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Manual and automatic extraction and high-performance liquid chromatographic determination of a spicamycin derivative, KRN5500, in rat plasma

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Abstract

A sensitive reliable method for the extraction, separation and quantitation of KRN5500 (I), a spicamycin derivative, from rat plasma was developed. It involves solid-phase extraction of the drug using a Bond Elut C_{18} cartridge and reversed-phase HPLC on a YMC-Pack ODS column with an ultraviolet detector. The intra- and inter-assay coefficients of variation by manual ($n=10$) and automatic ($n=5$) extraction were less than 9 and 13% and 6 and 8%, respectively. The limit of quantitation of each extraction procedure was 2 ng potency/ml. This extraction method may thus be considered useful for monitoring I in animals following its administration.

Keywords: Spicamycin derivative; KRN5500

1. Introduction

Spicamycin, a nucleoside antibiotic containing fatty acids differing in chain length and produced by *Streptomyces alanosinicus* 879-MT_3, was isolated as a differentiation inducer of HL-60 and M1 myeloid leukemic cells [1,2]. Spicamycin shows potent antitumor activity towards human stomach cancer SC-9 and human breast cancer MX-1 in a human tumor xenograft model. Several semi-synthetic spicamycin analogues (SPMs) differing in fatty acid moiety chain length, have been examined for structure–antitumor activity relationships [3,4]. KRN5500, a spicamycin derivative I, shows the greatest activity towards human colon cancer COL-1 in a human tumor xenograft model and the highest therapeutic index among spicamycin derivatives [5]. The antitumor activity of I, which is greatest in the human colon cancer xenograft model, exceeded that of mitomycin C. The structures of I and SPK-65, the internal standard (I.S.), are shown in Fig. 1.

This study describes a sensitive method for determining I in rat plasma using reversed-phase HPLC with UV detection. The method was shown to be useful for preclinical studies and to be applicable to monitoring of I in patients.

2. Experimental

2.1. Animals

Male Sprague–Dawley rats were obtained from Japan (Shizuoka, Japan). They were housed at least

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one week before experimentation and maintained under specific pathogen-free conditions, fed irradiated basal CE-2 diet (Clea Japan, Tokyo, Japan) and given water ad libitum.

2.2. Chromatographic system

The HPLC system consisted of a Hitachi L-6210 pump (Tokyo, Japan) and Hitachi autosampler AS-4000. A Hitachi L-4200 UV–Vis variable-wavelength detector was used to detect peaks at 264 nm. Peak-height data were obtained and evaluated with a Hitachi chromato-integrator D-2500. The YMC-Pack ODS A-312 column 6.0×150 mm (Kyoto, Japan) was maintained at 35°C with a Hitachi 655A-52 column oven. The mobile phase was 74% methanol and run at a flow-rate of 1.0 ml/min.

2.3. Robotic system

A Zymark BenchMate II workstation (Zymark, Hopkinton, MA, USA) was used to extract I from rat plasma, the procedure being programmed by original software.

2.4. Chemicals and reagents

Compound I and SPK-65, I.S., were synthesized by Kirin Brewery Pharmaceutical Research Laboratory (Gunma, Japan). All other chemicals were of analytical-reagent grade. Bond Elut C_{18} 1210-2025 cartridges were purchased from Varian (Harbor City, CA, USA).

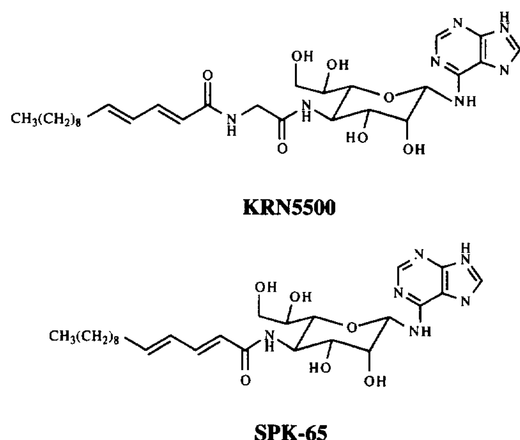

Fig. 1. Chemical structures of KRN5500 and SPK-65, the internal standard in this study.

2.5. Sample extraction

The present sample extraction can be performed either manually ($n=10$) or automatically using the Zymark BenchMate II workstation ($n=5$). In this study, a plasma sample (1 ml) was diluted with 2 ml methanol and then 5 ml 50 m*M* sodium phosphate buffer (pH 6.0), it was then passed through a Bond Elut C_{18} cartridge previously conditioned with 2 ml methanol and then 2 ml water. After successive washings with 6 ml water and 2 ml 5% methanol, the desired fraction was eluted with 2 ml methanol. The eluate was dried under a stream of nitrogen and redissolved with 50 μl of I.S. methanol solution containing 200 ng I.S. (4 μg/ml). A 40-μl aliquot was injected into the HPLC system.

