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Development of a simplified, sensitive high-performance liquid chromatographic method using fluorescence detection to determine the concentration of UCN-01 in human plasma

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Abstract

UCN-01 is a naturally derived anticancer agent isolated in the culture broth of actinomyces streptomyces. We have developed a sensitive high-performance liquid chromatographic method for the determination of UCN-01 in human plasma. UCN-01 was isolated from human plasma after intravenous administration, by using 100% ice-cold acetonitrile liquid–liquid phase extraction. Liquid chromatographic separation was achieved by isocratic elution on a phenyl analytical column. The mobile phase consisted of acetonitrile–0.5 *M* ammonium acetate (45:55) with 0.2% triethylamine added as a modifier. The UCN-01 peak was identified from other peaks using fluorescence excitation energy and emission energy wavelengths of 310 and 410 nm, respectively. Retention time for UCN-01 was 4.2±0.5 min. The UCN-01 peak was baseline resolved, with nearest peak at 2.6 min distance. No interfering peaks were observed at the retention time of UCN-01. Peak area amounts from extracted samples were proportional over the dynamic concentration range used: 0.2 to 30 μg/ml. Mean recoveries of UCN-01 at concentrations of 0.5 and 25 μg/ml were 89 and 90.2%, respectively. Relative standard deviations for UCN-01 calibration standards ranged from 1.89 to 2.31%, with relative errors ranging from 0.3 to 11.6%. Assay precision for UCN-01 based on quality control samples of 0.50 μg/ml was ±4.86% with an accuracy of ±5.7%. For drug extracted from plasma the lowest limit of detection was 0.1 μg/ml, with the lowest limit of quantitation being 0.2 μg/ml. This method is suitable for routine analysis of UCN-01 in human plasma at concentration from 0.2 to 30 μg/ml.

Keywords: UCN-01

1. Introduction

UCN-01, 7-hydroxystaurosporine, is a potent anticancer agent isolated in the culture broth of actinomyces streptomyces [1] (Fig. 1). In numerous in vitro and in vivo studies UCN-01 has demonstrated antitumor activity in a variety of tumor cell lines representing both solid and hematological malignan-

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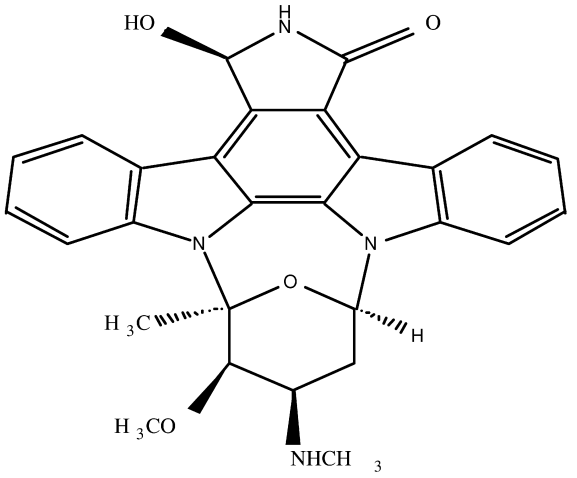

Fig. 1. Chemical structure of UCN-01 (7-hydroxystaurosporine) (M_r=483).

cies [2–6]. The mechanism of action was originally postulated to be potent, selective Ca^{2+}-dependent inhibition of protein kinase C. UCN-01 has inhibitory activity at multiple points along the protein kinase C (PKC) cell signaling pathway such as inhibition of cyclin dependent kinase (CDK) 2, decreased expression of cyclin A and D_1, induction of p21 and inducing the dephosphorylation of pRB that all contribute to cell cycle arrest in G_1 phase [7,8]. As a single agent UCN-01 has had promising antitumor activity, however it may have more benefit in combination therapy.

For instance, several studies have suggested that the combination of cisplatin and UCN-01 demonstrates enhanced cytotoxicity compared to cisplatin alone [9,10]. The mechanism of this augmentation is not clear, but suggests that after cisplatin induced DNA damage, UCN-01 mediated abrogation of the G_2 cell check point shortens the DNA repair time leading to enhanced cell kill [9–12]. Preclinical studies of UCN-01 in combination with camptothecin derivatives, etoposide, and 5-fluorouracil have demonstrated similar results [5,6,13–15]. Results from these preclinical studies have lead to clinical trials of UCN-01 in combination with a variety of other anticancer agents.

