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Development of a highly sensitive high-performance liquid chromatographic method for measuring an anticancer drug, UCN-01, in human plasma or urine

Noriaki Kurata, Tomoko Kuramitsu, Hiromi Tanii, Eiichi Fuse, Takashi Kuwabara*, Hiroyuki Kobayashi, Satoshi Kobayashi

Drug Development Research Laboratories, Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka-ken 411, Japan

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Abstract

We have established a highly sensitive high-performance liquid chromatographic method for the determination of an anticancer drug, UCN-01, in human plasma or urine. Using a fluorescence detector set at an excitation wavelength of 310 nm and emission monitored at 410 nm, there was a good linearity for UCN-01 in human plasma ($r=0.999$) or urine ($r=0.999$) at concentrations ranging from 0.2 to 100 ng/ml or 1 to 400 ng/ml, respectively. For intra-day assay, in plasma samples, the precision and accuracy were 1.8% to 5.6% and −10.0% to 5.2%, respectively. For inter-day assay, the precision and accuracy were 2.0% to 18.2% and 2.4% to 10.0%, respectively. In urine samples, the intra- and inter-day precision and accuracy were within 3.9% and ±2.7%, respectively. The lower limit of quantification (LLOQ) was set at 0.2 ng/ml in plasma and 1 ng/ml in urine. UCN-01 in plasma samples was stable up to two weeks at −80°C and also up to four weeks in urine samples. This method could be very useful for studying the human pharmacokinetics of UCN-01. © 1998 Elsevier Science B.V.

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1. Introduction

UCN-01 (7-hydroxystaurosporine, KW-2401, Fig. 1) is being developing as an anticancer drug with potent and selective inhibition of protein kinase C [1,2]. Recently, several researchers have reported that UCN-01 induces preferential G(1)-phase accumulation in in vitro human cell lines [2–6]. In order to clarify the pharmacokinetics of UCN-01, a high-performance liquid chromatographic (HPLC) method for the determination of UCN-01 is needed. In this paper, we have developed a highly sensitive HPLC method for the determination of UCN-01 in human plasma or urine samples, using a fluorescence detector.

*Corresponding author.

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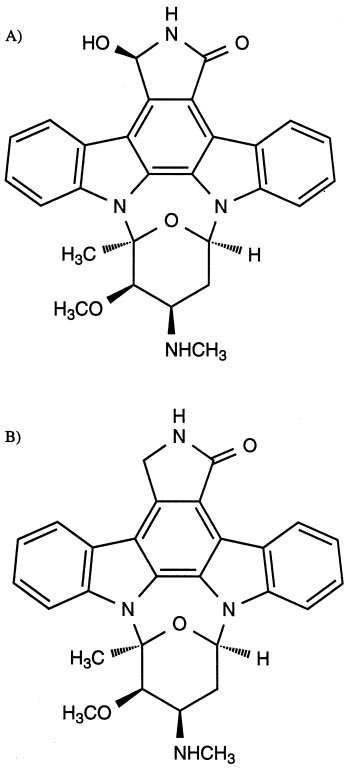

Fig. 1. Chemical structures of (A) UCN-01 and (B) staurosporine (as I.S.).

2. Experimental

2.1. Chemicals and reagents

UCN-01 and staurosporine as internal standard (I.S., Fig. 1) were produced by the fermentation technique in our laboratories as described previously [7]. Triethylamine and polyoxyethylene (20) sorbitan monolaurate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile was HPLC grade from Kanto (Tokyo, Japan). Human plasma and urine were obtained from healthy volunteers in our laboratories with their informed consent. In order to avoid adsorption of UCN-01 to any containing vessels, 0.5% polyoxyethylene (20) sorbitan monolaurate was added to human urine. UCN-01 and I.S. were separately dissolved at concentrations of 1 mg/ml in dimethyl sulfoxide (Kanto chemical) and stored at 4°C. UCN-01 stock solution was diluted to 2 μg/ml for plasma and 20 μg/ml for urine with acetonitrile and then this acetonitrile solution was diluted with plasma or urine containing 0.5% polyoxyethylene (20) sorbitan monolaurate to give designated concentrations before use. I.S. stock solution was diluted to 10 ng/ml for plasma and 50 ng/ml for urine using acetonitrile.

2.2. Sample preparation

Under natural light, UCN-01 was chemically unstable in buffered solution [8]. As a precaution, plasma and, especially, urine samples were handled in the dark as much as possible after deproteinization.

Fifty μl of plasma in a polypropylene microtube was deproteinized by adding I.S. solution in ice-cold acetonitrile (100 μl). The solution was mixed for 30 s, and then stood on ice for 10 min protected from light. After centrifugation (10 000 *g*, 10 min, 4°C), 20 μl of supernatant was injected onto the HPLC system. In order to avoid adsorption of UCN-01 in urine to the containing vessel, surfactant was added to the pooled urine before sample preparation. Twenty μl urine in a polypropylene microtube was mixed with I.S. solution in ice-cold acetonitrile (180 μl). After mixing, the mixture was centrifuged and 20 μl of supernatant was injected onto the HPLC system. The HPLC samples for plasma and urine were kept below 10°C before injection.

