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Simultaneous determination of a new anticancer agent (NB-506) and its active metabolite in human plasma and urine by high-performance liquid chromatography with ultraviolet detection

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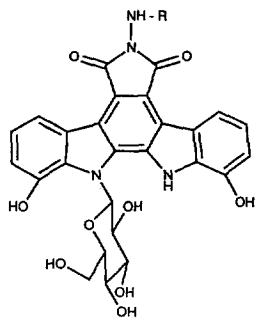
Abstract

A high-performance liquid chromatographic method with ultraviolet detection has been developed to quantify NB-506 and its active metabolite in human plasma and urine. This method is based on solid-phase extraction, thereby allowing the simultaneous measurement of the drug and metabolite with the limit of quantification of 0.01 μg/ml in plasma and 0.1 μg/ml in urine. Standard curves for the compounds were linear in the concentration ranges investigated. The range for the drug in plasma was 0.01–2.5 μg/ml, and for the metabolite 0.01–1 μg/ml. In urine, the range for both compounds was 0.1–10 μg/ml. The method was validated and applied to the assay of plasma and urinary samples from phase I studies.

1. Introduction

NB-506, 6-N-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6H)-dione (I, Fig. 1), has antitumor activity and is currently being developed as an anticancer agent. This compound was derived from a novel indolocarbazole antibiotic produced by an actinomycete [1–3]. Compound I acts as a potent inhibitor of topoisomerase I, and also inhibits the activity of



R = CHO I

R = H II

R = SO_2CH_3 III (I.S.)

Fig. 1. Structure of NB-506 (I), metabolite (II) and the internal standard (III).

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DNA polymerase α and RNA polymerase II [4–6].

Initial studies have shown that mice mainly produce metabolite II (Fig. 1) which shows antitumor activity. The metabolite may be formed in humans and can contribute to the pharmacological effects after administration of I. Thus, it became essential to quantify I and II in human plasma and urine for characterizing their pharmacokinetic parameters in phase I clinical trials.

A high-performance liquid chromatographic (HPLC) assay has been developed for simultaneous analysis of I and II in the biological fluids, using compound III (Fig. 1) as the internal standard (I.S.). This assay involves a solid-phase extraction procedure for sample clean-up. The method was validated and found to be reliable for quantifying I and II in the concentration ranges of 0.01–2.5 μg/ml and 0.01–1 μg/ml, respectively, in plasma, and 0.1–10 μg/ml in urine. The limit of quantification (LOQ) for both compounds in plasma was 0.01 μg/ml and in urine was 0.1 μg/ml. Plasma and urinary samples were assayed by the method presented here to evaluate pharmacokinetic profiles of I and II in the phase I clinical study.

2. Experimental

2.1. Reagents and materials

Compounds I, II and III were synthesized by Banyu Tsukuba Research Institute (Tsukuba, Japan). Acetonitrile, methanol, ethanol (all HPLC grade), N,N-dimethylformamide (DMF) and glycine (both reagent grade) were purchased from Wako (Osaka, Japan). Glycine-HCl buffer was used as a stabilizer for I in plasma and urine. Water was purified with a Milli-Q system (Millipore-Japan, Tokyo, Japan). Bond-Elut C_8 cartridges (100 mg, 1 ml) were purchased from Uniflex (Tokyo, Japan).

2.2. Apparatus

For plasma assay, a Beckman (Beckman-Japan, Tokyo, Japan) HPLC system consisting of a module 126 pump, a 507 autosampler with a column oven and sample cooler, a 116 UV detector and a GT-104 degasser (GL Science, Tokyo, Japan) was used. Data were acquired and processed by a Beckman System Gold program on an IBM PS/2 microcomputer originally included in the system. A Shiseido (Tokyo, Japan) Superiorex ODS (250 mm × 4.6 mm, 5 μm) column was employed for analysis and an Applied Biosystems (Chemco, Tokyo, Japan) RP-18 Newguard (15 mm × 3.2 mm, 7 μm) was used as a guard column. The HPLC system for the analysis of urine was virtually identical to that for the plasma assay except that an AS-8020 autosampler (Tosoh, Tokyo, Japan) and a UV-8020 detector (Tosoh) were used.

