ELSEVIER

Journal of Chromatography B, 759 (2001) 247–257

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

# Quantitative determination of perifosine, a novel alkylphosphocholine anticancer agent, in human plasma by reversed-phase liquid chromatography–electrospray mass spectrometry

Eunhee W. Woo[a], Richard Messmann[b], Edward A. Sausville[b], William D. Figg[a,*]

[a]*Clinical Pharmacokinetics Section, Medicine Branch, National Cancer Institute, Bethesda, MD 20852, USA*
[b]*Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD 20852, USA*

Received 13 October 2000; received in revised form 23 April 2001; accepted 23 April 2001

**Abstract**

A sensitive and selective reversed-phase LC–ESI-MS method to quantitate perifosine in human plasma was developed and validated. Sample preparation utilized simple acetonitrile precipitation without an evaporation step. With a Develosil UG-30 column (10×4 mm I.D.), perifosine and the internal standard hexadecylphosphocholine were baseline separated at retention times of 2.2 and 1.1 min, respectively. The mobile phase consisted of eluent A, 95% 9 m*M* ammonium formate (pH 8) in acetonitrile–eluent B, 95% acetonitrile in 9 m*M* ammonium formate (pH 8) (A–B, 40:60, v/v), and the flow-rate was 0.5 ml/min. The detection utilized selected ion monitoring in the positive-mode at $m/z$ 462.4 and 408.4 for the protonated molecular ions of perifosine and the internal standard, respectively. The lower limit of quantitation of perifosine was 4 ng/ml in human plasma, and good linearity was observed in the 4–2000 ng/ml range fitted by linear regression with $1/x$ weight. The total LC–MS run time was 5 min. The validated LC–MS assay was applied to measure perifosine plasma concentrations from patients enrolled on a phase I clinical trial for pharmacokinetic/pharmacodynamic analyses. Published by Elsevier Science B.V.

*Keywords:* Perifosine; Alkylphosphocholine

## 1. Introduction

Perifosine (Fig. 1a), a synthetic alkylphosphocholine signal transduction modulator, is a novel antitumor agent currently under clinical development at the National Cancer Institute (NCI), Bethesda, MD, USA. Patients with relapsed or refractory neoplasms are being treated with escalating doses of perifosine by oral administration. Alkylphosphocholines (APCs), derived from alkylphospholipids, lack the glycerol backbone of alkylphospholipids and therefore do not serve as substrates for many of the lysophopholipid metabolizing cellular enzymes, allowing for higher tissue and tumor concentrations of the APCs than those of alkylphospholipids [1,2]. Their cytotoxic efficacy often increases with the alkyl-chain length, with at least 16 carbons being a

*Corresponding author. Building 10, Room 5A01, 9000 Rockville Pike, Medicine Branch, NCI/NIH, Bethesda, MD 20892, USA. Tel.: +1-301-4023-622; fax: +1-301-4028-606.
*E-mail address:* wdfigg@helix.nih.gov (W.D. Figg).

0378-4347/01/$ – see front matter Published by Elsevier Science B.V.
PII: S0378-4347(01)00231-6

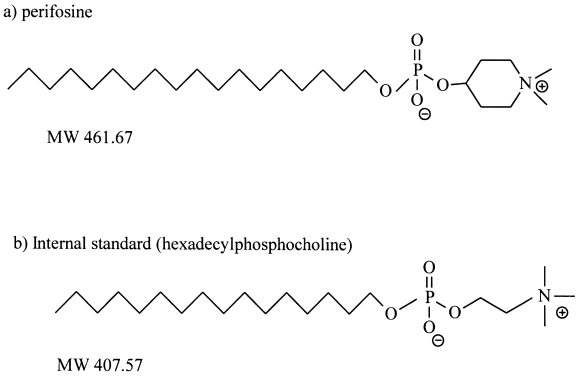


Fig. 1. Chemical structures of (a) perifosine and (b) hexadecylphosphocholine, internal standard.

minimum structural requirement [3,4]. Perifosine, an APC analog with an 18 carbon chain, modulates signal-transduction pathways, inhibits protein kinase C, induces apoptosis in human leukemia cell lines but not in normal vascular endothelial cells, and enhances radiation-induced apoptotic effect [5–8]. Perifosine has entered clinical evaluation since miltefosine (hexadecylphosphocholine, Fig. 1b), the first APC to enter clinical trials, failed to show activity probably because of gastrointestinal toxicities [9,10].

