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

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Abstract

A sensitive and specific liquid chromatographic method using solid-phase extraction with Sep-pak cartridges has been developed for the determination of Casiopeina IIgly and validated over the linear range 2.5–50 μg/ml in rat plasma. The analysis was performed on a Symetry C_{18} (5 μm) column with a Phenomenex C_{18} precolumn. The mobile phase was methanol–water (58:42, v/v). The column effluent was monitored at 273 nm. The results showed that the assay is sensitive at 2.5 μg/ml. Maximum intra-day coefficient of variation was 11.47%. The recovery based upon addition of internal standard to rat plasma was 80.98%. The method was used to perform preclinical pharmacokinetic studies in rat plasma and was found to be satisfactory.

Keywords: Validation; Casiopeina IIgly

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=5*R*,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-tm,1,10-phen or 2,9-dm,4,7-dph,1,10-phen and O–N=aminoacidate [VIII], are under the name of Casiopeinas® and patented [1–3]. These compounds show cytotoxic and antineoplastic activity in vitro and in vivo; stability constant and structural data are reported [4–8]. Also, a study of the superoxide dismutase activity (SOD) of Casiopeinas is reported [9]. Studies of the mechanism of action are in progress. A high-performance liquid chromatography (HPLC) method is reported for Casiopeina IIIi [10].

Casiopeina IIgly (when N–N=4,7-dm-1,10-phenantroline, N–O=glycine; Fig. 1) is a potentially useful antineoplastic agent and its structure is previously reported [4,14]. It is very active against L1210 leukemia cells, also kills cells by apoptosis [11], induces a weak recombigenic action and has the

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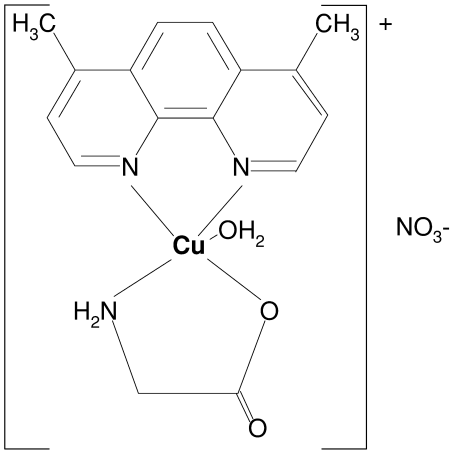

Fig. 1. Chemical structure of Casiopeina IIgly. Aqua[(4,7-dimethyl-1,10-phenanthroline, glycine copper II)nitrate].

ability to degrade DNA in vitro under a range of conditions. This work is part of a global project for the design and development of antitumor molecules named Casiopeinas at the National University of Mexico.

In order to determine the preclinic pharmacokinetic parameters in rats, a sensitive and specific method of assay is 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 IIgly. The method was validated according to procedures and acceptance criteria based on USP XXIV guidelines [12–16] and recommendations of the CDER-FDA [17,18].

2. Experimental

2.1. Materials and reagents

Casiopeina IIgly was obtained in our laboratory following the procedure reported in previous patents [1,2]. Pooled Wistar rat plasma samples were used for validation of the method. Methanol was HPLC grade and other reagents were analytical grade. Prednisone (100 μg/ml, USP reference standard) was used as the internal standard. It was added to academic solutions (calibration samples in mobile phase) and to control rat plasma samples. The relative peak area (drug peak/internal standard peak) was analyzed.

2.2. Animals

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

2.3. Chromatographic conditions

The HPLC equipment used was a Shimadzu pump model LC10ADVP (Kyoto, Japan), a Shimadzu variable-wavelength UV absorbance detector model SPD10ADVP, an automatic injector Shimadzu model SIL10ADVP fitted with a a 50 μl sample loop (Cotati, CA, USA), a Shimadzu system controller model SCL10AVP (Kyoto, Japan) and an integrator chromatography data station, (Shimadzu Class VP Version 5.0, Shimadzu, 1999), stationary phase was a stainless steel analytical column of 250×4.6 mm I.D. (Waters Associates, Milford, MA, USA) packed with Symetry C_{18} (particle size 5 μm), preceded by a C_{18}, 5 μm precolumn (Security Guard, Phenomenex guard cartridge system).The mobile phase was water–methanol (42:58) at a flow-rate of 0.7 ml/min. The analyses were performed at room temperature. The absorbance at 273 nm was recorded.