2.3. Chromatographic conditions

A mobile phase consisting of acetonitrile–methanol–water (19:15:66, v/v/v) was delivered at a flow-rate of 1 ml/min for plasma assay. The sample cooler was set at 4°C, and the column oven was maintained at 40°C. The UV detector was monitored at 305 nm with a time constant of 2 Hz. The autosampler was operated so as to have a run time of 20 min. Under these conditions, the retention times of I, II and I.S. were 9.8, 11.9 and 17.8 min, respectively.

For the analysis of urine, HPLC elution conditions were slightly modified. A mixture of acetonitrile–methanol–water (15:24:61, v/v/v) was passed through for 15 min at a flow-rate of 1 ml/min, after which a methanol–water mixture (80:20, v/v) was pumped for 5 min at a flow-rate of 1.2 ml/min to eliminate hydrophobic substances retained on the column. Then, the flow-rate of the initial mobile phase was set at 1.2 ml/min for 5 min and then to 1 ml/min for 5 min, thus reconditioning the column for the next analysis. The autosampler was run at a 35-min cycle. Compounds I, II and I.S. had retention times of 9.1, 11.5 and 15.4 min, respectively.

2.4. Standard solutions

A stock solution of I was prepared in ethanol (1 mg/ml) and of II in DMF (1 mg/ml). These solutions were further diluted with ethanol to

yield standard solutions with concentrations of 0.05, 0.125, 0.25, 1.25, 5 and 12.5 μg/ml for plasma assay, and concentrations of 1, 2.5, 10, 25 and 100 μg/ml for urinary assay. The I.S. solution with a concentration of 10 μg/ml was prepared similarly by diluting the solution of III in DMF (1 mg/ml) and was used for all analyses.

2.5. Standard curves

The standard curve for I in plasma was constructed by spiking blank plasma with known amounts of I in the concentration range of 0.01–2.5 μg/ml, and for II in the range of 0.01–1 μg/ml. The standard solutions of I (100 μl) and II (100 μl) were placed in a culture tube, and the solvent was evaporated. To enhance the solubility of I and II in plasma, ethanol (20 μl) was added to the residue. To the resulting syrup, blank plasma (0.5 ml) and glycine-HCl buffer (0.2 *M*, pH 2.8, 0.5 ml) were added, following which the mixture was vortex-mixed. After addition of the I.S. solution (20 μl), the mixture was vortex-mixed and then centrifuged (1800 *g*, 5 min, 4°C). The supernatant was added to a Bond-Elut C_8 cartridge pre-treated with methanol (1.2 ml), water (1.2 ml) and glycine-HCl buffer (0.02 *M*, pH 2.8, 1 ml). The cartridge was washed with water (1 ml) and a methanol–water mixture (1:9, v/v, 1 ml). The washing solvent was eliminated from the cartridge by centrifugation. The analytes and I.S. were eluted with ethanol (1 ml), and elution was completed by centrifugation. The combined eluate was evaporated, and the residue was reconstituted in a 1:1 methanol–water mixture (200 μl). The solution was centrifuged (1800 *g*, 5 min, 4°C) to remove particles coming from the cartridge. The supernatant was filtered through a 0.45-μm filter (GL Science). A 40-μl portion of the filtrate was injected onto the HPLC system.

The same procedure was used to generate standard curves for I and II in urine in the concentration range of 0.1–10 μg/ml with slight modifications. As a stabilizer, glycine-HCl buffer (0.5 *M*, pH 3) was employed. The standard solutions of I (50 μl), II (50 μl) and I.S. (50 μl) were added to a mixture of blank urine (0.5 ml) and the buffer (0.5 ml). The solution was processed according to the method described for plasma, and the resulting sample was added to a Bond-Elut C_8 cartridge pre-conditioned with methanol (1.2 ml) and water (1.2 ml). The cartridge was washed with water (1.2 ml) and a methanol–water mixture (35:65, v/v, 1 ml). The solid-phase extraction followed by the sample processing was the same as the method for the plasma treatment except that the extract was reconstituted in a methanol–water mixture (60:40, v/v, 200 μl) and that the injection volume was 50 μl.

Plots of the peak-height ratio of either I or II and I.S. against the concentration of the added compound were used to calculate the linear regression equation, giving linear standard curves in the concentration ranges.

2.6. Preparation of plasma and urinary samples

Plasma was separated from heparinized blood samples by centrifugation at 1500 *g* for 10 min at 4°C. Plasma and urinary samples were stored at −80°C until assay. After thawing, these samples were treated following the procedures described for standard curves, but for the assay of urine, ethanol (100 μl) corresponding to the volume of the standard solutions was added just before the sample treatment.

