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Determination of the anticancer drug KW-2170, a pyrazoloacridone derivative, and its metabolites in human and dog plasma by high-performance liquid chromatography using an electrochemical detector

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Abstract

KW-2170, 5-(3-aminopropyl) amino-7,10-dihydroxy-2-(2-hydroxethyl)-aminoethyl-6H-pyrazolo [4,5,1-de] acridin-6-one dihydroxychloride, is a novel anticancer agent under clinical development. We have established a highly sensitive method which can simultaneously quantitate KW-2170 and its two metabolites, a carboxylic (M1) and hydroxylated (M2) derivative involving the 5-position, in human and dog plasma. KW-2170 and its metabolites were extracted from plasma using a weak cation-exchange cartridge and then determined by HPLC using an electrochemical detector (ED). Over the concentration range 0.1–50 ng/ml, precision and accuracy of intra- and inter-day assay were within 11% in human plasma. In dog plasma, they were within 17% at the lower quantitation limit and within 11% at other concentrations. These three compounds were stable during the assay procedure, freeze–thawing cycles and during long-term storage. Using this methodology, the pharmacokinetics of KW-2170 in a dog could be monitored over 24 h. This method is suitable for evaluation of the detailed pharmacokinetics of KW-2170 and its metabolites in humans and dogs.

Keywords: KW-2170; Pyrazoloacridone derivatives

1. Introduction

KW-2170, 5-(3-aminopropyl) amino-7,10-dihydroxy-2-[(2-hydroxethyl)-aminoethyl]-6H-pyrazolo [4,5,1-de] acridin-6-one dihydrochloride, is a novel anticancer drug with DNA-intercalating activity (Fig. 1A) [1,2]. KW-2170 exhibits significant antitumor activity against various human tumor xenografts in nude mice and has superior antitumor activity against adriamycin (ADM)-resistant human ovarian carcinoma A2780/ADM which highly expresses P-glycoprotein in vitro and in vivo [2]. A Phase I clinical trial of KW-2170 in patients with refractory malignant tumors is now underway in both Japan and the US [3,4].

In order to determine plasma concentrations in the clinical trials, a highly sensitive method for determining KW-2170 has been developed using high-performance liquid chromatography with electro-

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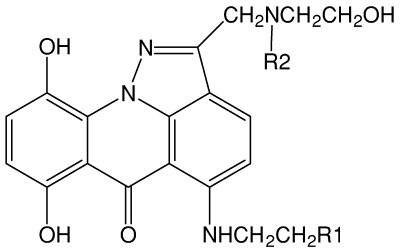

	(A)	(B)	(C)	(D)
R1	-CH_2NH_2	-COOH	-CH_2OH	-CH_2NH_2
R2	-H	-H	-H	-CH_2CH_3

Fig. 1. Chemical structures of KW-2170 base (A), M1 (B), M2 (C) and I.S. base (D); KW-2170 and I.S. are the two hydroxychloride salt forms.

chemical detection (HPLC–ED) [5]. The hydroquinone structure of KW-2170 allows electrochemical detection to be used and a lower quantitation limit of 0.1 ng/ml was achieved with good reproducibility using 0.25 ml human plasma. Although this method was useful for measuring plasma concentrations of unchanged KW-2170 in Japanese clinical trials, unknown peaks which interfered with the measurement of unchanged drug were observed and thought to be due to metabolites of KW-2170. The structures of these metabolites were identified by LC–MS as the carboxylic derivative of the aminomethyl group at the 5-position of the parent drug (M1, Fig. 1B) and the hydroxylated derivative of the aminomethyl group at the 5-position of the parent drug (M2, Fig. 1C). Therefore, in the present study, we have developed and validated a method to allow the simultaneous quantification of KW-2170 and its metabolites in human and dog plasma by HPLC–ED.