2.4. Preclinical pharmacokinetics assay in rats

Wistar rats, male, body weight 300 g, (n=10) were administered a single dose of 8.0 mg Casiopeina IIgly/kg body weight in a solution of 5% glucose. Blood samples were collected at 0, 15, 30, 60 and 120 min postdose. Two rats were bled per each time point. Plasma was prepared by centrifugation of heparin-treated blood. Casiopeina IIgly concentration was analyzed in 1 ml of rat plasma and treated as sample preparation. Calibration curves were run with the samples, 2.5–50 μg Casiopeina IIgly/ml.

2.5. Sample preparation

To 1 ml of rat plasma was added 0.5 ml of methanol and the mixture was shaken for 20 s and

centrifuged for 5 min at 5000 *g*. The supernatant was transferred to a clean tube and 0.5 ml of 20 m*M* 1-hexanesulfonic acid (sodium salt, pH 4.0) was added. The mixture was shaken for 15 s. The cartridge Sep-pak C_{18}, 500 mg, 3 ml (Waters Associates) was first conditioned with 4 ml of methanol and then with 1 ml of 20 m*M* 1-hexanesulfonic acid (sodium salt, pH 4.0) with a vacuum manifold system (Visiprep Supelco, cat. 5-7030, Bellefonte, PA, USA). The shaken mixture was added to the prepared cartridge, and was washed with 1 ml of 20 m*M* 1-hexanesulfonic acid (sodium salt, pH 4.0) and 1 ml of water. The cartridge was eluted with 1.5 ml of methanol. The solvent was evaporated under N_2. The residue was reconstituted in 0.5 ml of 100 μg/ml internal standard (prednisone in mobile phase), followed by vigorous stirring to dissolve. An aliquot of 250 μl was put in vials and 50 μl injected into the HPLC system.

2.6. Calibration curves in mobile phase (academic solutions)

Casiopeína IIgly (10.0 mg) was diluted in the mobile phase in a 10 ml volumetric flask, and the required concentrations (2.5–50 μg/ml) were prepared in mobile phase at a dilution of 5.0 ml.

2.7. Calibration curves in rat plasma

Casiopeina IIgly (10.0 mg) was diluted in a small amount of mobile phase (less than 5% of the total volume), and then diluted to 10 ml in rat plasma in a volumetric flask (1.0 mg/ml); the required concentrations (2.5–50 μg/ml) were prepared in rat plasma at a dilution of 5.0 ml.

2.8. Stability studies

For stability studies, control rat plasma and mobile phase solutions were spiked with Casiopeina IIgly (1 ml) in eppendorf tubes, at 6 °C (during 24 h) and −20 °C (during 30 days); each determination was performed in duplicate. After 30 days each tube was treated in accordance to the sample preparation.

3. Results and discussion

Chromatograms of plasma are shown in Fig. 2. Retention time for Casiopeina IIgly was 4.6 min. No interfering peaks from plasma were detected at the retention time of Casiopeina IIgly. The probable compounds of decomposition are 4,7-dimethyl-1,10-phenantroline–Cu and glycine–Cu. This was corroborated by an experiment in which the proposed dissociated compounds were analyzed and the retention time found for 4,7-dimethyl-1,10-phenanthroline–Cu was 3.73 min and the retention time for glycine–Cu was 3.28 min. These times were compared with those obtained in the experiment with the original drug.

