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Development and validation of a liquid chromatographic method for Casiopeina IIIi® in rat plasma

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Abstract

A sensitive and specific liquid chromatographic method using extraction with zinc sulfate has been developed for the determination of Casiopeina IIIi and validated over the linear range 5–100 μg/ml in 1 ml of rat plasma. The analysis was performed on a Symmetry C_{18} (5 μm) column. The mobile phase was methanol: 0.01 *M* phosphate buffer pH 6.5 (40:60, v/v). The column effluent was monitored at 262 nm. The results showed that the assay is sensitive at 5 μg/ml. Maximum intra-day coefficient of variation was 10.6%. The recovery obtained in plasma was 87.2%. The method was used to perform protein binding studies by equilibrium dialysis in rat plasma and was found to be satisfactory.

Keywords: Antineoplastic; Casiopeina IIIi

1. Introduction

A series of mixed chelated copper(II) complexes of general formulae Cu(N–N)(O–N)NO_3 or Cu(N–N)(O–O)NO_3 (when N–N=4,7-dph,1,10-phen and O–N=aminoacidate (I), N–N=1,10-phen, or 4,7-dm,1,10-phen and O–N=aminoacidate (II), N–N=2,2-bipy or 1,10-phen and O–O=acac or salal (III), N–N=4,4-dm 2,2-bipy or 2,2-bipy and O–N=aminoacidate (IV), N–N=5R,1,10-phen (R=Me, Cl, OH, NO_2) and O–N=aminoacidate (V), N–N=5,6-dm,1,10-phen and O–N=aminoacidate (VI), N–N=3,4,7,8-dph,1,10-phen or 2,9-dm, 4,7-dph,1,10-phen and O–N=aminoacidate (VII), have been registered under the name of CASIOPEINAS® [1–3].

These compounds have shown cytotoxic and antineoplastic activity in vitro and in vivo [4–7]; stability constants and structural data have been reported [8–11]. Also, a study of the activity dismutasa superoxide (SOD) of Casiopeinas has been reported [12]. Studies of mechanisms of action are in progress.

Casiopeina IIIi (when N–N=4,4-dm,2,2-bipy, O–

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O=acac) is a potentially useful antineoplastic agent [13]. It is very active against L1210 leukemia cells and also kills cells by apoptosis [14], induces a weak recombinogenic action and has the ability to degrade DNA in vitro under a range of several cultures [15]. This work was realized as part of a global project for the design and development of antitumor molecules named Casiopeina at the National University of Mexico.

In order to determine the protein binding and the preclinical pharmacokinetics parameters in rats, a sensitive and specific method of assay was needed in order to measure the drug in plasma. This paper describes a rapid, specific, reliable and sensitive analytical method based on reversed-phase liquid chromatography for the quantitation of Casiopeina IIIi. The method was validated according to procedures, and acceptance criteria based on USP XXIII guidelines [16] and recommendations of Shah et al. [17], Bresolle et al. [18] and Braggio et al. [19].

2. Experimental

2.1. Materials and reagents

Casiopeina IIIi (Fig. 1) was obtained in our laboratory following the procedure reports in Patents [1,2]. Pooled rat plasma samples were used for the validation method.

Methanol was HPLC grade and other reagents were analytical grade. Naproxen was used as internal standard, it was added to academic solutions in a concentration of 10 μg/ml (calibration samples in mobile phase and control rat plasma samples). The relative peak area (drug peak/internal standard peak) was analyzed.

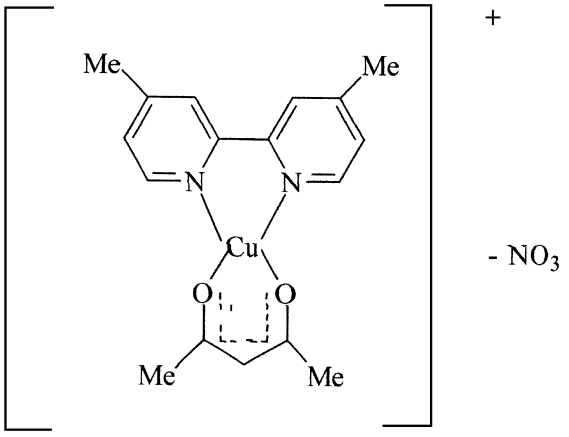

Fig. 1. Chemical structure of Casiopeina IIIi.