3. Results and discussion

When plasma containing I was allowed to stand at room temperature, the drug was decomposed by ca. 25% in 4 h. In urine, it was degraded by ca. 10% under similar conditions. Initial studies indicated that I was stable in organic solvents and buffer solutions with pH values of 3–4, but somewhat unstable in solutions with higher pH values. On acidification of plasma and urine by addition of an equal volume of glycine-HCl buffer, the drug stability during the sample treatment was maximized. Because of larger inter-subject differences in pH values of urine, a buffer solution with more strength was added for the stabilization of I in urine. Com-

pound II and I.S. were stable under the above conditions. Compounds I, II and I.S. in the sample solutions were stable long enough for overnight analysis when maintained at 4°C in the autosampler.

Among several C_{18} columns, a Superiorex ODS column was selected for the analysis, because it provided better chromatographic separation between the peaks of I and II. Prior to the use of a new column, conditioning with the mobile phase for more than 5 h was necessary to produce steady retention times and symmetrical peaks of the compounds to be analyzed.

The UV spectra of I and II in the mobile phase indicated the presence of two major bands with the maximum at 248 nm and 305 nm. The latter band had slightly lower intensity. However, owing to its longer wavelength, a choice of the latter for detection was expected to offer better selectivity and cleaner backgrounds in chromatography. Hence, the UV detector was monitored at the wavelength of 305 nm for the quantification.

Fig. 2 shows chromatograms of blank plasma and urine obtained from healthy volunteers, blank samples spiked with I, II and I.S., and a post-dose sample of plasma (2.769 μg/ml for I and 0.017 μg/ml for II) and urine (5.50 μg/ml for I and 0.11 μg/ml for II) taken from a patient participating in the phase I study. No interferences from endogenous substances were found in the blank plasma. Although minor endogenous components were observed in chromatographic backgrounds in the assay of blank urine, they appeared to have no influence on the precision of the method. The analysis of the pre-dose samples from the patients sometimes showed more complicated chromatographic profiles, but no interferences were encountered. This indicates that the assay is specific.

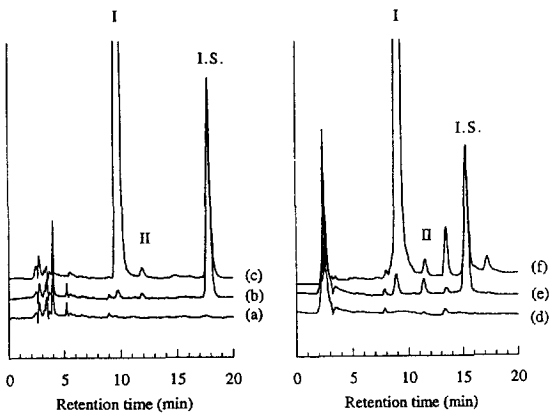

Fig. 2. Representative chromatograms of human plasma and urinary samples: (a) blank plasma, (b) spiked plasma (I and II, 0.01 μg/ml; I.S., 0.4 μg/ml), (c) plasma collected from a patient 15 min after the beginning of infusion at a dose of 120 mg/m^2, (d) blank urine, (e) spiked urine (I and II, 0.1 μg/ml; I.S., 1 μg/ml), (f) urine collected from a patient 0–9 h after the beginning of infusion at a dose of 120 mg/m^2.

The intra-day precision, expressed by coefficient of variation (C.V.), accuracy and recovery for the assay of I in plasma and urine are summarized in Table 1, and those for II in Table 2. The precision and accuracy were examined by replicate analyses ($n = 6$) of the samples prepared by the method described for standard curves. The recovery was determined by comparison of the peak-height of I and II extracted from both fluids spiked with known amounts to that obtained by the injection of the same amounts of the respective compounds.

The recoveries of I and II from plasma were relatively low (68%–87% for I and 63%–77% for II), whereas those of I and II from urine were high (101%–110% for I and 90%–109% for II) as shown in Tables 1 and 2. However, the recoveries were practically independent of concentration, and their variation did not affect the C.V.s in the analysis. The C.V. for I at the lowest concentration in plasma was 8.6%, and that for II was 13%. The C.V.s for the compounds at the other concentrations did not exceed 10%. When the LOQ was defined as 0.01 μg/ml for both compounds, the method for the plasma assay provided good reproducibility. Similarly, for the urinary assay, the LOQ for I and II was determined to be 0.1 μg/ml.

