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Simultaneous determination of the peptide-mitomycin KW-2149 and its metabolites in plasma by high-performance liquid chromatography

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Abstract

A gradient high-performance liquid chromatographic (HPLC) method is described for the quantification of KW-2149 and its two major metabolites in plasma. The method involves a sample clean-up by solid-phase extraction on C_{18} columns, separation of the respective compounds by HPLC on a YMC ODS-AQ column (5-μm particle size, 150×6 mm I.D.), using a methanol–water gradient system as an eluent, and measurement by UV absorbance detection at 375 nm. The limits of quantitation were 10 ng/ml for KW-2149 and M-16, and 15 ng/ml for M-18. Recoveries from plasma were higher than 92% on C_{18} extraction columns. Intra-day precision, expressed as %C.V., was between 1.4 and 6.5%. Intra-day accuracy ranged from 94 to 107%. Precision and accuracy of variability of inter-assays increased somewhat; however, were still within acceptable ranges. The ability of the method to quantify KW-2149 and two major metabolites simultaneously, with precision, accuracy and sensitivity, make it useful in monitoring the fate of this new mitomycin in cancer patients. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Peptide-mitomycin; KW-2149

1. Introduction

Mitomycin C (MMC) is a valuable antitumor antibiotic in the treatment of gastrointestinal, breast and lung carcinomas, and it is also used in the intravesical therapy of superficial transitional cell carcinoma of the bladder [1,2]. However, its clinical usefulness has been limited by side effects, particularly, severe bone marrow suppression. New analogues of MMC have therefore been investigated for many years to develop a compound with a broader antitumor spectrum but less myelosuppressive than MMC [3–5].

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KW-2149 (7-*N*-[2-(γ-L-glutamylamino)ethyldithioethyl] mitomycin C) (**I**) is a new water-soluble MMC analogue [6] currently undergoing clinical trials in Europe [7]. It has a broad spectrum of antitumor activity similar to MMC against a range of experimental murine tumors and human tumor xenografts in vivo, but is less myelosuppressive [8] and lacks cross resistance against MMC-resistant tumors in vitro and in vivo [9–12]. In vitro studies have demonstrated that KW-2149 is 10–100 times more cytotoxic than MMC on a molar basis to 23 human cancer cell lines [10]. These data confirm the activity of KW-2149 as an agent with equal or superior activity to MMC. The activities of KW-2149 and its metabolites, M-16 (**II**) and M-18 (**III**), have been compared using cell lines with different types of resistance [12]. M-18 was significantly the effective to the parent compound against the human ovarian carcinoma cell lines used.

An HPLC method has been developed for the determination of KW-2149 based on an earlier technique for MMC [13]. However, the simultaneous determination of KW-2149 and its main metabolites is impeded by the large differences in hydrophobicity in the analytes (Fig. 1). KW-2149 is a MMC analogue with a disulfide containing side-chain at C-7; M-16 and M-18 are the methyl sulfide form and the symmetrical disulfide dimer, respectively. This study describes the methodology and validation of an HPLC assay for the simultaneous determination of KW-2149, M-16 and M-18 in human plasma. Following sample preparation by solid-phase extraction, a gradient mobile phase was used to separate KW-2149 from its metabolites and reduce the analysis time.

2. Experimental

2.1. Materials and reagents

KW-2149, M-16 and M-18 were kind gifts from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). Methanol was HPLC grade and obtained from Acros Organics (Geel, Belgium). All water used in the analyses was purified with a Vel HQ-5 purification system (Leuven, Belgium). Stock solutions of standards were prepared by dissolving the standard compounds in a small volume of methanol, then adjusted to a concentration of 100 μg/ml with a 60:40 (v/v) mixture of methanol and water and stored at 4°C. A range of working standard solutions were prepared freshly as required by diluting stock solutions. Alltech (Deerfield, IL, USA) extraction