2.2. Animals

Male Wistar rats (250–300 g) were kept under clean conventional conditions and had access to food and water ad libitum.

2.3. Chromatographic conditions

A high performance liquid chromatograph apparatus was used with a Shimadzu pump Model LC10AS (Kyoto, Japan), a Shimadzu variable-wavelength UV absorbance detector (Model SPD10AV), a Shimadzu automatic injector (Model SIL 10A) fitted with a 50-μl sample loop (Cotati, CA, USA), a Shimadzu system controller Model SCL 10A (Kyoto, Japan) and an integrator chromatography data station (Shimadzu Class VP Version 5.0, Shimadzu, 1999), a stainless steel analytical column of 250×4.6 mm I.D. (Waters Associates, Millford, MA, USA) packed with Symmetry C_{18} (particle size, 5 μm). The mobile phase of 0.01 *M* sodium phosphate buffer (pH 6.5)/methanol (60:40) was kept at a flow-rate of 1 ml/min. The analyses were carried out at 20–24 °C. The absorbance at 262 nm was recorded at a sensitivity of 1.0 AUFS (absorbance units full scale) in the programmed parameters. A C_{18}, 5 μm precolumn (Security Guard, Phenomenex guard cartridge system) was used.

2.4. Sample preparation

To 1 ml of plasma in a 5-ml centrifuge tube was added 0.1 ml of zinc sulfate (10% w/v) [20]. The mixture was shaken for 15 s in a vortex, then 1 ml of mobile phase was added to Naproxen (concentration of 10 mg/l, in the stock solution) followed by vigorous stirring for 15 s, and centrifugation for 10 min at 5000 *g*.

The supernatant was filtered through a green 25-mm syringe filter 0.2 μm (Acrodisc, Gelman Sci-

ences). An aliquot of 250 μl was put in the vials and 50 μl injected into the HPLC system.

2.5. Calibration curves in mobile phase

Casiopeina IIIi (10.0 mg) was diluted in water in a 10-ml volumetric flask, and the required concentrations (5–100 μg/ml) were prepared in mobile phase at a dilution of 5.0 ml.

2.6. Calibration curves in rat plasma

Casiopeina IIIi (10.0 mg) was diluted in a little water (less than 7% of the total volume), and then diluted to 10 ml in rat plasma in a volumetric flask (1.0 mg/ml); the required concentrations (5–75 μg/ml) were prepared in rat plasma at a dilution of 5.0 ml.

2.7. Calibration curves in phosphate solution (64 mM, pH 7.4)

Casiopeina IIIi (10.0 mg) was diluted in 10 ml of phosphate solution. The required concentrations (5–50 μg/ml) were prepared in phosphate solution at a dilution of 5.0 ml.

2.8. Protein binding studies

In a preliminary study, equilibrium dialysis was used to determine protein binding in rat plasma. To avoid problems with equilibrium dialysis equipment with five cells, it was validated before analysis with no adsorption of Casiopeina IIIi at the membrane; it was freely permeable. Shift volume was also analyzed (the water which passed over the buffer to plasma, but the displaced volume is less than 10%) and blank errors (the membranes do not have any soluble impurity which could give some absorption at the analyzed wavelength) [21,22]. Teflon cells and Spectra/Por® 2 dialysis membranes were used with molecular mass cut-offs of 12–14 000 Da (Spectrum, USA). Membranes were washed in distilled water for approximately 30 min, another 30 min in methanol, and they were put in phosphate buffer solutions of pH 7.4, one day before use. The equilibrium time was 5 h; to find this time, 1 ml of phosphate buffer solution (64 m*M*, pH 7.4) was placed in the narrowest cell of dialysis equipment and in the wider cell was placed 1 ml of rat plasma with 50 μg/ml of Casiopeina IIIi. The concentration in both cells was analyzed several times (0, 30, 60, 90, 180, 250, 320, 400 and 480 min) using the methodology mentioned in Section 2.4 and HPLC (Fig. 2).