2.6. Calibration curve

Standard solutions containing I (1.22–1250 ng potency/ml) in plasma were prepared and treated as above. Peak-height ratios of I to that of I.S. were used to obtain plasma calibration curves. The calibration curve was plotted as a double reciprocal plot to regression in a straight line, because the calibration curve of I has a wide concentration range and becomes shallow at high concentrations.

2.7. Assay validation

The assay was validated for intra- and inter-assay precision, accuracy and recovery of frozen plasma samples. Several batch concentrations of I in rat plasma were prepared, 2, 5, 10, 50, 200, 1000 ng potency/ml (2, 10, 50, 250, 1000 ng potency/ml in case of automatic extraction). Each plasma sample was placed in a polypropylene tube. The samples were frozen in liquid nitrogen and stored at −80°C until use.

Intra- and inter-assay variability were assessed based on repeated measurement of a prepared concentration of aliquots of the same sample within-day and between-day assays, respectively ($n=10$ and $n=5$ for manual and automatic extraction, respec-

tively). Compound I was determined from a daily standard curve.

Accuracy was assessed based on observed concentrations of the same samples for intra- and inter-assay precision determinations. Means of determined concentrations were calculated and percentage differences of the means from expected values were computed. Recovery was determined by adding known amounts of I standards to prepared concentrations.

2.8. Animal studies

Rats were assigned randomly to groups corresponding to plasma sampling times. There were three animals per sampling time. Compound I (200 μg potency/kg) was administered intravenously through the tail vein. Arterial blood was collected into a heparinized glass tube from the abdominal aorta under light ether anesthesia at 5, 15, 30 min and 1, 2, 4, 6, 8 h after injection. Blood was centrifuged at 400 *g* for 10 min at room temperature to separate plasma which was then transferred to polypropylene microtubes and frozen in liquid nitrogen and stored at −80°C until use.

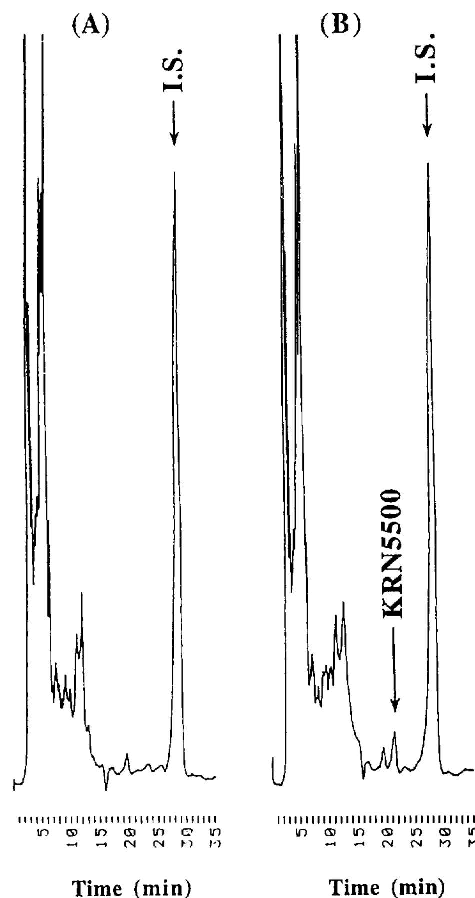

Fig. 2. Typical chromatograms of KRN5500 and SPK-65, internal standard (I.S.). (A) Blank rat plasma. (B) Rat plasma spiked with KRN5500 (5 ng potency/ml) and I.S. (4 μg/ml).

3. Results and discussion

3.1. HPLC separation and typical calibration curve

Typical chromatograms are shown in Fig. 2. Compound I and I.S. peak separation was good. A calibration curve was obtained for the direct quantitation of I based on peak height. A typical calibration curve is shown in Fig. 3. This method has an effective quantitative range from 1.22 to 1250 ng potency/ml. A high-degree of linearity was obtained over the entire calibration curve.

3.2. Validation

The reproducibility of this method was examined using rat plasma spiked with standard I solution. Intra- and inter-assay coefficients of variation (C.V.) evaluated from 2 to 1000 ng potency/ml are shown in Table 1. C.V. values of intra- and inter-assay in manual and automatic extraction were 0.72 to 8.82% and 3.45 to 12.99%, respectively. Inter-assay C.V. values were larger than generally expected and reflected intra-assay and assay-to-assay differences. Variability in inter-assay C.V. of manual extraction due to intra-assay variability ranged from 21 to 68% and the rest to assay-to-assay differences. At absolute concentrations, assay-to-assay differences are small. On the whole, the accuracy of automatic extraction improved more than manual extraction, possibly since sample treatment is more accurate as a result of robotic operation. The recoveries of prepared concentrations from the sample were estimated from the ratio of peak height of the plasma sample to that of the standard solution and were found to be