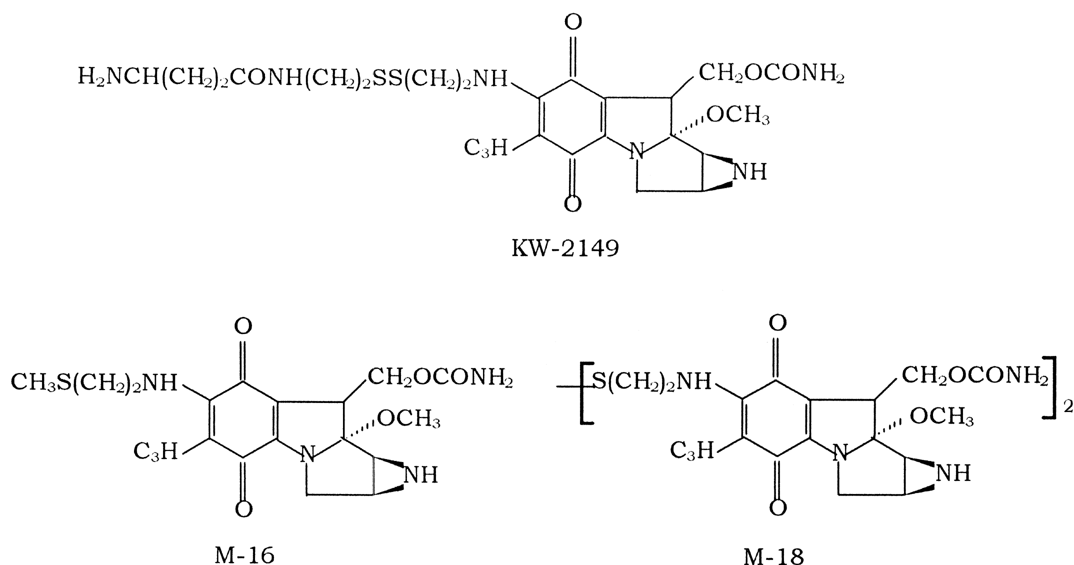

Fig. 1. Structures of KW-2149, M-16 and M-18.

cartridges packed with 500 mg of C_{18} sorbent, with a 1-ml capacity, were used in the sample clean-up procedure. XAD-2 solid-phase extraction columns were prepared by filling empty cartridges with 500 mg of Amberlite XAD-2 of particle size of 100–200 μm (Brunschwig Chemie, B.V. Amsterdam, The Netherlands).

2.2. Instrumentation

A modular system from Shimadzu (Kyoto, Japan) was used for the HPLC analysis. The system consisted of two LC-6A liquid chromatograph pumps, a SPD-6AV variable-wavelength UV–Vis detector, a SiL-6B automatic injector, a SCL-6B system controller and a C-R6A chromatopac integrator. Separations were carried out on a YMC-Pack ODS-AQ analytical column (150×6 mm I.D., YMC Co., Kyoto Japan) connected to a guard column (15×3.2 mm I.D., 5 μm) and the system was operated at ambient temperature. To prepare the mobile phase, 50 m*M* potassium dihydrogen phosphate buffer, adjusted to pH 6.5 with 1 *M* sodium hydroxide, was used. A two-solvent gradient was employed to separate KW-2149 and its metabolites. Eluens A and B consisted of methanol–buffer of 58:42 (v/v) and 67:33 (v/v), respectively. The column was equilibrated with eluent A, and after injection of 50 μl of sample, a linear gradient was started. Gradient conditions are shown in Table 1. The time between injections was 25 min. All solvents were sonicated for 15 min to remove air bubbles and dissolved air. The flow-rate of the mobile phase was 1.0 ml/min, and the eluent was monitored at a wavelength of 375 nm.

2.3. Sample processing

After thawing at room temperature, the plasma sample was vortex-mixed for 10 s and centrifuged for 5 min at 2000 *g* to remove fibrin. A 500-μl aliquot of supernatant was pipetted into the extraction column, which was preconditioned by flushing four times with 1 ml of methanol followed by 4 ml of distilled water with an approximate flow-rate of 2.5 ml/min. Solid-phase extraction was performed on a vacuum manifold system holding 20 extraction cartridges. The column was protected against drying-out before applying the plasma samples. The sample aliquot was passed through the bed at a flow-rate of 1 ml/min, under a slight vacuum. The columns then were flushed five times with 1 ml of water, and dried out by increasing the vacuum. Adsorbed compounds were eluted from the column using four consecutive washings of 1 ml of methanol, taking care to break the vacuum between each of these four elutions. The combined eluates were evaporated to dryness in a vacuum centrifuge (approximately 4–5 h for 20 samples). The residue was reconstituted with 0.5 ml of a 60:40 (v/v) mixture of methanol and water. After centrifugation at 2000 *g* for 5 min, 100 μl of supernatant was transferred into a vial placed on the sample rack of an auto-sampler (operating at room temperature) and 50 μl was injected into the HPLC system.