The inter-day variability of this analytical system was assessed by comparison of the slopes of the standard curves constructed on five different days. The C.V. of the slopes for I in plasma was 2.7%, for II in plasma it was 8.9%, for I in urine 1.9%, and for II in urine 6.2%. This demonstrates that the assay for I is more stable

Table 1
Intra-day precision, accuracy and recovery for the assay of I in human plasma and urine

Spiked concentration (μg/ml)	Measured concentration (mean ± S.D., μg/ml)	Coefficient of variation (%)	Accuracy (%)	Recovery (%)
Plasma				
0.010	0.011 ± 0.001	8.6	106	68
0.025	0.023 ± 0.001	5.9	91	74
0.050	0.049 ± 0.001	2.7	98	76
0.250	0.258 ± 0.004	1.5	103	78
1.000	1.045 ± 0.014	1.3	105	87
2.500	2.591 ± 0.069	2.7	104	86
Urine				
0.10	0.10 ± 0.00	2.7	97	107
0.25	0.23 ± 0.00	1.9	92	110
1.00	1.01 ± 0.01	1.3	101	105
2.50	2.45 ± 0.06	2.4	98	102
10.00	10.16 ± 0.07	0.7	102	101

and repeatable than that for II. A more direct basis for this assay stability was found in the analysis of quality control (Q.C.) samples.

These Q.C. samples were prepared by spiking blank plasma and urine with I at high and low

period of 6 months. The results are given in Table 3. For both Q.C. samples, the CVs were ≦5.3%, indicating the assay repeatability. Furthermore, the Q.C. data show the long-term storage stability of I for at least 6 months in the

Table 2
Intra-day precision, accuracy and recovery for the assay of II in human plasma and urine

Spiked concentration (μg/ml)	Measured concentration (mean ± S.D., μg/ml)	Coefficient of variation (%)	Accuracy (%)	Recovery (%)
Plasma				
0.010	0.011 ± 0.001	12.8	107	66
0.025	0.023 ± 0.002	9.9	93	63
0.050	0.050 ± 0.001	2.2	100	66
0.250	0.255 ± 0.004	1.5	102	69
1.000	1.050 ± 0.025	2.4	105	77
Urine				
0.10	0.11 ± 0.00	3.8	110	91
0.25	0.23 ± 0.01	4.0	93	90
1.00	1.02 ± 0.01	1.3	102	90
2.50	2.50 ± 0.12	4.7	100	91
10.00	10.56 ± 0.07	0.6	106	109

Table 3
Inter-day variability for the assay of quality-control plasma and urinary samples spiked with I

Spiked concentration (μg/ml)	n	Measured concentration (mean ± S.D., μg/ml)	Coefficient of variation (%)
Plasma			
0.025	18	0.027 ± 0.001	5.3
1.000	18	0.978 ± 0.044	4.5
Urine			
0.20	18	0.20 ± 0.01	2.6
5.00	18	4.88 ± 0.13	2.7

prepared for each set of assays of the clinical samples, and Q.C. samples were also analyzed. The plasma assay showed that humans, in contrast to mice, produced a very small amount of metabolite II, which could be quantified at a few time points in several subjects. The amounts of I and II excreted in urine were also very small. Although the results of the clinical study together with pharmacokinetic parameters determined by this assay will be reported in detail elsewhere [7], as an example, some plasma and urinary concentrations of I and II are shown in Table 4.

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Table 4
Plasma and urinary concentrations (μg/ml) of I and II after a single dose of 120 mg/m^2 of I by one-hour infusion to three subjects

Time after the start of infusion (h)	Subject A		Subject B		Subject C	
	I	II	I	II	I	II
Plasma						
1	3.651	0.010	3.428	0.014	4.122	0.019
2	0.216	N.D.	0.134	N.D.	0.208	N.D.
3	0.173	N.D.	0.095	N.D.	0.154	N.D.
7	0.077	N.D.	0.047	N.D.	0.064	N.D.
13	0.035	N.D.	0.024	N.D.	0.031	N.D.
25	0.013	N.D.	0.011	N.D.	0.012	N.D.
Urine[a]						
9	5.50	0.11	0.69	N.D.	1.34	N.D.
25	1.30	N.D.	0.23	N.D.	0.33	N.D.

N.D. stands for not detected.
[a] Urine samples are collected at 0–9 and 9–25 h after the start of infusion.

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