Pre-clinical pharmacology studies of UCN-01 have suggested a rapid plasma clearance and large volume of distribution [4,16,17]. However, the initial phase I study demonstrated a significantly different pharmacokinetic profile [17]. This variability in pharmacokinetics has been attributed to species-specific differences in protein binding, specifically the binding of UCN-01 to α_1-acid glycoprotien [2,17]. Due to the extensive protein binding of this drug, and the desire to use this agent in combination with a variety of other drugs the possibility of alterations in pharmacokinetics caused by displacement reactions exists. Since this type of interaction may influence the pharmacokinetic profile for either agent, a sensitive analytical technique is required to describe the disposition of UCN-01. To date two studies have reported high-performance liquid chromatographic (HPLC) methods for quantification of UCN-01 [18,19].

Previously, Kurata et al. reported a HPLC method with fluorescence detection for determining UCN-01 plasma concentrations used for pre-clinical studies [18]. This HPLC method had a reported dynamic range adequate for the quantification of plasma concentrations achieved in animal studies, but was well below that necessary for dose ranging trials in humans. Bauer et al. recently reported another HPLC method using UV detection for determining UCN-01 in human plasma. This method was validated for quantification using a dynamic range of 200 to 20 000 ng/ml for extracted plasma samples and used in an NCI phase I trial [19].

In both of these HPLC methods, a mobile phase gradient was required for elution, with analytical run times of 20 to 30 min per sample. In this paper we report a simplified, highly sensitive HPLC method with a 10-min run time for the quantification of UCN-01 in human plasma.

2. Experimental

2.1. Chemicals and reagents

UCN-01, 100% pure, was obtained from the Developmental Therapeutics Program of the National Cancer Institute. (Rockville, MD, USA). HPLC-grade acetonitrile, dimethylsulfoxide (DSMO), ammonium acetate, triethylamine, and staurosporine (internal standard) were purchased from Sigma (St. Louis, MO, USA). Purified deionized water (HPLC grade) was obtained from the Milli-Q Academic

Quantum Purification System (Millipore, Bedford, MA, USA).

Salvaged human plasma used for the preparation of controls and standards was obtained from the University of Texas M.D. Anderson Cancer Center Blood Bank. Drug-free fresh human plasma used in protein binding experiments only was collected from healthy drug-free volunteers within our laboratories with their informed consent.

2.2. Standard stock solutions

A UCN-01 stock solution was prepared by dissolving 20 mg of drug in 20 ml acetonitrile to a final concentration of 1 mg/ml, which was then divided and stored as 1-ml aliquots at −40°C prior to further dilution with acetonitrile. For the internal standard, staurosporine, stock solution was prepared by first dissolving 1 mg of drug in 1 ml DSMO yielding a final concentration of 1 mg/ml. This was then stored at −40°C prior to final dilution to 0.1 μg/ml with acetonitrile.

2.3. Sample preparation

UCN-01 was isolated from human plasma using a liquid–liquid extraction method similar to that previously reported by Kurata et al. [18]. Briefly, 20 μl (2 μg) of the internal standard, staurosporine, was added to 1 ml of the plasma standard or sample, then vortex-mixed for 30 s. A 250-μl volume of this standard or sample was added to 750 μl of ice-cold acetonitrile in a 1.5-ml amber microcentrifuge vial and the vortex-mixed for 30 s. These vials were allowed to stand in an ice water bath for 10 min, then centrifuged at 12 000 *g* for 10 min. The supernatant was removed, placed into a borosilicate glass disposable tube, placed on a heating block at 40°C and evaporated under nitrogen for 15 to 20 min until dry. Samples were then reconstituted with 250 μl of acetonitrile and 30 μl was injected on column for analysis. Samples were kept at room temperature prior to injection.

2.4. Extraction efficiency

To determine extraction efficiency of UCN-01 from plasma, human plasma samples were spiked with UCN-01 to achieve either a final concentration of 0.5 μg/ml or 25 μg/ml, then extracted. Peak areas of UCN-01 obtained from extracted human plasma samples were compared with peak areas of unextracted standards that were prepared in 1 ml of mobile phase (acetonitrile–0.5 *M* ammonium acetate, 45:55, with 2% triethylamine as a final concentration) and injected directly onto HPLC system. Six samples were analyzed at each concentration.

Extraction efficiency was calculated using the following equation: (peak area for the extracted sample/peak area for the unextracted neat sample)·100=percent extraction efficiency.