Animal plasma samples were prepared from blood taken from the Wistar rat species, plasma was collected from ≥5 animals and pooled prior to storage at −20 °C for <1 month. Plasma (1.0 ml) adjusted to pH 7.4 with carbogen gas (5% CO_2, 95% O_2) and spiked with different concentrations of Casiopeina IIIi was dialyzed against an equivalent volume of phosphate buffer (64 m*M*, pH 7.4) made isotonic with NaCl at 37 °C for 5 h, until equilibrium was reached according to a prestudy.

All experiments were carried out in quintuplicate. Three concentrations of Casiopeina IIIi in rat plasma (10, 25 and 50 μg/ml) were analyzed and prepared as mentioned in Section 2.6.

After 5 h, the solutions of phosphates and plasma were analyzed by HPLC. The plasma samples of rats were analyzed for total drug concentration using the method described in Section 2.4, phosphate solution samples were filtered by Acrodisc filters and then injected into the HPLC.

Calibration curves in rat plasma, prepared at 5, 10, 25, 50, 75 μg/ml of Casiopeina IIIi and calibration curves in phosphate solution prepared as mentioned before, at a concentration of 5, 10, 20, 40 and 50 μg/ml of Casiopeina, with the dialyzed samples in plasma and phosphate solution were run at HPLC.

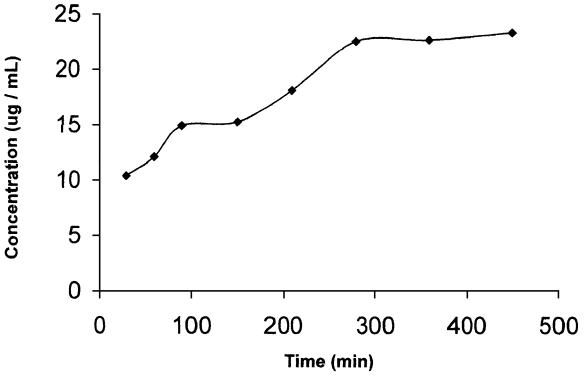

Fig. 2. Equilibrium time for Casiopeina IIIi with the dialysis methodology.

The unbound and bound drug concentration were determined. The unbound fraction (fu) of drug was calculated as the ratio of concentrations between phosphate buffer solution and the total concentration in plasma. It was assumed that albumin is the only protein binding involved

$$\text{fu}\,(\%) = C_{\text{buffer}}/C_{\text{total}} \times 100$$

where $C_{\text{total}} = C_{\text{bound}} + C_{\text{unbound}}$.

2.9. Stability studies

For stability studies, control rat plasma and buffer phosphate (64 m*M*, pH 7.4) solutions were spiked with Casiopeina IIIi at 6 and 37 °C; each determination was carried out in triplicate.

2.10. Pharmacokinetics application (rat)

Wistar rats ($n = 18$) were administered with a single i.v. dose of 4.5 mg Casiopeina IIIi/kg body wt. in a solution of 1% NaCl. Blood samples were collected at 0, 10, 15, 20, 30, 45, 50, 90 and 120 min postdose. Two rats were bled once per time point.

Plasma was prepared by centrifugation of blood and plasma samples of Casiopeina IIIi were analyzed in 1 ml of rat plasma, described in Section 2.4. Calibration curves (5–100 μg Casiopeina IIIi/ml) were made with the same described extraction methodology and were run with the samples into HPLC.