Due to the absence of a chromophore or an electroactive group in the molecule, conventional high-performance liquid chromatography (HPLC) assay of perifosine using absorption or electrochemical detection is not feasible. Hexadecylphosphocholine has been determined by gas chromatography but this method requires chemical derivatization [11]. Although light-scattering detection has been used in the HPLC of phospholipids [12], it is generally not selective and requires careful calibration, making mass spectrometry (MS) the preferred on-line detection method for phospholipids because of its superior sensitivity and specificity. A reversed-phase HPLC column offers enhanced selectivity in the separation of phospholipids where only subtle differences exist in their structure such as two extra carbon atoms or a double bond in the alkyl chain [12]. Knebel et al. recently [13] reported a sensitive normal-phase HPLC assay using tandem mass spectrometric detection with a limit of quantitation of 4 ng/ml for perifosine in human plasma. In their study, a normal-phase column (Chromsep 5 μm SI 100×3 mm) was used since reversed-phase columns resulted in unsatisfactory retention of perifosine on the columns. However, perifosine and the internal standard were almost co-eluting and peak-tailing was observed with the normal-phase column. In addition, the performance of the column started to degrade after approximately 150 injections, resulting in increased peak-tailing and decreased signals. Furthermore, the plasma samples were prepared by solid-phase extraction equipped with an automatic sample processor (RapidTrace; Zymark, Hopkinton, MA, USA), and its applicability is limited by the expense. Therefore, a simple sample preparation procedure would be highly desirable.

To support the clinical pharmacokinetic and pharmacodynamic analyses, a sensitive analytical method for quantitating perifosine in human plasma was needed. In this report, we describe a validated and cost-effective method to quantitate perifosine in human plasma using reversed-phase liquid chromatography interfaced to electrospray ionization mass spectrometry (LC–ESI-MS). Our method simplified the sample preparation by utilizing acetonitrile precipitation with no evaporation step, required small sample volume, used a reversed-phase column to enhance peak selectivity with improved peak shape, and used a less expensive single-quadrupole MS instrumentation, while still achieving the same range of quantitative concentration compared with previously reported LC–MS–MS assay.

## 2. Experimental

### 2.1. Chemicals and materials

Perifosine, D-21266, 1,1-dimethyl-4-[(octadecyloxy)hydroxyphosphinyl]-piperidinium inner salt, was a generous gift from Asta Medica (Frankfurt, Germany). The internal standard (I.S.), hexadecylphosphocholine, 2-{[(hexadecyloxy)hydroxyphosphinyl]oxy} - *N*, *N*, *N* - trimethylethylammonium inner salt, was obtained from Sigma (St. Louis, MO, USA). The HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate was obtained from Sigma. Deionized water was generated in the laboratory with a Hydro Reverse Osmosis system (Durham, NC, USA) con-

nected to a Milli-Q UV Plus filtration system (Marlborough, MA, USA). Drug-free heparinized human plasma was purchased from Biological Specialty (Lansdale, PA, USA) and centrifuged at 2000 *g* for 10 min to remove fibrous material prior to use. Clinical blood samples were obtained, with informed consent, in heparinized Vacutainer Plus tubes from patients at the NCI, and centrifuged at 2000 *g* for 10 min at 4°C to obtain plasma. All other reagents were of analytical or HPLC grade. Seal/leak-free screw cap polyethylene tubes (1.5 ml) were purchased from Sarstedt (Newton, NC, USA). Autosampler vials (polypropylene, 0.75 ml) and PTFE/silicon caps were purchased from Scientific Resources International (Reno, NV, USA). HPLC mobile phase was filtered through a 0.45-μm nylon filter obtained from Whatman (Clifton, NJ, USA) under a laboratory vacuum. A polyethylene volumetric flask was obtained from Fisher Scientific (Pittsburgh, PA, USA).