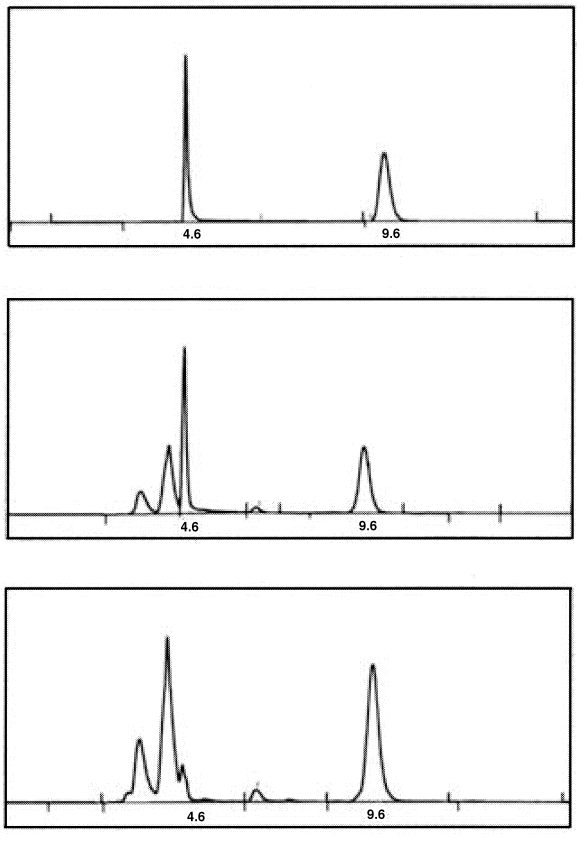

Fig. 2. (A) Casiopeina IIgly (50 μg/ml) in mobile phase (retention time 4.6 min) with internal standard (prednisone 100 μg/ml, retention time 9.6 min). (B) Rat plasma spiked with Casiopeina IIgly (50 μg/ml) retention time 4.6 min. (C) Rat plasma with heparin (retention time 6.6 min) and prednisone (internal standard, retention time 9.6 min).

Table 1
Standard curve of Casiopeina IIgly over the range 2.5 to 50 μg/ml in 1 ml of rat plasma, triplicate assays

Theoretical concentration (μg/ml)	Relative peak area, curve 1	Relative peak area, curve 2	Relative peak area, curve 3	Mean	Standard deviation	C.V. %
2.5	0.111	0.106	0.111	0.109	0.002	2.16
5	0.132	0.130	0.149	0.137	0.009	6.22
10	0.219	0.193	0.230	0.214	0.016	7.25
25	0.437	0.367	0.316	0.373	0.050	13.29
50	0.857	0.883	0.780	0.840	0.044	5.21

A linear relationship (r=0.9933, r^2=0.9867) was found when the relative peak area of Casiopeina IIgly was plotted against various concentrations from 2.5 to 50 μg/ml, (2.5, 5.0, 10.0, 25.0 and 50.0 μg/ml, in 1 ml of rat plasma, three curves in 1 day) as shown in Table 1. The standard curve in rat plasma and academic solutions were run in mobile phase.

The inter-assay precision of the validation was estimated from quality control samples prepared on the same day in five determinations for each concentration using the different stock solutions (n=15). The corresponding coefficient of variation (C.V.) of the quality control samples (low=4 μg/ml, medium=20 μg/ml and upper=40 μg/ml) was from 2.45% to 11.02%.

Intra-day and inter-day precision and accuracy of the method, assessed by analyzing quality control samples, are shown in Table 2. It can be seen that the maximum within-day coefficient of variation was 13.06% at 20 μg/ml.

The recoveries of Casiopeina IIgly was determined by comparing the relative peak area from plasma spiked with known amounts of the compound (2.5, 5.0, 10.0, 25.0 and 50.0 μg/ml) using the described extraction procedure vs. the relative peak area from the same series prepared in mobile phase and injected. Each sample was determined in triplicate. The mean recovery of Casiopeina IIgly in plasma averaged 80.98% (n=15). The heparin used in the plasma samples showed a retention time of 6.6 min. (Fig. 2C).