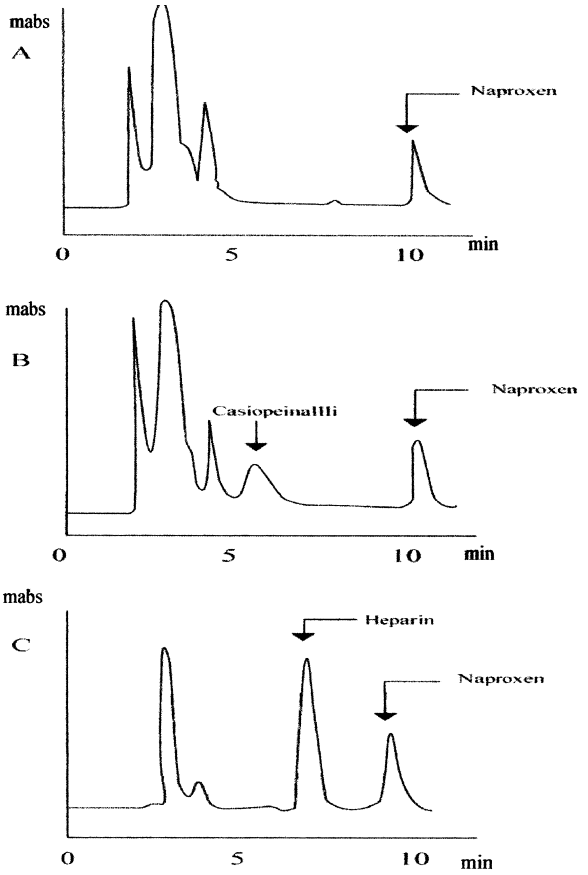

Fig. 3. (A) Chromatogram of blank plasma with internal standard, Naproxen, retention time 0.9 min. (B) Plasma spiked with Casiopeina IIIi (25 μg/ml), retention time 5.0 min. (C) Plasma spiked with heparin (retention time 8.7 min) and Naproxen (internal standard).

3. Results and discussion

Chromatograms of plasma are shown in Fig. 3. The retention time for Casiopeina IIIi was 5.0 min. No interfering peaks from plasma were detected at the retention time of Casiopeina IIIi. The probable decomposition compounds are 4,4′-dimethyl 2,2′-bipyridin-Cu and acetylacetonate-Cu. This statement was corroborated by an experiment in which the dissociated compounds proposed were analyzed and the retention time found for 4,4′-dimethyl 2,2′-bipyridin-Cu was 2.3 min and the retention time for acetylacetonate-Cu was 6.0 min. These retention times were compared with those obtained in the experiment with the original drug.

A linear relationship ($r = 0.9988$) was found when the relative peak area of Casiopeina IIIi was plotted against various concentrations from 5.0 to 100 μg/ml.

The intra-assay precision of the validation was estimated from calibration curves in rat plasma (5, 10, 25, 50, and 100 μg/ml of Casiopeina IIIi) prepared separately the same day in triplicate.

The corresponding mean determination coefficient (r^2) of the linear regression analysis was 0.9978, and the C.V. was from 1.7 to 9.1%. Inter-day precision was estimated from the calibration curves in rat plasma worked in triplicate on three different days.

Intra-day and inter-day precision of the method,

assessed by analyzing samples with plasma spiked with known amounts of Casiopeina IIIi, are given in Table 1. It can be seen that the maximum within-day coefficient of variation was 10.6% at 5 μg/ml.

The recoveries of Casiopeina IIIi were determined by comparing the relative peak area from plasma spiked with known amounts of the compound (5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml) using the extraction procedure described versus the relative peak area from the same series prepared in mobile phase and injected into HPLC. Each sample was determined in triplicate. The mean recovery of Casiopeina IIIi in plasma was on average 87.2%. The heparin used as anticoagulant in plasma samples showed a retention time of 8.7 min (Fig. 3C).

The LOQ of 5 μg/ml was defined as the sample concentration from spiked rat plasma resulting in the minimum concentration in the validated curve.

The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times the noise level. A value of 150 ng/ml was determined.

The stability of Casiopeina IIIi before and after sample pre-treatment was determined. After 48 h at 6 °C, 96% of Casiopeina IIIi was still present in rat plasma. In buffer phosphate solutions, there was 97% Casiopeina IIIi at 37 °C after 24 h and plasma spiked with Casiopeina IIIi at 37 °C was determined to be 97.5% for 24 h.

The precolumn filter was changed after 60 samples and the column was used for 45 samples with Casiopeina IIIi plasma extraction; after this, it needs to be washed with warm water (approximately 100 ml) and methanol/water (1:1) (approximately 100 ml). The life of the column was about 750 injections.

In protein binding studies in rat plasma at concentrations of 50, 25 and 10 μg/ml, a lower percentage (12.9%) was observed at a high concentration (50 μg/ml) and a high percentage (83.9 and 77.2%) at 25 and 10 μg/ml (Fig. 4).