Table 1
Gradient program

Time (min)	Flow (ml/min)	Percent A	Percent B
0.0	1.0	100	0
15.0	1.0	0	100
16.0	1.0	100	0
25.0	1.0	100	0

2.4. Validation

To assess linearity, drug-free plasma samples were mixed with serial dilution samples of stock solutions to prepare the six nominal concentration ranges of 10–1000 ng/ml for KW-2149, M-16 and 15–1000 ng/ml for M-18. For replicas, stock solutions used for calibration were prepared from different weighing and a weighting factor of $1/x$ was used for regression. Calibration curves were constructed by plotting peak area ratios of each analyte against concentration. Reproducibility was determined by analyzing three aliquots of each spiked plasma sample on five different days. Concentrations in clinical and quality control samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve.

The inter-day and intra-day reproducibility of the assay were assessed by performing replicate plasma analyses of all three compounds in quality control samples of four different concentrations. The procedure was repeated on three different days to

determine inter-day repeatability, and intra-day repeatability was assessed by treating spiked samples in six replicas on the same day. The accuracy was evaluated as percent error [(mean of measured-mean of added)/mean of added] × 100, while the precision was given by the inter-day and intra-day coefficients of variation (C.V.). A precision (C.V. ≤ 15%) and an accuracy (relative error ≤ 15%) are considered acceptable.

The limit of quantitation was defined as the lowest concentration of analyte determined with acceptable precision and accuracy under the stated experimental conditions. It corresponds to sample concentrations of KW-2149, M-16 and M-18 resulting in peak areas of ten times the noise level (S/N). This determination was performed by repeated analysis of spiked plasma samples ($n = 10$).

To establish the recovery of KW-2149, M-16 and M-18 after sample extraction, drug-free plasma was spiked with all three analytes to give final concentrations of 50, 400 and 1000 ng/ml, respectively, and the assay procedure was performed as previously described. The extraction efficiency (recovery) was determined by comparing peak areas from drug-free plasma spiked with known amounts of drugs vs. peak areas of the same concentrations prepared in the mobile phase and injected directly onto the analytical column. Each sample was determined in triplicate.

To evaluate the specificity of the method, plasma was spiked separately with KW-2149, M-16 or M-18 and subjected to the assay procedure. The retention times of endogenous substances in plasma were compared with those of the compounds of interest. The specificity of the method was assessed to evaluate the influence of the matrix from different plasma samples originating from healthy volunteers and patients.

2.5. Stability study

To study the stability in the biological matrix, control plasma samples were spiked with KW-2149 and the two metabolites separately at different concentrations and treated with oxidized glutathione. The short-term stability was assessed after storage under both normal laboratory conditions (20°C) and at 4°C. The long-term stability in frozen plasma (−20°C) was determined by periodic analysis over 1 month; prior to analysis, samples were brought to room temperature and vortex-mixed. Stability was evaluated by comparing peak areas from initial and subsequent determinations. In addition the spiked plasma samples were subjected to three freeze–thaw cycles, after which analysis was performed and peak area was compared to the mean response, obtained from non-frozen samples analyzed immediately after preparation. The stability of all analytes in the injection solutions within the autosampler was determined by periodically injecting replica preparations of processed samples at various time intervals.

2.6. Clinical study

The analytical method described was used to determine the plasma concentrations of KW-2149 and its metabolites in a patient after a continuous infusion of 60 mg/m^2 of KW-2149. Venous blood samples were collected at frequent intervals for 8 h, and immediately treated with oxidized glutathione (GSSG) to prevent the opening of the disulfide bridge of KW-2149 and M-18. The presence of oxidized glutathione or gluthathione (GSSG and GSH) in the samples does not cause interfering peaks during the chromatographic analysis of KW-2149 and its metabolites. Blood samples were cen-

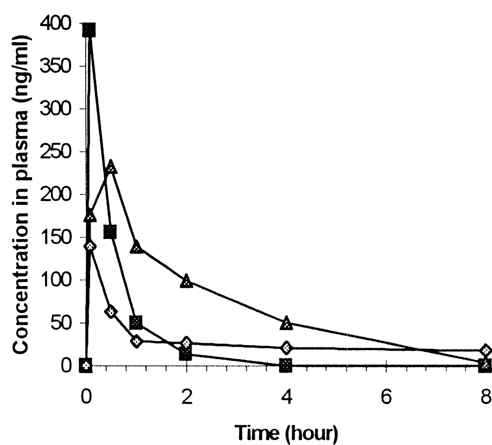

Fig. 2. Concentration–time curves for KW-2149 (♦), M-16 (■) and M-18 (▲) in a patient plasma following an infusion of KW-2149.

trifuged for 5 min and the plasma frozen at −20°C until HPLC analysis. The plasma concentration–time profiles of analytes are presented in Fig. 2. For pharmacokinetic studies, the area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal rule. Maximum concentration and the time to reach maximum concentration are the real time values.