2.3. HPLC equipment

The HPLC system consisted of Shimadzu LC Workstation software, CLASS-LC10/M10A, (Shimadzu, Kyoto, Japan), a Shimadzu LC-6A or a Hitachi 638-30 (Hitachi, Tokyo, Japan) pump, a HITACHI L-7200 or a 638-08 autosampler (Hitachi) and a Jusco FP-920 fluorescence detector (Jusco, Tokyo, Japan). The fluorescence detector was set at an excitation wavelength of 310 nm and emission monitored at 410 nm. The pre-column used was a Guard-Pak Puresil C_{18} (Nihon Millipore, Yonezawa, Japan) and the analytical column used was an AM-312 ODS column (150×6.0 mm I.D., S-5mm, 120 Å) (YMC, Kyoto, Japan). The column was used at room temperature.

2.4. Separation

For the sample assay, an isocratic mobile phase was used, acetonitrile–0.1% triethylamine in 0.05 *M* phosphate buffer (pH 7.3) (50:50, v/v). We chose the flow-rate of 1 ml/min in order to separate the UCN-01 peak from other peaks arising from biological samples. The retention times of UCN-01 and I.S. were 11 min and 17 min in both plasma and urine samples.

2.5. Quantification

To confirm the specificity of this HPLC method, the plasma or urine samples obtained from five or six individuals were treated as described above and HPLC chromatograms were compared. The range of calibration curves was 0.2 ng/ml to 100 ng/ml for plasma or 1 ng/ml to 400 ng/ml for urine, respectively. Calibration curves were obtained by plotting the UCN-01 concentrations and the ratios of the UCN-01 peak height to internal standard peak height (I.S. ratios) using a linear least squares method with a weight of $1/(\text{I.S. ratio})^2$. The studies of the precision and accuracy for intra-day assay were conducted at concentrations of 0.2, 5 and 100 ng/ml in plasma ($n=5$), and at concentrations of 1, 3, 60 and 300 ng/ml in urine ($n=5$). The studies of the precision and accuracy for inter-day assay were conducted over three days at concentrations of 0.2, 5 and 100 ng/ml in plasma, and at concentrations of 3, 60 and 300 ng/ml in urine, respectively. We have conducted the inter-day assay validation for urine at three times the low limit of quantitation (LLOQ) according to the criteria of Shah et al. [9], with one concentration near the LLOQ. However, the precision and accuracy in plasma are very important from the standpoint of practical use of the method. Phase I studies of anticancer drugs are conducted in cancer patients. The starting clinical dose is low and the plasma concentrations are predicted to be low at the dose and the monitoring of plasma concentrations of the tested drugs is considered to determine the dose escalation. Therefore, the inter-day validation at the LLOQ in plasma was studied to confirm accurately the confidence of the assay procedure of UCN-01.

The stability of UCN-01 at −80°C was checked at concentrations of 0.5 ng/ml and 10 ng/ml in plasma, and 60 ng/ml and 300 ng/ml in urine. Furthermore, the effects of freezing–thawing on the stability were examined at concentrations of 0.5 ng/ml and 10 ng/ml in plasma, and 3 ng/ml and 60 ng/ml in urine, respectively, after two freeze–thaw cycles. These cycles were enough for the estimation of the UCN-01 concentration because we reanalyze the sample only once if necessary.

3. Results and discussion

We have developed an HPLC method for the determination of UCN-01 concentrations in human plasma or urine, using a fluorescence detector. In order to minimize tailing of UCN-01, triethylamine was added to the isocratic mobile phase. The samples were deproteinized by adding ice-cold acetonitrile. Figs. 2 and 3 represent typical chromatograms of plasma and urine, respectively, and show that there were no interfering peaks in another five individual plasma blanks and six individual urine blanks (data not shown). UCN-01 eluted with a retention time of about 11 min in both plasma and urine samples. The calibration curve for plasma ranged from 0.2 ng/ml to 100 ng/ml and was linear ($y=0.0620x-0.0005$, $r=0.999$) with confidence intervals of $0.0598<\text{slope}<0.0642$ and $-0.1012<y\text{-intercept}<0.1002$. The precision and accuracy for intra- or inter-day assay are presented in Table 1. For intra-day assay, the precision and accuracy at concentration of 0.2 ng/ml in plasma were 5.6% and

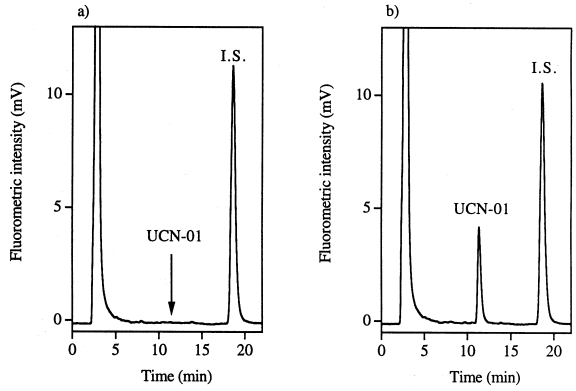

Fig. 2. Chromatograms of (a) human plasma blank and (b) human plasma spiked with 5 ng/ml UCN-01.