2.5. Protein binding experiments

To investigate the role and variability of UCN-01 plasma protein binding the following experiments were conducted. Briefly, Soerensen phosphate buffer (pH 7.4, 0.067 *M* KH_2PO_4 and Na_2HPO_4 and NaCl) was prepared from analytical-grade reagents. Fresh human drug-free plasma was generated from blood donated by normal volunteers. Regenerated cellulose membranes of M_r cut-off 12 000–14 000 were presoaked in Soerensen phosphate buffer to be used in each dialysis macro-well (Spectrum Labs., Rancho Dominguez, CA, USA). UCN-01 was added to the above plasma to achieve final concentrations of 10 and 30 μg/ml, respectively. Five samples of each concentration were analyzed in each experiment, and each experiment was repeated in triplicate. After completing the appropriate time-analysis studies, samples were incubated for at least 36 h to reach equilibrium. Upon completion of the dialysis experiments, volumes on each side of the membrane were measured to correct for possible volume shifts.

2.6. HPLC apparatus and chromatographic conditions

The HPLC system consisted of Millennium Chromatography Manager 32 version 3.05.01 for integration and management of the Alliance 2690 Separations module, and Waters 474 fluorescence detector (Waters, Milford, MA, USA). The fluorescence detector was set at an excitation wavelength of 310 nm and emission was monitored at 410 nm with a signal gain set at 1. Separation was achieved at

ambient temperature (27°C) using an ODS column, 4 μm particle size, 150×3.9 mm, 60 Å pore size (Nova-Pak Phenyl, Waters) with a C_{18} pre-column.

2.7. Separation

This reversed-phase HPLC assay employed isocratic elution. The mobile phase consisted of acetonitrile–0.5 *M* ammonium acetate (45:55) with 0.2% triethylamine. For separation, a constant flow-rate of 1 ml/min was used and the total run time was 10 min. The retention times of the internal standard, staurosporine, and UCN-01 were 6.8±0.4 and 4.2±0.3 min, respectively, for samples extracted from human plasma.

2.8. Quantification

As part of the validation for this analytical method, salvaged human plasma was spiked with authentic UCN-01 and extracted as described above. A five-level standard curve, covering a dynamic linear range from 0.2 to 30 μg/ml, was employed for instrument calibration. Three calibration standard curves, completed on 3 consecutive days, were obtained by analyzing the five standards (0.2, 1, 10, 20, and 30 μg/ml) in triplicate each day. The best-fit calibration line was determined for each curve by sum-squared linear regression analysis of the calibration data using Millennium Chromatography Manager 32 version 3.05.01 (Waters). The inter-day analysis of accuracy and precision included back calculations of the concentration for all standard samples using the slope and intercept from each curve for each day. The peak area responses from all standards were fit to each of the three curves to estimate the nominal value for each standard concentration, with acceptable precision being <15% variability within and across each standard curve (Table 1). Intra-day analysis of assay precision and accuracy was completed at concentrations of 0.5 μg/ml (n=25) and 25 μg/ml (n=10) using extracted plasma samples. The same relative standard deviation (RSD) (<15%) was set as the threshold for acceptance of intra-day reproducibility (Table 1). The lower limit of detection (LLD) was defined as the lowest concentration with a peak area response having a signal-to-noise ratio of 3:1. The lower limit of quantification (LLQ) was the lowest concentration that could reliably be reproducibly measured with <15% variability.

2.9. Stability study

The stability of UCN-01 in plasma when stored for up to 3 months at −40°C was evaluated at concentrations of 0.5 and 25 μg/ml. In addition, the effects of freezing and thawing on the stability of UCN-01 were evaluated in plasma at the same concentrations for three freeze–thaw cycles. For these studies salvaged human plasma was spiked with UCN-01, then frozen at −40°C. At appropriate intervals from the day of preparation (24, 48, 72 h, 1 week, 2 weeks and 3 months) these samples were then thawed and analyzed. Peak areas from these samples were then compared to results obtained from identical samples prepared fresh on the day of analysis.

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

Nominal concentration (μg/ml)	Measured concentration (μg/ml)	RSD (%)	Accuracy (%)
Intra-day assay[a]			
0.5	0.51±0.1	2.97	97.2
25	24.9±0.5	2.09	99.5
Inter-day assay[b]			
0.2	0.21±0.1	3.98	96.4
10	10.6±0.2	2.37	93.3
30	29.3±0.3	1.14	97.6

[a] Mean±SD (n=10).
[b] Mean±SD (n=3).