3. Results and discussion

Typical chromatograms obtained from plasma spiked with standard solutions, and patient plasma samples before and after an infusion of KW-2149, are shown in Fig. 3A–C. The analysis of random plasma samples ($n>6$) showed that there was no interference from endogenous substances at the retention times of KW-2149 and its metabolites, and mutual interference between KW-2149 and its metabolites was not detected. However, the plasma obtained from the hospital blood bank exhibited more endogenous noise compared to that observed with volunteer plasma. Chromatographic separation was performed favorably within 15 min with appropriate development of the gradient mobile phase. Retention times were 5.7, 8.6 and 14.8 min for KW-2149, M-16 and M-18, respectively.

A weighted linear regression of the peak area ratios versus standard concentrations was performed for the three analytes using a weight of 1/[concentration]. The observed peak area ratios were linear over the concentration range of 10–1000 ng/ml for KW-2149 and M-16, and 15–1000 ng/ml for M-18 in human plasma. The average results of intra- and inter-day assays of standard curves showed good reproducible linearity. The values of r^2 ($\geq$0.995) and consistency in slope value (Table 2) demonstrate that the standard curve is reliable over the standard concentration range.

The values obtained during the three-day validation for precision and accuracy are summarized in Table 3. The method proved to be accurate and precise. For all three analytes, average accuracy at five concentrations ranged from 94 to 107%; intra-day precision ranged from 1.4 to 6.5%. Some additional variation was observed as a result of performing the assay on different days (inter-day precision ranged from 3.9 to 7.4%).

The extraction capacities of two different solid adsorbent materials (XAD-2 and C_{18}) for KW-2149, M-16 and M-18 from plasma, were compared using methanol as elution solvent (Table 4). The selectivity of the adsorbents was evaluated by comparing chromatograms of plasma samples under the same conditions. Low and inconsistent recoveries were obtained with XAD-2. In addition, the recovery with the XAD-2 columns, was significantly affected by changing the spiked concentration. The minimum recovery was found at the minimum concentration, and the recovery increased with the concentration. Taking into account adsorbent selectivity and analyte recovery, the C_{18} extraction column was finally selected and used through the whole analytical procedure.

The limit of quantitation based on an acceptable accuracy and precision was 10 ng/ml in plasma for KW-2149 and M-16 and 15 ng/ml for M-18, deviating less than ±8% from the respective nominal values. The estimated precision for the limit of quantitation was within 7.5%. Lower concentrations of the compounds could be detected (100 pg/ml–10 ng/ml); however, C.V. and relative error increased subsequently beyond 15%. Reduction of the I.D. of the column from I.D. 6.0 mm down to 1.0 mm resulted in lowering of the LLOQ for spiked plasma samples. Unfortunately, handling of patient samples then appeared to be troublesome with reduced I.D. columns with respect to peak shape of KW-2149 and M-18.

Stock solutions of KW-2149 and its two metabolites were stable for at least one month, without detectable decomposition. After storage at 20°C, the different concentration decreasing of KW-2149 and its metabolites was observed for KW-2149, M-16 and M-18 at the concentration of 100 ng/ml in plasma within 6 h. At 4°C degradation was not observed. KW-2149 and its metabolites were stable for 1 month at −20°C, and compared to the reference values there was no statistical difference. At least three freeze-thaw cycles can be tolerated for plasma samples without losses higher than 15%. Indeed, after the third cycle, losses of less than 10% were observed. These values are summarized in Table 5. At room temperature, KW-2149 and its two

