amount of the analyte (CasIII-ia) that generated a response with respect to the blank in a ratio of 10 to 1 was verified; this concentration corresponds to the limit of quantification since it was evaluated that it complied with precision and accuracy criteria.

Limit of detection (LOD): determination of the concentration that generated a response with respect to the response of blanks in a ratio of 3 to 1 was evaluated.

Intra-day and inter-day relative standard deviation (RSD)

The analysis was conducted in quintuplicate, at three concentrations high, medium, and low-quality (control points), corresponding to 15, 35, and 75 µg/mL.

Stability studies

For stability studies, the rabbit blood control and methanol solutions with Cas III-ia (1 mL) were added in Eppendorf tubes, at both 4 °C (for 96 h) and room temperature, both with and without light protection (for 96 h). Each determination was performed in duplicate, and the samples were treated in accordance with how they were prepared.

Preclinical pharmacokinetics

Healthy male New Zealand rabbits (n=10), weighing between 2.0 and 3.0 kg, were used in the study. A 10.0 mg/kg Cas III-ia dose was prepared in 30 ml of a saline solution and methanol mixture (10:1) and then administered via marginal ear vein for 60 minutes at slow infusion (0.5 mL/min). The blood samples were collected in microtubes through a cannula inserted into the marginal ear vein, first prior to administration (blanck sample) and then at 80, 90, 100, 110, 120, 140, 160, 180, 210, 240, 270, and 300 minutes post admin istration. At the end of the extraction of each sample (200 µL), the cannula was flushed with an equal volume of heparinized solution. The blood samples were then immediately stored at 4 °C until analysis.

Results and discussion

Representative chromatograms of the whole rabbit blood are shown as a blank chromatogram (Fig. 2), the chromatogram of total blood spiked with acetaminophen as internal standard (**a**), and heparin (**b**) (Fig. 3), and chromatogram for Cas III-ia with a retention time of 10.6 min (Fig. 4). No interfering peaks were detected in the blood during the retention time for Cas III-ia.

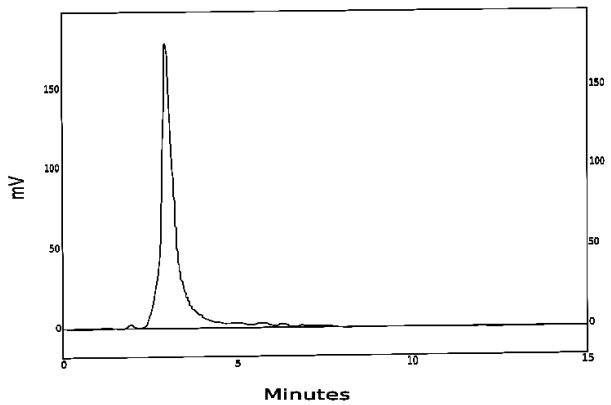

Fig. 2. Blank chromatogram in whole rabbit blood.

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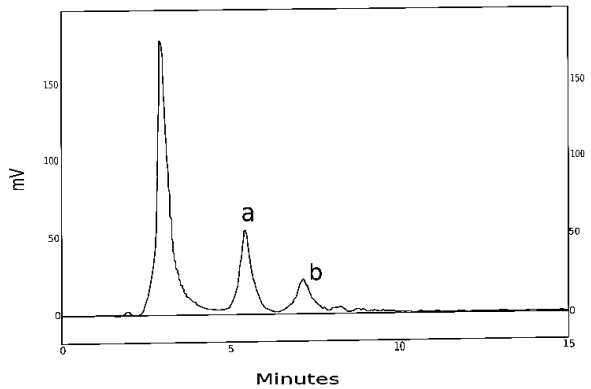

Fig. 3. Chromatogram of total blood spiked with acetaminophen as internal standard **(a)**, and heparin **(b)**.