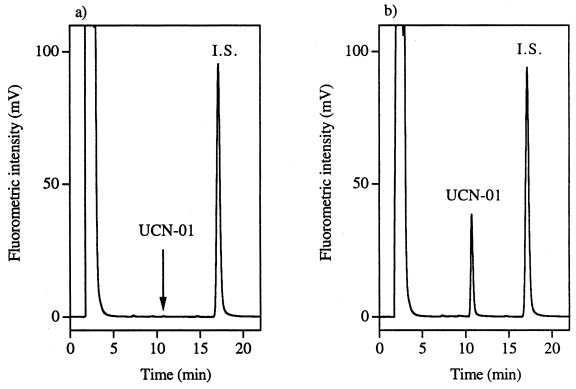

Fig. 3. Chromatograms of (a) human urine blank and (b) human urine spiked with 100 ng/ml UCN-01.

−10.0%, respectively, therefore the LLOQ in plasma was 0.2 ng/ml. The precision and accuracy at other concentrations were appropriate for the assay of UCN-01. For inter-day assay, variations in the precision and accuracy were within 18.2% and ±10%, respectively.

For urine samples, after the addition of 0.5% polyoxyethylene (20) sorbitan monolaurate to urine to prevent adsorption to the containing vessels, the concentrations of UCN-01 were determined. The calibration curve for urine ranged from 1 ng/ml to 400 ng/ml and was linear ($y=0.00389x+0.00129$, $r=0.999$) with confidence intervals of $0.00385<$ slope<0.00394 and $-0.00679<y$-intercept$<$

Table 1
Precision and accuracy of UCN-01 in human plasma

Concentration added (ng/ml)	Concentration found (ng/ml)	%R.S.D. (%)	Accuracy (%)
Intra-day assay[a]			
0.2	0.18±0.01	5.6	−10.0
5	4.91±0.09	1.8	−1.8
100	105±5	4.7	5.2
Inter-day assay[b]			
0.2	0.22±0.04	18.2	10.0
5	5.12±0.20	3.9	2.4
100	106±2	2.0	6.4

%R.S.D. represents the percent relative standard deviation.
[a] Mean±S.D. ($n=5$).
[b] Mean±S.D. ($n=3$).

Table 2
Precision and accuracy of UCN-01 in human urine

Concentration added (ng/ml)	Concentration found (ng/ml)	%R.S.D. (%)	Accuracy (%)
Intra-day assay[a]			
1	1.00±0.07	7.0	0.0
3	2.95±0.08	2.7	−1.7
60	61.5±0.6	1.0	2.5
300	293±2	0.7	−2.3
Inter-day assay[b]			
3	2.92±0.07	2.4	−2.7
60	59.5±2.3	3.9	−0.8
300	293±2	0.7	−2.3

%R.S.D. represents the percent relative standard deviation.
[a] Mean±S.D. ($n=5$).
[b] Mean±S.D. ($n=3$).

0.00937. The precision and accuracy for intra- or inter-day assay are presented in Table 2. The results of the intra- and inter-day assay showed that the LLOQ in urine was 1 ng/ml and there was good precision and accuracy in urine at other concentrations.

The results of the stability of UCN-01 in plasma or urine stored at −80°C showed that the percentages of UCN-01 remaining were more than 91.0%. Moreover, after two freeze–thaw cycles, UCN-01 was stable in both plasma and urine (Table 3).

In conclusion, this HPLC method for measuring UCN-01 in human plasma and urine is highly sensitive, i.e., the LLOQ was 0.2 ng/ml in plasma and 1 ng/ml in urine. The stability of UCN-01 in plasma and urine was also good. This method has been validated for the quantification of UCN-01 in human plasma and urine and will be very useful for studying the human pharmacokinetics of UCN-01. Phase I studies of UCN-01 are ongoing in Japan and the US and we can successfully monitor plasma or urine concentrations of UCN-01.

Table 3
Stability of UCN-01 in human plasma and urine

	Percentage remaining
Stored at −80°C	
in plasma for two weeks (n=3)	
0.5 ng/ml	94.3±0.9
10 ng/ml	91.0±2.5
in urine for four weeks (n=2)	
3 ng/ml	100
60 ng/ml	100
300 ng/ml	101
After two freeze–thaw cycles	
in plasma (n=3)	
0.5 ng/ml	96.7±3.3
10 ng/ml	94.2±5.3
in urine (n=2)	
3 ng/ml	90.2
60 ng/ml	95.5

Each value represents the mean±S.D. or mean.

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