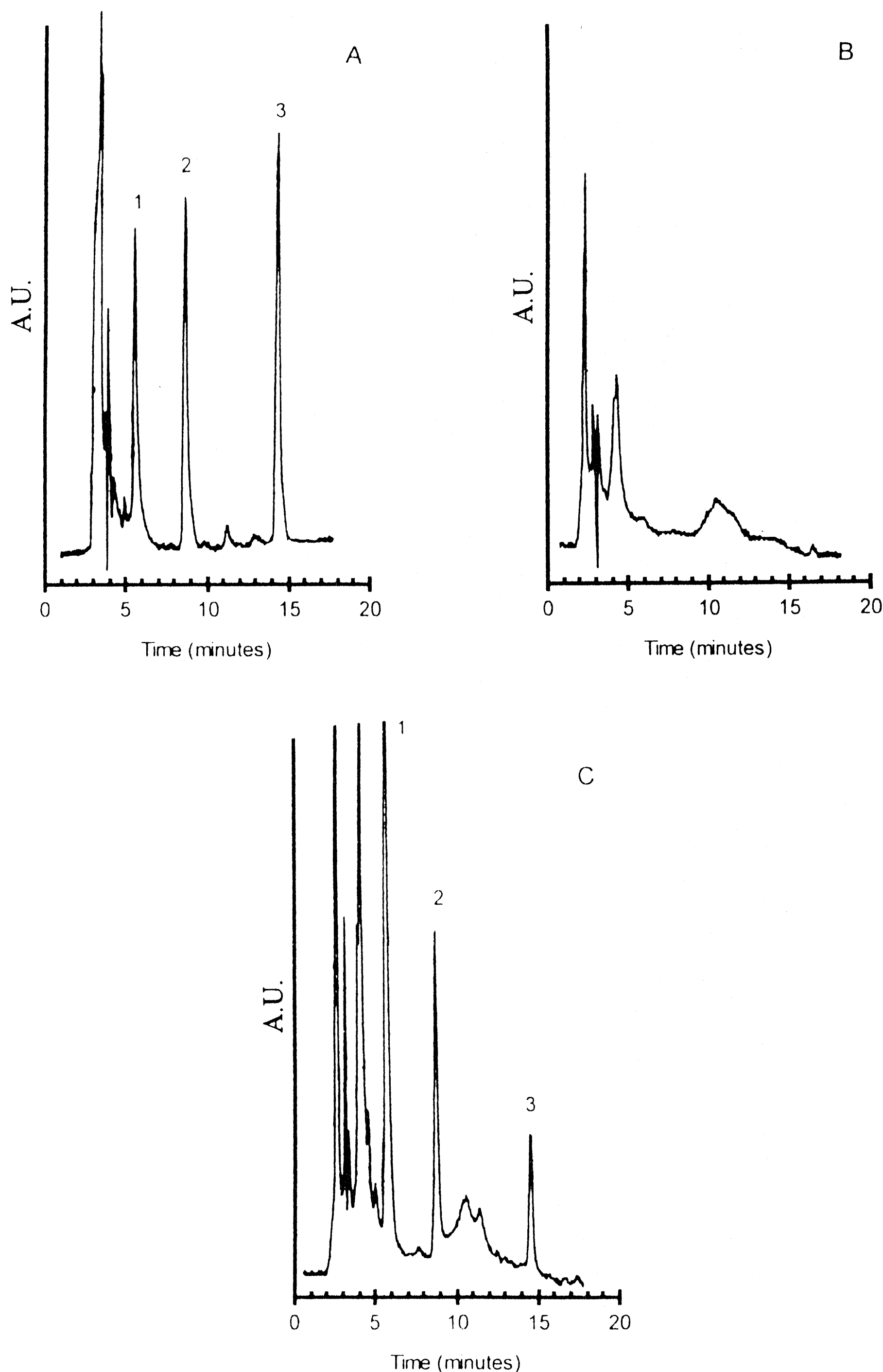

Fig. 3. HPLC chromatograms for (A) drug free plasma spiked with a standard mixture, (B) plasma sample collected from a patient before drug administration and (C) patient plasma sample (5 min) after an infusion of KW-2149 (peaks corresponding 390 ng/ml of KW-2149, 175 ng/ml of M-16 and 150 ng/ml of M-18). Peaks: (1)=KW-2149, (2)=M-16, (3)=M-18. Chromatographic conditions: column: YMC ODS-AQ (150×6.0 mm I.D., 5 μm), mobile phase: 58% methanol followed by linear gradient to 67% methanol within 15 min. The chromatograms are shown with a full scale of 8 mV.

Table 2
Calibration curves for the HPLC assay of KW-2149, M-16 and M-18[a]

Compound	Linear regression equation ($y = a + bx$) Slope, b (S.D.)[b]	Intercept, a (SD)	Correlation, r^2
I	117.9 (2.6)	−26.9 (3.9)	1
II	158.7 (1.8)	−89.5 (2.3)	0.998
III	163.5 (4.2)	56.9 (5.2)	0.997

[a] Results based on peak area with detector: gain = ×1, attenuation = 3.
[b] Standard deviation ($n = 5$).