2. Experimental

2.1. Chemicals and reagents

KW-2170, M1, M2 and KF31133 (internal standard, I.S., Fig. 1D) were synthesized in our institute. Bond Elut® CBA (50 mg/1 ml, a weak cation exchanger) cartridges were purchased from Varian (Harbor City, CA, USA). Acetonitrile, methanol and dichloromethane were of HPLC grade from Kanto (Tokyo, Japan). Sodium-1-octanesulfonate was a reagent specially prepared for ion-pair chromatography (Nacalai Tesque, Kyoto, Japan). Purified water was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The other reagents were of analytical grade and commercially available. Human blood and plasma were obtained from healthy volunteers following their written informed consent. Plasma from male beagle dogs was purchased from Toyota Tsusho (Tokyo, Japan). Dog blood was obtained from a healthy male beagle dog (LRE, Covance Research Products Kalamazoo, MI, USA). KW-2170, M2 and the I.S. were separately dissolved at concentrations of 1 mg/ml in water, and M1 was dissolved in 0.1 *M* hydrochloric acid and each solution was stored at 4 °C.

2.2. Sample preparation

Bond Elut CBA was pretreated before use with 3 ml dichloromethane, 2 ml acetonitrile and 2 ml water. Human or dog plasma (0.25 ml) was diluted with 0.75 ml 20 m*M* KH_2PO_4 containing 4 mg/ml of ascorbic acid and 0.5 ng/ml of I.S. to maintain an appropriate pH (about 5.0). The plasma sample was then applied to a cartridge to extract KW-2170, M1, M2 and the I.S. Then the cartridge was washed with 2 ml water and 1 ml acetonitrile and the compounds of interest were eluted with 0.75 ml methanol–trifluoroacetic acid (99:1, v/v) by centrifugation (60 *g*, 5 min, 4 °C). The eluate was evaporated to dryness under a stream of nitrogen and reconstituted with 0.2 ml HPLC mobile phase. The insoluble residue was removed by centrifugation (2000 *g*, 10 min, 4 °C) and 0.1 ml of the supernatant was injected into the HPLC system. The recoveries of KW-2170, M1, M2 and the I.S. were approximately 85, 60, 75 and 75%, respectively, for human plasma and approximately 100, 70, 75 and 80%, respectively, for dog plasma.

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of a L-7100 pump (Hitachi, Tokyo, Japan), an AS-4000 autosampler (4 °C, Hitachi) and a Class-LC10 (Shimadzu, Kyoto, Japan) or D-7000 (Hitachi) data processor. To stabilize the baseline, a pulse damper (HPD-1, GL Sciences, Tokyo, Japan) and degasser (Shodex Degas KT-17, Showa Denko, Tokyo, Japan) were used. The

ECD-300 detector (Eicom, Kyoto, Japan) and analytical column (YMC-Pack pro C_{18}, 4.6×150 mm, I.D., 5 μm, YMC, Kyoto, Japan) were maintained at 25 °C using an ATC-300 column oven (Eicom). ECD-300 was equipped with a grassy carbon electrode (WE-GC, Eicom) and the applied potential was set at 350 mV. The analytes were separated using an isocratic solvent of McIlvaine's buffer (pH 4.0) containing 1 m*M* EDTA·2 Na and 2.5 m*M* sodium 1-octanesulfonate– acetonitrile (78:22, v/v) at a flow-rate of 1 ml/min.

2.4. Calibration curve, method validation and stability

Samples for calibration curves were prepared from human or dog plasma spiked simultaneously with KW-2170, M1 and M2 at concentrations of 0.1, 0.4, 2, 10 and 50 ng/ml ($n=2$) for each assay. The peak height ratio of each compound to the I.S. was plotted against the added concentration. The regression equation was calculated by the linear least-squares method with weighting ($1/y^2$). The intra-day assay validation was conducted at concentrations of 0.1 (the lower limit of quantitation, LLOQ), 2 and 50 ng/ml ($n=5$) and the precision and accuracy were evaluated. The inter-day assay validation was conducted at 0.2, 2 and 40 ng/ml, each one sample was measured per assay, for three times. For intra- and inter-day assay validation, precision (relative error) and accuracy (relative standard deviation) were calculated as follows:

$$\text{RSD (\%)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

where observed concentrations were used for the calculation of standard deviation and mean.