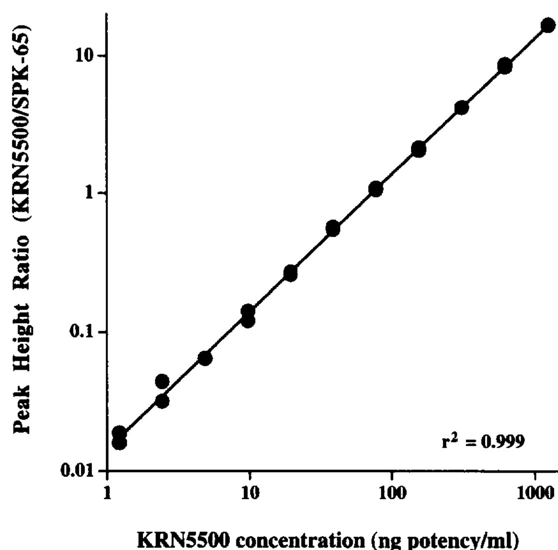

Fig. 3. Typical calibration curve of KRN5500 in rat plasma.

74–93% (manual) and 88–109% (automatic) (Table 2). The limit of quantitation (LOQ) for I, i.e. the lowest concentration on the standard curve at acceptable reproducibility, accuracy and precision and detector sensitivity, was 2 ng potency/ml. These data, shown in Table 3, demonstrate that the present method can be used as a means for precise intra- and inter-assay determinations in various pharmacokinetic experiments.

3.3. Animal study

Blood samples were obtained following intravenous administrations of 200 μg potency/kg I to rats. The plasma concentration–time profile determined by manual extraction is shown in Fig. 4. The plasma concentration curve declined very rapidly and was below the detection limit of determination after 4 h.

4. Conclusion

The present method is clearly useful for monitoring I in animals. Similar chromatograms were obtained using human plasma spiked with standard I solution (data not shown). It should prove applicable to the monitoring of I in patient plasma.

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Table 1
Intra-assay and inter-assay precision and accuracy of KRN5500 (ng potency/ml) determinations in rat plasma samples by manual extraction (n=10).

KRN5500 concentration (ng potency/ml)		C.V. (%)	Accuracy (%)
Prepared	Observed±S.D.		
Intra-assay			
2	1.920±0.169	8.82	4.02
5	5.241±0.331	6.32	4.83
10	10.14±0.46	4.49	1.43
50	53.54±0.41	0.76	7.08
200	197.6±1.1	0.57	1.22
1000	990.7±7.2	0.72	0.93
Inter-assay			
2	2.010±0.261	12.99	0.52
5	4.822±0.485	10.06	3.56
10	9.450±0.694	7.35	5.50
50	49.83±2.80	5.61	0.33
200	204.0±4.6	2.26	2.02
1000	999.6±34.5	3.45	0.04

Table 2
Recovery of KRN5500 from rat plasma by manual and automatic extraction

Prepared concentration (ng potency/ml)	Recovery (mean±S.D.) (%)	
	Manual extraction	Automatic extraction
2	92.51±14.56	99.49±2.72
5	89.62±7.08	–[a]
10	90.20±7.20	109.0±2.39
50	82.12±4.23	89.79±4.91
200	78.27±2.86	94.85±2.99[b]
1000	73.62±1.99	87.80±2.64

[a] Not investigated.
[b] Prepared to 250 ng potency/ml.

Table 3
Intra-assay and inter-assay precision and accuracy of KRN5500 (ng potency/ml) determinations in rat plasma samples by automatic extraction (n=5).

KRN5500 concentration (ng potency/ml)		C.V. (%)	Accuracy (%)
Prepared	Observed±S.D.		
Intra-assay			
2	1.97±0.06	2.88	1.42
10	9.48±0.22	2.31	5.19
50	49.69±2.86	5.76	0.63
250	254.6±8.5	3.32	1.84
1000	1085.4±34.4	3.17	8.54
Inter-assay			
2	2.04±0.08	4.09	1.96
10	9.83±0.77	7.82	1.70
50	49.95±2.51	5.02	0.10
250	260.2±4.9	1.89	4.07
1000	1079.3±21.0	1.94	7.93

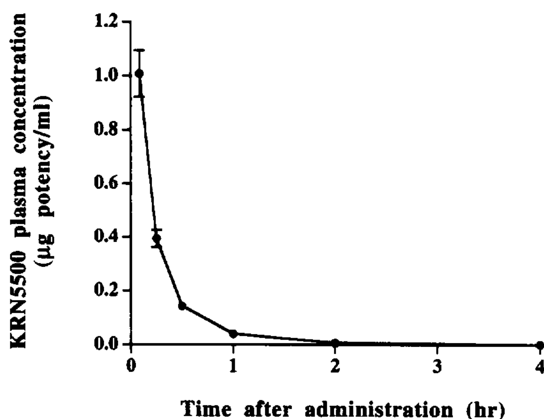

Fig. 4. KRN5500 plasma concentration profile following intravenous 200 μg potency/kg KRN5500 administration to rats. Each point shows mean±S.D. (n=4).

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