Table 3
Intra- and inter-assay variability and precision of KW-2149, M-16 and M-18 in spiked human plasma

Compound	Concentration (ng/ml)		C.V. (%)	Relative error (%)
	Added	Analyzed (mean±SD)		
Intra-assay				
KW-2149	10[a]	9.7±0.6	6.2	−3.0
	50	49.3±2.9	5.9	−1.4
	200	208.0±10.2	4.9	4.0
	400	399.0±10.6	2.7	−0.3
	1000	1006.0±14.1	1.4	0.6
M-16	15	15.0±0.7	4.7	0.0
	50	48.0±3.0	6.3	−4
	200	198.0±12.6	6.4	−1.0
	400	400.0±16.5	4.1	0
	1000	996.8±30.9	3.1	−0.3
M-18	15	16.0±1.0	6.3	6.7
	50	49.0±3.1	6.3	−2.0
	200	206.0±13.3	6.5	3.0
	400	394.0±22.2	5.6	−1.5
	1000	1025.0±21.0	2.1	2.5
Inter-assay				
KW-2149	10	9.8±0.7	7.1	−2.0
	50	51.2±3.8	7.4	2.4
	200	191.0±13.2	6.9	−4.5
	400	398.0±27.5	6.9	−0.5
	1000	983.0±42.5	4.3	−1.7
M-16	15	16.0±0.8	5.0	6.7
	50	47.0±3.5	7.5	−6
	200	194.0±13.2	6.8	−3.0
	400	389.0±19.5	5.0	−2.8
	1000	977.0±38.1	3.9	−2.3
M-18	15	16.0±0.7	4.4	6.7
	50	53.0±3.9	7.4	6
	200	210.0±15.0	7.1	5
	400	380.0±26.3	6.9	−5.0
	1000	1034.0±42.7	4.1	3.4

[a] LLOQ.

Table 4
Extraction efficiencies of KW-2149, M-16 and M-18 from human plasma

Compound	Concentration (ng/ml)	Recovery (mean±S.D.) (%)	
		XAD-2	C_{18}
KW-2149	50	82±11.0	92±5.8
	400	87±12.0	94±3.6
	1000	91±3.5	98±2.3
M-16	50	68±7.1	94±5.7
	400	71±5.6	97±3.3
	1000	83±2.7	102±2.4
M-18	50	58±9.5	93±8.5
	400	68±7.8	95±7.0
	1000	95±3.1	96±5.1

metabolites were stable in the injection solution, at two concentrations, and the percentage recovery ranged from 92 to 95% during the course of this stability experiment (6 h).

The validated method for plasma was used to study the pharmacokinetics of KW-2149, M-16 and M-18 in one patient following an infusion of 60 mg/m^2 of KW-2149 (submitted for publication). The plasma concentration–time profile over a period of 0–8 h is presented in Fig. 2. The lag times for detectability of all three analytes was 5 min. Plasma concentrations of KW-2149 were measurable for 2 h; those of M-16 and M-18 for 8 h. A maximum plasma concentration (C_{max}) of KW-2149 of 390 ng/ml was reached 5 min post-dose, and the $AUC_{0-\infty}$ was 230 ng h/ml. The maximum plasma concentrations of M-16 and M-18 were 232 ng/ml and 140 ng/ml after 5 min and 0.5 h post-dose, respectively, with $AUC_{0-\infty}$ values of 569 ng h/ml and 263 ng h/ml.

4. Conclusions

This study describes the determination of KW-2149 and its major active metabolites, M-16 and M-18, in human plasma using solid-phase extraction and reversed-phase HPLC. The method has a high selectivity, reproducibility and accuracy. With limits of quantification of 10 ng/ml for KW-2149 and M-16 and 15 ng/ml for M-18, KW-2149 and its metabolite concentrations in human plasma can be analysed. By using a gradient mobile phase system, the total run time is reduced to 25 min for each analysis. The application of the method has been successfully demonstrated by the measurement of plasma concentrations in a patient following the administration of KW-2149 by continuous infusion.

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Table 5
Stability of KW-2149 and its metabolites in human plasma

Compound	Recovery (mean±SD) (%) (n=3)			
	25°C (6 h)	4°C (6 h)	−20°C (1 month)	Freeze-thaw cycles
KW-2149	80.0±7.3	95.4±6.3	98.0±7.9	92.5±2.4
M-16	84.2±3.6	96.9±2.6	96.0±6.3	101.5±9.4
M-18	70.4±5.8	93.0±5.4	94.5±7.8	90.5±8.2

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