$$\text{RE (\%)} = \frac{\text{Mean} - \text{added concentration}}{\text{Added concentration}} \times 100$$

where the observed concentrations were used for the

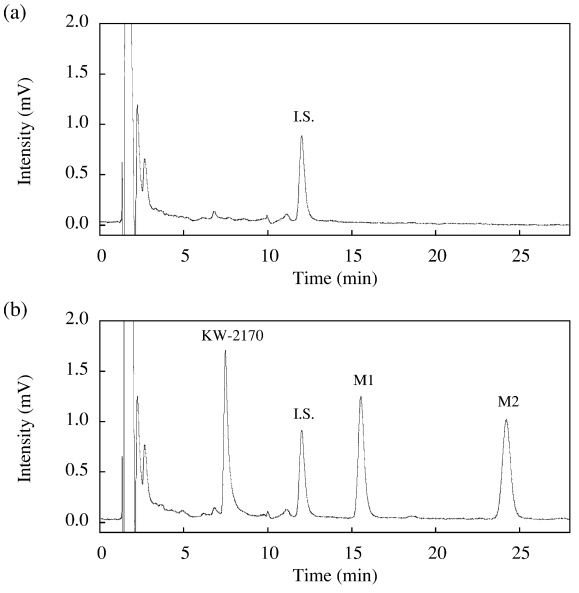

Fig. 2. Typical HPLC–ED chromatograms of human plasma: control plasma from a healthy Japanese volunteer (a), plasma sample spiked with 2 ng/ml of KW-2170, M1 and M2 (b).

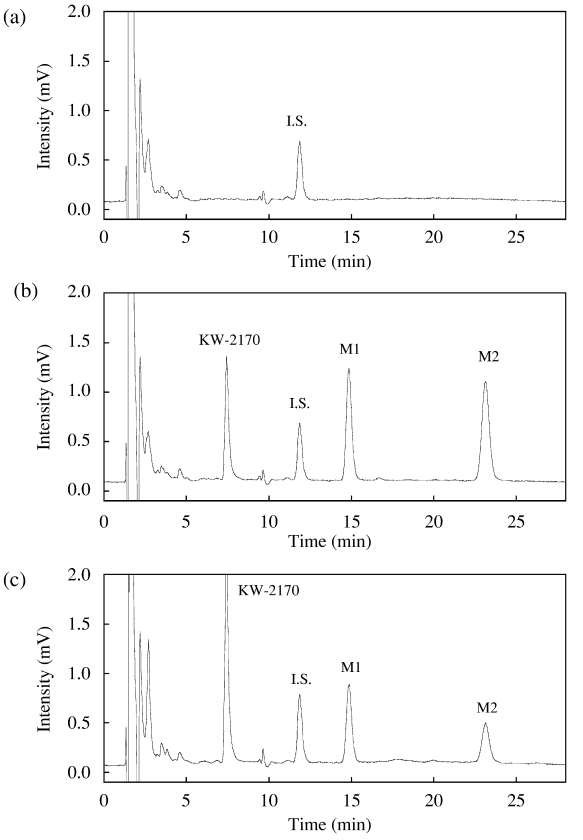

Fig. 3. Typical chromatograms of dog plasma: blank plasma from male beagle dogs (a), a plasma sample spiked with KW-2170, M1 and M2 (b) and a plasma sample 0.5 h after intravenous administration of KW-2170 to a male beagle dog at a dose of 0.3 mg/kg (c).

mean and added concentration represented the defined concentration of compounds.

The stability of each compound in human or dog plasma was assessed after standing on ice or at room temperature, after freeze–thawing at −80 °C and during cryopreservation. In the stability study, each compound was added separately to prevent any potential degradation products of one compound interfering with the determination of the other compounds. The remaining ratio to the initial value (actual value) was calculated. The stability of each compound in human or dog blood was also evaluated by measuring its concentration in plasma separated from the blood as described in Section 2.2.

2.5. Pharmacokinetics in a dog

KW-2170 was dissolved in saline to give a 0.6 mg/ml solution. This preparation was administered as a bolus intravenous injection (0.5 ml/kg) to a male beagle dog (LRE, 1 year old) and blood samples were collected in heparinized tubes at 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24 h after dosing. Then blood samples were centrifuged to obtain plasma and specimens were stored at −80 °C until analysis.