3. Results and discussion

We developed and validated a HPLC method using fluorescence detection for the determination of UCN-01 content in human plasma. Previously published methods for the extraction and analysis of UCN-01 in human plasma have required gradient elution necessitating the use of more complex chromatographic systems. These methods have also required longer sample run times or complicated sample manipulations schemes to quantify UCN-01 concentrations achieved in patients. The method described here uses a uncomplicated buffered mobile phase, isocratic elution and a straightforward liquid–liquid extraction. The linearity of this method requires no sample dilutions to quantify plasma UCN-01 concentrations in the dynamic range of 0.2 to 30 μg/ml.

The retention time for UCN-01 was at 4.2±0.3 min and staurosporine at 6.8±0.4 min in human plasma (Fig. 2). The peak of interest was baseline resolved with no other peak within 2.6 min of UCN-01 elution. Using salvaged pooled human plasma, peaks interfering with the quantification of either UCN-01 or the internal standard could be detected (Fig. 3). This methodology was linear over the dynamic range 0.2 to 30 μg/ml for UCN-01 extracted from plasma. The linear equation, derived from a five-level standard curve, for the determination of UCN-01 plasma concentration was: UCN-01 concentration=[$(4.16 \cdot 10^5)$·observed area response]. The linear correlation coefficient (r^2) for this standard curve was 0.997, with an overall assay RSD of <3%.

UCN-01 peak areas were proportional throughout the dynamic plasma concentration range (0.2 to 30 μg/ml) measured. Mean recoveries of UCN-01 from plasma at the two concentrations evaluated, 0.5 and 25 μg/ml, were 89 and 90.2%, respectively. RSDs of the UCN-01 plasma standard concentration calculated values at the concentrations evaluated in the standard curve ranged from 1.14 to 5.12% with relative errors ranging from 0.7 to 12.33%.

Assay precision, based on an extracted UCN-01 standard plasma sample of 0.5 μg/ml, was ±4.56% with an overall accuracy of 94.3%. Using this method the UCN-01 the LLD was 0.1 μg/ml, with an LLQ of 0.2 μg/ml.

Results of the stability studies of UCN-01 indicate when stored at −40°C, UCN-01 is stable in human plasma for up to 3 months. Moreover, after three freeze–thaw cycles, when protected from light,

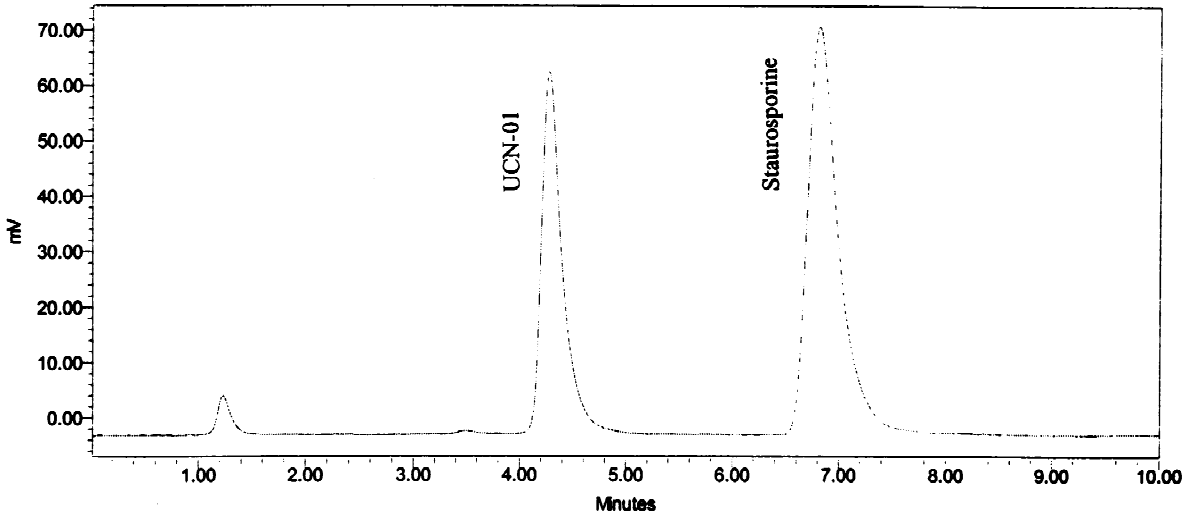

Fig. 2. Chromatogram of salvaged human plasma spiked with 2 μg/ml staurosporine and UCN-01 1 μg/ml. Separation was achieved using the above method. UCN-01 was detected by fluorescence using excitation and emission wavelengths of 310 and 410 nm, respectively. Retention time for UCN-01 was 4.1 min and 7.1 min for staurosporine, the internal standard. Using this method these compounds are easily resolved with no interfering peaks.