### 2.2. Equipment

The LC–MS experiments were carried out with a HP 1100 LC–MS system (Agilent Technology, Palo Alto, CA, USA). The system consisted of a G1312A binary pump, a mobile phase vacuum degassing unit, a G1329A autosampler, a temperature controlled column compartment, and a HP 1100 single quadrupole mass spectrometric detector equipped with an electrospray source. The original autosampler seat and needle sets were replaced with polyetheretherketone (PEEK)-based needle seat and needle assembly and Tefzel rotar seal (Agilent Technology) was used in injector valve to avoid carry-over. A needle wash with methylene chloride–isopropanol (1:1, v/v) on an uncapped glass autosampler vial was used to wash the needle tip just prior to the injection. Data were acquired and integrated by HPLC–MS ChemStation software Rev. A. 07.01 run by HP Vectra 150/PC under the Windows NT operating system. The HPLC column was a Develosil C30-UG (10×4 mm, 5 μm particle size) (Phenomenex, Torrance, CA, USA) attached with a column inlet filter (3 mm×0.5 μm; Varian, Walnut Creek, CA, USA). PEEK tubing of 0.005 in. I.D. (Upchurch Scientific, Oak Harbor, WA, USA) was used to connect the column to the pump and the mass spectrometer with minimal tubing length to avoid extra-column volume (1 in.=2.54 cm).

### 2.3. Chromatographic and mass spectrometric conditions

Isocratic elution was carried out with a mobile phase consisting of eluent A, 95% 9 m*M* ammonium formate (pH 8) in acetonitrile–eluent B, 95% acetonitrile in 9 m*M* ammonium formate (pH 8) (A–B, 40:60, v/v) with a flow-rate of 0.5 ml/min. The 9 m*M* ammonium formate was adjusted to pH 8 using ammonium hydroxide. The analytical column was kept at ambient temperature. The sample injection volume was 20 μl. The LC effluent was interfaced to the ESI-MS system without splitting.

The MS system was operated in the positive ion mode and the conditions were optimized to generate maximum analyte signal. The MS conditions were as follows: drying gas flow 13 l/min, nebulizer pressure 40 p.s.i. (275 790.4 Pa), drying gas temperature 350°C, capillary voltage 3000 V, fragmentor 100 V, multiplier gain 5. Nitrogen from liquid nitrogen was used as a nebulizing and drying gas. Selected ion monitoring (SIM) was accomplished with the ion at $m/z$ 462.4 for perifosine and 408.4 for the internal standard as protonated molecular ions, respectively. Both ions had a dwell time of 289 ms per ion and were monitored in the high-resolution mode. After data acquisition, the SIM chromatograms were integrated using the HP ChemStation software. The total LC–MS run time was 5 min.

### 2.4. Preparation of standard and quality control samples

A stock solution of perifosine was prepared by dissolving perifosine in methanol–water (1:1, v/v) in a polypropylene volumetric flask to yield a final concentration of 100 μg/ml and stored at −20°C. Working standard solutions were prepared from the stock solution in drug-free heparinized human plasma. Plasma calibration standards of 4, 10, 25, 100, 250, 500, 1000 and 2000 ng/ml of perifosine were obtained by serial dilution with the human plasma.

To prepare human plasma quality control (QC) samples, an independent stock solution of perifosine was prepared as described for the calibration stan-

dard curve, except by separate weighting of perifosine. QC samples with concentrations of 12, 800 and 1600 ng/ml were prepared by appropriate serial dilution of the working stock solution with human plasma. In addition, QC samples at 4000 and 8000 ng/ml were prepared from the stock solution as dilution QC to cover concentrations exceeding the upper limit of standard curve. The dilution QC samples were diluted 10-fold using heparinized human plasma just prior to analysis. The QC samples were kept at −20°C until analysis. A stock solution of hexadecylphosphocholine (1 mg/ml) was prepared in methanol–water (1:1) and stored at −20°C. A working internal standard solution was prepared by diluting the stock solution to yield 500 ng/ml concentration in acetonitrile.