3. Results and discussion

We have developed a highly sensitive method for quantification of KW-2170 and its two metabolites (M1 and M2, Fig. 1) using HPLC–ED. Fig. 2 shows typical chromatograms of human plasma after solid-phase extraction using Bond Elut® CBA. There were no endogenous peaks interfering in the determination of each compound in plasma from six healthy Japanese volunteers (data not shown). Typical chromatograms of dog plasma after solid-phase extraction are shown in Fig. 3. As in human plasma, no interference peaks were observed in dog plasma.

The precision (RE) and accuracy (RSD) of the intra-day assay ($n=5$) and the inter-day assay ($n=3$)

Table 1
Precision and accuracy of the determination of KW-2170 and its metabolites in human plasma

	Compound	Added conc. (ng/ml)	Found conc. (ng/ml)	RSD[a] (%)	RE[b] (%)
Intra-day[c]	KW-2170	0.1	0.09205±0.00608	6.6	−8.0
		2	1.877±0.107	5.7	−6.2
		50	47.04±1.57	3.3	−5.9
	M1	0.1	0.09930±0.00387	3.9	−0.7
		2	1.906±0.013	0.7	−4.7
		50	46.79±0.55	1.2	−6.4
	M2	0.1	0.09216±0.00861	9.3	−7.8
		2	1.969±0.037	1.9	−1.6
		50	45.51±0.47	1.0	−9.0
Inter-day[d]	KW-2170	0.2	0.2030±0.0122	6.0	1.5
		2	2.210±0.110	5.0	10.5
		40	44.26±1.38	3.1	10.7
	M1	0.2	0.2054±0.0150	7.3	2.7
		2	2.174±0.133	6.1	8.7
		40	44.06±1.61	3.6	10.2
	M2	0.2	0.2036±0.0119	5.9	1.8
		2	2.174±0.140	6.4	8.7
		40	43.85±1.30	3.0	9.6

[a] Relative standard deviation.
[b] Relative error.
[c] Mean±SD ($n=5$).
[d] Mean±SD ($n=3$).

in human plasma were within ±11% for all compounds (Table 1). In dog plasma, they were within ±17% at 0.1 ng/ml and within ±11% at other concentrations (Table 2). Overall, each compound showed a good linearity over the concentration range of 0.1–50 ng/ml and precision and accuracy exhibited good reproducibility.

The stability of KW-2170, M1 and M2 in human blood and plasma are summarized in Table 3. In human blood, KW-2170, M1 and M2 are stable for 2 h on ice. It is recommended that in clinical trials blood samples should be kept on ice and centrifuged within 2 h. KW-2170, M1 and M2 are stable in human plasma for 2 h on ice and at room temperature, after two cycles freeze–thawing and for 2 years when kept frozen at −80 °C. From these results, plasma samples were stored at −80 °C. The short-term stability at −20 °C was also evaluated because some clinical sites were not equipped with a freezer for storage of samples at −80 °C and results showed that all compounds were stable for 10 days at −20 °C.

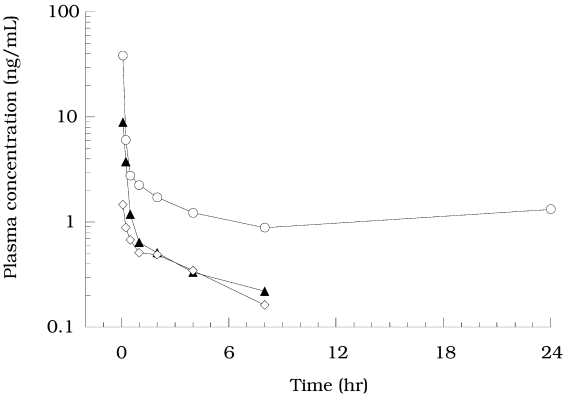

Fig. 4. Plasma concentrations of KW-2170 (open circle), M1 (closed triangle) and M2 (open diamond) after intravenous administration of KW-2170 to a male beagle dog (0.3 mg/kg, $n=1$).