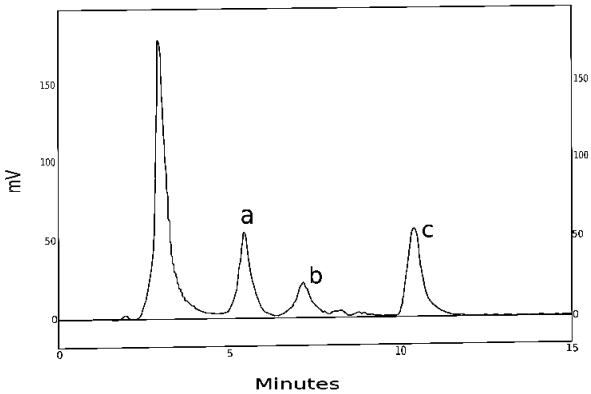

Fig. 4. Chromatogram of total blood spiked with internal standard **(a)**, heparin **(b)**, and Cas III-ia **(c)**, retention time 10.6 min.

The linearity system relationship (r^2=0.9996) was found when the relative peak area for Cas III-ia was plotted against concentrations ranging from 10 to 120 μg/mL (10.0, 20.0, 40.0, 60.0, 80.0, and 120.0 μg/mL), curves corresponding to triplicate assays (Table 3 and Fig. 5).

Table 3. Cas III-ia system linearity, corresponding to triplicate assays.

Theoretical con- centration (μg/mL)	**Relative peak area**	**Relative peak area**	**Relative peak area**	**Mean**	**SD**	**RSD%**
	Curve 1	**Curve 2**	**Curve 3**			
10	0.9	0.9	0.9	0.9	0.00	0
20	2.0	2.0	2.0	2.0	0.00	0
40	3.7	3.7	3.7	3.7	0.00	0.0
60	5.6	5.6	5.5	5.6	0.06	1.04
80	7.3	7.3	7.3	7.3	0.00	0
120	10.8	10.8	10.8	10.8	0.00	0

RSD: relative standard deviation, SD: standard deviation

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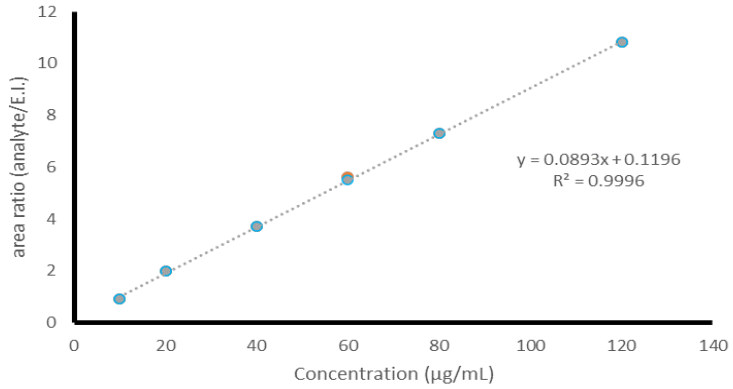

Fig. 5. Average-curve for CasIII-ia in the system, corresponding to triplicate assays.

A relationship (r^2=0.9954) was found when the relative peak area for Cas III-ia was plotted against concentrations ranging from 10 to 120 µg/mL (10.0, 20.0, 40.0, 60.0, 80.0, and 120.0 µg/mL) in 600 µL of rabbit blood, with the curves corresponding to triplicate assays (Table 4 and Fig. 6).

Table 4. Cas III-ia linearity in whole rabbit blood, corresponding to triplicate assays.

Theoretical con- centration (µg/mL)	Relative peak area	Relative peak area	Relative peak area	Mean	SD	RSD%
	Curve 1	Curve 2	Curve 3			
10	0.9	0.8	0.9	0.9	0.04	5.0
20	1.8	1.8	1.8	1.8	0.01	0.5
40	3.6	3.5	3.5	3.5	0.00	0.2
60	5.2	5.3	5.4	5.3	0.03	0.7
80	6.9	6.9	7.0	6.9	0.07	1.1
120	9.6	9.7	9.6	9.6	0.04	0.4

RSD: relative standard deviation, SD: standard deviation

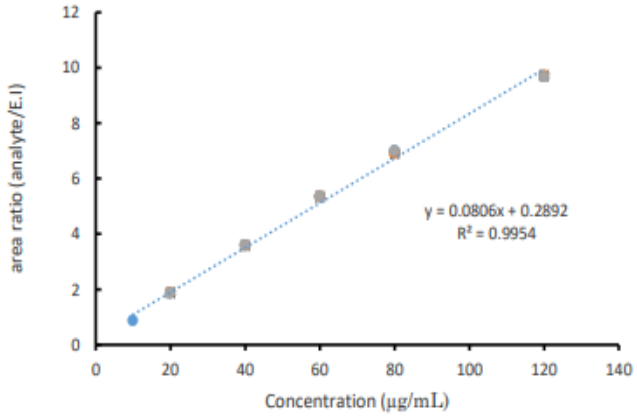

Fig. 6. Average-curve for CasIII-ia in whole rabbit blood, corresponding to triplicate assays.