### 2.5. Sample preparation

A 100-μl aliquot of human plasma standard, QC, blank or sample was pipetted into a screw cap polyethylene vial, and 100 μl of acetonitrile containing 500 ng/ml of hexadecylphosphocholine (internal standard) was added to precipitate the plasma proteins. The mixture was vortex-mixed briefly, then shaken for 2 min on an automatic shaker (Electronic Shaker, IKAK 501 digital; IKA Labortechnik, Wilmington, NC, USA). After a 5-min centrifugation (16 000 *g*) at room temperature, 100 μl of supernatant was transferred to an autosampler vial which already containing 100 μl of 9 m*M*, pH 8 ammonium formate to keep the final acetonitrile content less than 50%. A 20-μl volume of the reconstituted sample was injected for LC–MS analysis.

### 2.6. Validation procedure

#### 2.6.1. Calibration curve and linearity

The eight-point calibration curves were constructed by plotting peak area ratio ($y$) of perifosine to the internal standard versus perifosine concentrations ($x$). The regression parameters of slope, intercept and correlation coefficient were calculated by weighted ($1/x$) linear regression using the Sigma Plot software (Chicago, IL, USA). The concentrations of calibration standards, analyzed in duplicate, were then back calculated. The linearity was evaluated by comparing the correlation coefficient ($r^2$), residuals and errors between theoretical and back-calculated concentrations of calibration standard samples.

#### 2.6.2. Lower limit of quantitation

The lower limit of quantitation (LLQ) was evaluated by spiking perifosine at a concentration of 4 ng/ml with seven different lots of drug-free heparinized plasma from seven different individuals and assaying them in duplicate as unknowns against a standard curve. Precision and accuracy were required to be less than 20% at LLQ.

#### 2.6.3. Intra-assay and inter-assay precision and accuracy

Precision and accuracy were evaluated by determining the perifosine concentration in six replicates of three levels of QC samples and two levels of dilution QC samples daily for 3 different days. The QC concentrations were chosen to cover the entire range of the standard calibration curve at three times the LLQ (the low-level QC at 12 ng/ml), at 40% of the highest point of the standard curve (the mid-level QC at 800 ng/ml), and at 80% of the highest point of the standard curve (the high-level QC at 1600 ng/ml) [14]. Two QC samples at two- and four-times the upper limit of the standard curve (dilution QC samples at 4000 and 8000 ng/ml) were assayed to ensure that the dilution of clinical samples with human plasma did not affect accuracy and precision. The dilution QC samples were diluted 10-fold with heparinized human plasma prior to analysis and processed as other QC samples. Each run consisted of calibration standards in duplicate, QC and dilution QC samples in six replicates, blank plasma samples with and without internal standard in duplicate, was run daily for 3 different days to evaluate the assay performance.

The accuracy of the assay was evaluated by % deviation (DEV) from theoretical concentration using the formula: % DEV=100×(mean back calculated concentration−theoretical concentration)/theoretical concentration. Within- and between-assay precision were obtained by one-way analysis of variance (ANOVA) testing, reported as relative standard deviation (RSD) for each QC concentration. Acceptable accuracy and precision for the method

validation were set at less than 15% in both parameters at every concentration studied except for the LLQ where 20% is acceptable.

#### 2.6.4. Specificity

Ten blank plasma samples from 10 individuals were processed with and without the internal standard to evaluate the presence of interfering peaks.

#### 2.6.5. Recovery

For the recovery study, perifosine and the internal standard were spiked before and after protein precipitations to account for potential matrix effects on ionization efficiency in the electrospray. The recovery of perifosine from plasma was evaluated at six different concentrations in triplicate at 6, 12.5, 200, 400, 800 and 1600 ng/ml. The stock solution of perifosine was prepared in 50% MeOH in water and serially diluted to six different concentrations and used for spiking. The recovery of the internal standard was evaluated using 500 ng/ml in acetonitrile, the same concentration used in the assay.

#### 2.6.6. Stability

The bench-top stability was assessed in triplicate by leaving the QC samples of four different concentrations at room temperature for 0, 5, 12, and 24 h. Freshly prepared calibration standard samples were run along with the QC samples to assess the stability. Freeze–thaw stability was assessed over three cycles. QC samples at four different concentrations in triplicate were thawed at room temperature and refrozen at −20°C over three cycles and assayed. The stability of reconstituted samples in autosampler vials was assessed by assaying the previously assayed QC samples after leaving the QC samples in the autosampler chamber at ambient temperature for 24 and 72 h. Freshly processed standard samples were used to quantitate the QC samples. The freezer storage stability of perifosine in plasma at −20°C was evaluated by assaying QC samples in triplicate on day 0, week 8, week 16, and week 24. The concentration of perifosine after each storage period was determined against freshly prepared standard samples.