Results showed that protein binding in rat plasma was higher than 80% in concentrations of 25 and 10 μg/ml. More analyses must be carried out by this methodology in order to understand the behavior of Casiopeina IIIi in the presence of several plasma proteins.

Table 1
Intra-day and inter-day precision of the HPLC methodology in rat plasma samples

Theoretical concentration (μg/ml)	Experimental concentration (μg/ml)	C.V. (%)	Recovery (%)
Intra-day ($n=3$)			
5	4.20	9.2	84.0
10	8.52	1.7	85.2
25	22.13	1.4	88.5
50	44.50	3.1	89.0
100	85.10	3.9	85.1
			Average recovery 86.36
Inter-day ($n=6$)			
5	4.23	10.6	84.6
10	8.76	2.9	87.6
25	22.3	2.6	89.2
50	43.56	2.8	87.1
100	86.43	3.5	86.4
			Average recovery 87.0
Inter-day ($n=9$)			
5	4.35	9.3	87.0
10	8.92	4.3	89.2
25	23.12	3.4	92.5
50	43.87	4.7	87.7
100	85.67	2.8	85.7
			Average recovery 88.4
			Total average recovery 87.2

n is the number of samples used in standard deviation.

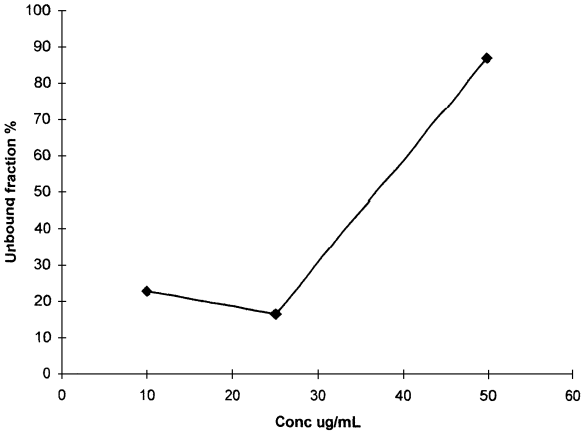

Fig. 4. Preliminary protein binding of Casiopeina IIIi, in rat plasma by equilibrium dialysis.

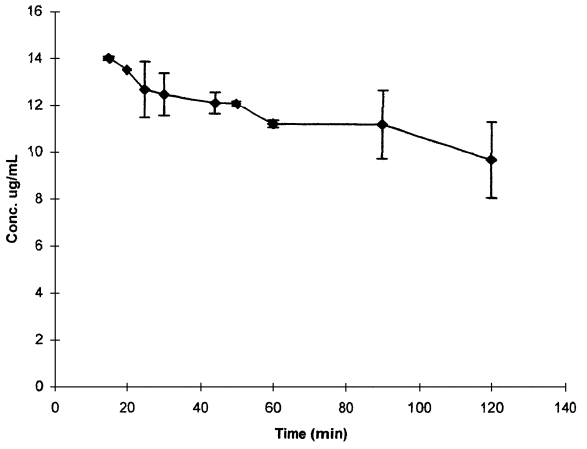

Fig. 5. Pharmacokinetics application of Casiopeina IIIi (Wistar rat), with SD and two determinations per point.

3.1. Pharmacokinetic results

No chromatographic interferences from any endogenous compounds were found.

Fig. 5 shows the plasma concentration–time profile of Casiopeina IIIi after the administration of 4.5 mg/kg. Apparently, the concentration C_o, at zero time, was 13.4 μg/ml, in a two-compartment model obtained by WINNONLIN software, and the beta half-life obtained was 276.1 min. In a preliminary study, the half-life obtained suggests that Casiopeina IIIi is distributed in several organs.

4. Conclusions

The method developed proved to be useful and reliable for the determination of Casiopeina IIIi in plasma. The pre-treatment procedure for the sample, involving direct precipitation with zinc sulfate, is fast and simple.

The method, validated for concentrations ranging from 5 to 100 μg/ml, had good repeatability and accuracy and low limits of quantification and detection. The recovery of Casiopeina IIIi was good enough, it is reproducible and constant over the entire ranging of the calibration line. This method is sufficiently sensitive to perform protein binding by equilibrium dialysis and can be applied in future preclinical pharmacokinetic studies.

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