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Both the intra-day and inter-day precision of the method applied, obtained via the analysis conducted on the samples, are shown in Table 3. The precision was estimated based on the control curve samples prepared on both the same day (n=15) and subsequent days (n=30) using different stock solutions. The corresponding relative standard deviation (RSD) was below 15 %, meeting the acceptance criteria.

The level of Cas III-ia recovery was determined by first identifying the relative peak observed for the whole blood spiked with the different amounts of the compound (15, 35, and 75 µg/mL, control points) using the extraction procedure described and then comparing this peak with the relative peak observed for the same series when prepared in the mobile phase and injected into the HPLC. It is necessary to specify that to prepare the control points, a clean pool of whole rabbit blood is used, which is obtained from rabbits that were not administered any compound. The level of Cas III-ia recovered from each sample was determined in quintuplicate (Table 5), with an average of 84.8 % intra-day (n=15) and 92.5 % inter-day (n=30) recovery % from the blood. Previous reports describe Casiopeinas® in plasma protein having been observed, in binding assays, to present a significantly higher accumulation in whole blood than in plasma, which is due to the significant binding to blood cells reported in red blood cell/plasma ratios (Ke/p) of over 2 for human blood and Beagle dogs at concentrations of 1 µg/mL [13,14]. Therefore, performing the extraction process in whole blood was more efficient for comparing the pharmacokinetic parameters with those of rabbit plasma.

Table 5. Accuracy and precision of the use of the HPLC method in whole rabbit blood.

Theoretical concentration (µg/mL)	**Average experimental concentration (µg/mL) (n=5)**	**Recovery (%)**	**RSD(%)**
Intra-day (n=15)			
15	12.3	82.0	5.1
35	31.6	90.3	1.1
75	61.7	82.3	0.8
		Average recovery	
		84.8	
Inter-day (n=30)			
15	12.4	83.0	2.2
35	33.9	97.0	0.9
75	73.2	97.7	1.5
		Average recovery	
		92.5	

RSD (relative standard deviation); LQC (low-quality control, 15 µg/mL); MQC (mid-quality control, 35 µg/mL); and HQC (high-quality control, 75 µg/mL).

Defined as the sample concentration obtained from spiked blood and which presents a peak area of ten times the noise level, the LOQ was observed at 5.08 µg/mL.

Defined as the sample concentration presenting a peak of three times the noise level, the LOD was observed at 3.5 µg/mL.

The determination of the stability of Cas III-ia both before and after the sample pre- treatment revealed that, after 96 h at 4 °C, 99.85 % of the Cas III-ia was still present in the rabbit blood. At room temperature and

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without light protection, 74.96 % of Cas III-ia was observed after 96 h, whereas, at room temperature and with light protection, 97.5 % of the blood was enriched with Cas III-ia after 96 h.

Pharmacokinetic results

No chromatographic interferences by any endogenous compounds were found. Fig. 7 shows the average pharmacokinetic profile of Cas III-ia terminal phase data, after administration by intravenous infusion in New Zealand rabbits (n=10), It was also found that the steady-state blood levels for Cas III-ia were 117.42± 3.26 µg/mL in a non-compartment model, was obtained by WINNOLIN software, while the half-life time was 53.92± 25.41 min.