The stability of KW-2170, M1 and M2 in dog

Table 2
Precision and accuracy of the determination of KW-2170 and its metabolites in dog plasma

	Compound	Added conc. (ng/ml)	Found conc. (ng/ml)	RSD[a] (%)	RE[b] (%)
Intra-day[c]	KW-2170	0.1	0.1093±0.0181	16.6	9.3
		2	1.970±0.019	1.0	−1.5
		50	49.32±0.75	1.5	−1.4
	M1	0.1	0.09449±0.00858	9.1	−5.5
		2	1.989±0.036	1.8	−0.5
		50	48.84±0.58	1.2	−2.3
	M2	0.1	0.1051±0.0131	12.5	5.1
		2	1.989±0.040	2.0	−0.5
		50	48.88±0.86	1.8	−2.2
Inter-day[d]	KW-2170	0.2	0.2035±0.0146	7.2	1.7
		2	1.884±0.199	10.6	−5.8
		40	40.88±1.33	3.3	2.2
	M1	0.2	0.1997±0.0041	2.1	−0.2
		2	1.929±0.197	10.2	−3.6
		40	41.63±1.34	3.2	4.1
	M2	0.2	0.2026±0.0031	1.5	1.3
		2	1.912±10.7	10.7	−4.4
		40	41.70±1.12	2.7	4.3

[a] Relative standard deviation.
[b] Relative error.
[c] Mean±SD ($n=5$).
[d] Mean±SD ($n=3$).

Table 3
Stability of KW-2170, M1 and M2 in human blood and plasma

Conditions	Added conc. (ng/ml)	Remaining ratio (%)[a]		
		KW-2170	M1	M2
In blood				
Standing on ice for 2 h	0.5	104	103	118
	10	99.5	131	120
Standing at RT[b] for 2 h	0.5	100	88.9	89.1
	10	96.7	97.0	99.6
In plasma				
Standing on ice for 2 h	0.2	102	110	98.6
	4	100	98.8	104
Standing at RT for 2 h	0.2	91.1	98.0	91.2
	4	92.5	93.1	96.9
Freeze–thawing 2 cycles	0.2	113	101	107
	4	91.7	97.1	90.3
Storage at −80 °C for 2 years	0.2	97.2	104	103
	4	97.1	97.3	110
Storage at −20 °C for 10 days	0.2	114	103	112
	4	102	107	93.4

[a] Mean ($n=2$).
[b] Room temperature.

blood and plasma is summarized in Table 4. The remaining ratio of KW-2170, M1 and M2 did not fall significantly in dog plasma after 1 h at room temperature, after two cycles of freeze–thawing and after 1 week of storage at −80 °C, and 2 h on ice in dog blood.

Plasma concentrations of KW-2170, M1 and M2 after intravenous administration of KW-2170 to a dog at a dose of 0.3 mg/kg (the maximum tolerated dose) are shown in Fig. 4. KW-2170 could be detected in plasma up to 24 h after dosing (1.28 ng/ml). The concentrations of both metabolites (M1 and M2) were lower than that of KW-2170. In the present study, we took samples only up to 24 h,

Table 4
Stability of KW-2170, M1 and M2 in dog blood and plasma

Conditions	Added conc. (ng/ml)	Remaining ratio (%)[a]		
		KW-2170	M1	M2
In blood				
Standing on ice for 2 h	1	87.8	106	109
	20	106	108	103
In plasma				
Standing at RT for 1 h	0.2	98.5	106	110
	4	111	105	99.0
Freeze–thawing 2 cycles	0.2	101	104	88.6
	4	90.2	97.2	94.9
Storage at −80 °C for 1 week	0.2	100	97.7	96.5
	4	102	102	99.4

[a] Mean ($n=2$).

however, it is predicted that plasma concentration of KW-2170 could be determined 168 h after dosing by extrapolation. A detailed pharmacokinetics study in dogs is planned using this HPLC–ED method.

The HPLC–ED method has been validated for the determination of KW-2170 and its metabolites (M1 and M2) in human and dog plasma and proved to be suitable for the investigation of its pharmacokinetics in humans and dogs. Phase I studies are ongoing in Japan and the US and we have been able to successfully monitor the plasma concentrations of these compounds in clinical samples [3,4].

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