The LOQ of 2.5 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 3.3

Table 2
Accuracy and precision of the HPLC method in quality control samples in rat plasma

Theoretical concentration (μg/ml)	Experimental concentration (μg/ml)	C.V. (%)	Recovery (%)
Intra-day (n=5)			
4	4.07	1.17	101.75
20	21.90	3.62	109.50
40	40.78	11.47	101.95
			Average recovery 104.33
Inter-day (n=10)			
4	3.90	2.45	97.00
20	17.97	11.02	89.85
40	33.80	8.82	84.50
			Average recovery 90.45
Inter-day (n=15)			
4	3.71	4.63	92.75
20	17.29	13.06	86.45
40	36.03	9.67	90.07
			Average recovery 89.75
			Total average recovery 94.87

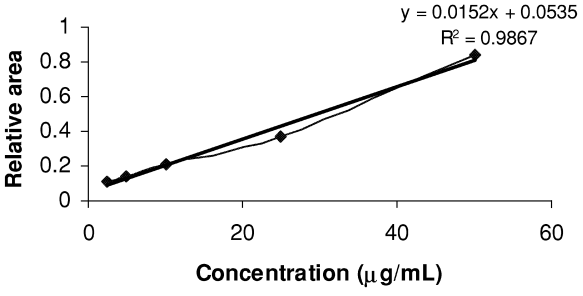

Fig. 3. Mean standard curve Casiopeina IIgly over the range 2.5 to 50 μg/ml in 1 ml of rat plasma.

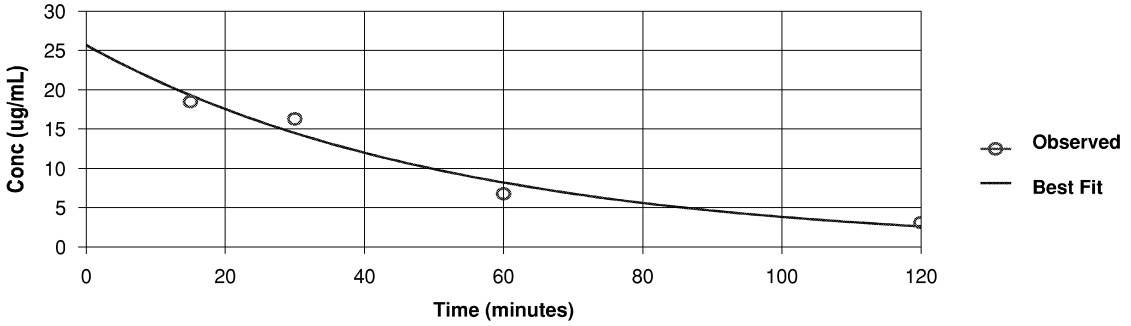

Fig. 4. Pharmacokinetics in rat plasma of Casiopeina IIgly.

times the standard deviation intercept/slope of the curve of calibration. A value of 0.52 μg/ml was determined.

The stability of Casiopeina IIgly before and after sample pre-treatment was determined. After 7 days at −20 °C, 95.6% of Casiopeina IIgly was still present in rat plasma. In solutions of mobile phase 97% was present after 24 h at 6 °C.

3.1. Pharmacokinetics results

No chromatographic interferences from any endogenous compounds were found. Fig. 3 shows the plasma concentration time profile of Casiopeina IIgly after the administration of 8.0 mg/kg. The concentration C_0, at zero time, was 25.67 μg/ml, the half-life obtained was 39.37 min, in this study the short half-life obtained suggests that Casiopeina IIgly is eliminated in a short time. The elimination from rats and mice is usually faster than man, therefore $t_{1/2}$ in man should be longer and is not yet known (Fig. 4).

4. Conclusions

This method proved to be useful and reliable for the determination of Casiopeina IIgly in plasma. It is fast and simple. The method, validated for concentrations ranging from 2.5 to 50 μg/ml, had good repeatability and accuracy and low limits of quantification and detection. The recovery of Casiopeina IIgly was reproducible and constant over the entire calibration range. This method is sufficiently sensitive to use in future preclinical pharmacokinetic studies.

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