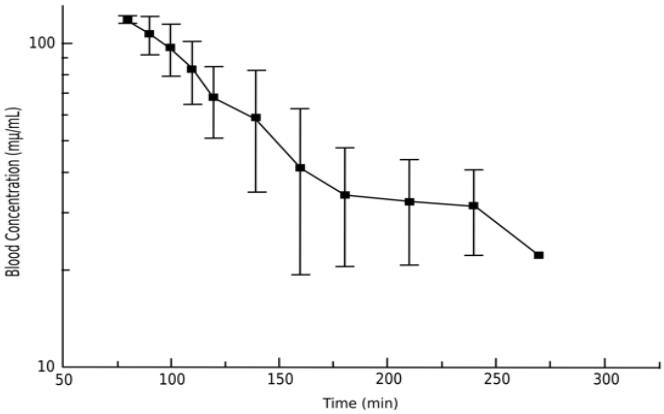

Fig. 7. Mean pharmacokinetic profile of Cas III-ia in whole blood (n= 10 New Zealand rabbit) after intravenous infusion.

The following pharmacokinetic parameters were obtained from the rabbit blood samples: (kel) = 0.0150 min-^{1}; half-life time ($T_{1/2}$) = 53.92 min; the apparent volume of distribution (Vd) = 202.81 mL; clearance (Cl) = 2.08 mL /min; and area under the curve (AUC)=23163.85 µg/mL.min. The results obtained reveal that the half-life time in rabbit blood (0.89 h) is shorter than that found in for rat blood (12.46 h) [3]. An elimination rate of 2.08 mL/min was observed for clearance in the rabbit blood samples, which was higher than that observed for rat blood (0.45 mL/min). According to the basic concepts of pharmacokinetics, the higher the elimination rate of a compound, the higher its clearance, and the smaller its distribution volume, the shorter its half-life. The volume of distribution data obtained for the different rabbits (202.81 mL), rats (0.462 L), and dogs (TMR= approximately 2 weeks) (data obtained from compartmental modeling program WINNOLIN) [3] species show that Cas III-ia presents a wide distribution in tissue. This suggests that, as reported for other Casiopeinas®, it has a wide distribution in tissues, such as blood, due to its high affinity with blood cells such as erythrocytes. [13] However, although different doses were administered intravenously in each species, the results obtained show that Cas III-ia presents low bioavailability across the three species of interest: 10 mg/kg administered to a rabbit sample=23163.85 µg/mL*.min; 10 mg/kg administered to a rat sample=22.27 µg/mL*min 3.5 mg/kg administered to a dog sample =40472.75 µg/mL.min [3]. Furthermore, a smaller distribution volume generates a higher concentration of the compound in the central circulatory system, and this is observed in the bioavailability data, since the rabbit presented a slightly higher bioavailability compared to that reported for the rat. (both species of rabbit and rat were administered the same dose of 10 mg/kg). These data indicate interspecies variability, due to both the body weight and physiological processes specific to each species. Perfusion is the flow of blood through blood vessels to multiple organs and tissues, therefore in rats, a greater perfusion could be related to their heart rate and blood pressure. Therefore, the greater the blood perfusion of multiple organs and tissues, the greater the volume of distribution of the compound in the organs of the rat, the lower the perfusion, the lower the volume of distribution of the compound in the rabbit [20]. In addition to perfusion, there is the pharmacokinetic parameter of binding to plasma proteins, with which it is known that only free drug can cross the membranes of tissues and organs. Depending on this binding will be the distribution in the different

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species. Pharmacokinetic scaling between species is necessary to optimize test doses in humans via allometric equations, in which biochemical, anatomical, and physiological similarities between animals can be generalized and expressed in mathematical models [17].

Conclusions

The development of the method proved to be useful and reliable for determining Cas III-ia levels in whole rabbit blood. The pre-treatment procedure applied on to the sample, which involved direct precipitation with zinc sulphate, was found to be fast and simple. The method, validated for concentrations in the range of 10 to 120 µg/mL, presented a simple, repeatability, accuracy, and low limits for both: quantification and detection. The recovery % of Cas III-ia was adequate and reproducible and constant over the range of the calibration curve. This method is sufficiently sensitive for performing pharmacokinetic evaluations and can, therefore, be applied at in the future in preclinical pharmacokinetic studies. The pharmacokinetic data obtained by the present study, especially when compared with the data obtained for the different species reported [3], contributes to the characterization of Cas III-ia. At a preclinical level, this data enables the bases to be established for extrapolating an adequate dose, via allometric studies, for future clinical studies. The data also contributes to the adequate design of dosing intervals' safety for humans.

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