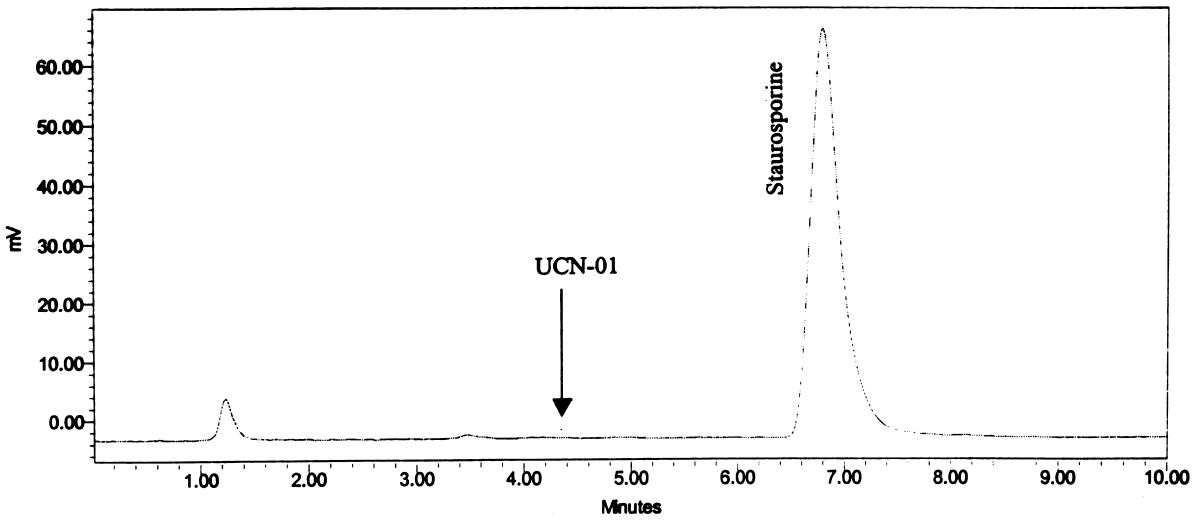

Fig. 3. Chromatogram of salvaged human plasma spiked with 2 μg/ml staurosporine alone (no UCN-01). Using this method this compound is easily resolved with no interfering peaks.

UCN-01 plasma samples show less than a 4% loss of drug (Table 2). In each instance the overall reduction in UCN-01 concentration was smaller than the RSD for the assay (<15%).

As previously reported, the protein binding studies confirmed that UCN-01 is highly protein bound. The mean percent of UCN-01 bound to human plasma proteins in our study was 95.4±3.1%. The degree of binding did not appear to be dependent on drug concentration, but was dependent on protein content. Additional studies have been conducted to further describe the protein binding characteristics on UCN-01 in humans (reported elsewhere).

In conclusion, we have reported a highly sensitive, liquid chromatographic method for quantifying UCN-01 in human plasma. The previously published UCN-01 HPLC assays using UV or fluorescence detection previously have required gradient elution and had significantly longer run times approximately 20–30 min [18,19]. This UCN-01 assay method has the benefit of implementing a simplified isocratic mobile phase, and a significantly decreased analytical run time. Our method also exploits a simplified extraction to improve sensitivity. By decreasing the sample injection volume and signal gain, we have been able to use fluorescence detection over a wider dynamic range than had been previously reported. This assay method has been validated in

Table 2
Stability and freeze–thaw results in human plasma

	Remaining % of nominal concentration
Stored at −40°C	
In plasma for 90 days (n=3)	
0.5 μg/ml	97.5±3.2
25 μg/ml	96.3±0.1
After three freeze–thaw cycles	
In plasma (n=3)	
0.5 μg/ml	96.7±0.9
25 μg/ml	99.6±1.0

human plasma and will be useful for conducting future preclinical and clinical trials of UCN-01 in combination anticancer drug regimens.

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