## 3. Results

### 3.1. Chromatography

The protonated molecular ions $[M+H]^+$ of perifosine and the internal standard were observed as base peaks with full scan ESI-MS (Fig. 2) and were used as SIM ions for quantitation. With 100 V fragmentation voltage, no fragmentation of perifosine $[M+H]^+$ was observed, which is crucial in maximizing the signal sensitivity. Other mass spectrometric parameters (capillary voltage, nebulizer pressure, and drying gas flow) were also optimized to obtain the maximum signal for perifosine.

Under optimized HPLC and MS conditions, perifosine and the internal standard were baseline separated with retention times of 2.2 and 1.1 min,

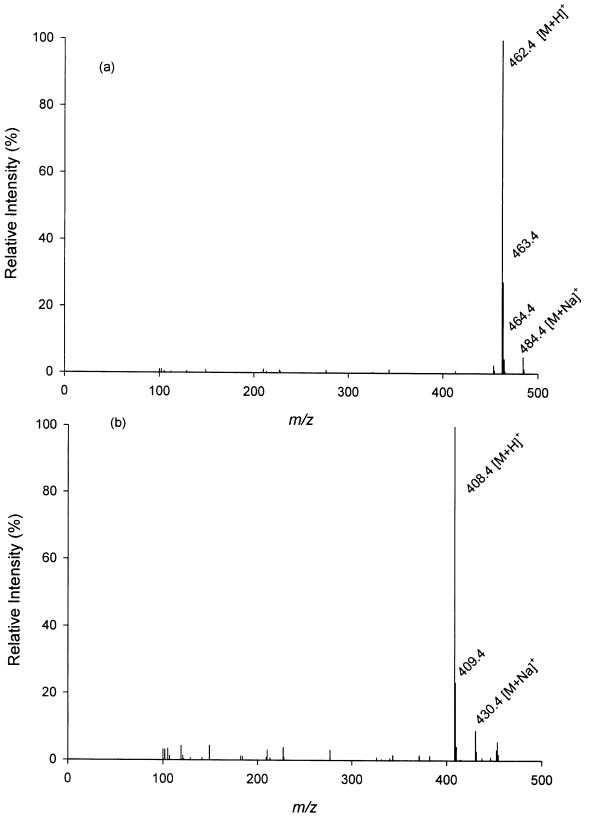


Fig. 2. Full scan mass spectra of (a) perifosine and (b) internal standard, hexadecylphosphocholine obtained by positive-mode LC–ESI-MS.

respectively (Fig. 3c and d). Since no late eluting compounds were observed, regeneration of the column using a gradient elution step was unnecessary. The total analysis time took only several minutes using isocratic elution. Blank human plasma from 10 individuals showed no significant interfering peaks at the retention times of perifosine and its internal standard (Fig. 3a and b). The peak with low ion count was observed around the retention time of the internal standard, but its intensity was insignificant compared to that of internal standard (less than 0.05%) and did not affect the assay accuracy and precision.

### 3.2. Calibration curves, linearity, and LLQ

The calibration curve was linear over the concentration range of 4 to 2000 ng/ml of perifosine in human plasma with correlation coefficients ≥0.9989 and consistent slope values when evaluated by weighed $(1/x)$ linear regression. Residuals were randomly distributed when plotted against the concentration. Table 1 shows the results of calibration accuracy in the 3-day validation study. The calibration curves were accurate with less than 6.4% deviation from the nominal values and precision was within 3.4%. At a perifosine plasma concentration of 4 ng/ml, the accuracy deviated within 13.7% and the precision was within 3.7%. Therefore, the LLQ of perifosine in human plasma was established at 4 ng/ml. A typical chromatogram of LLQ sample is shown in Fig. 3c.

### 3.3. Accuracy and precision

Table 2 shows the intra- and inter-assay accuracy and precision data. The method was found to be highly accurate with less than 4.1% deviation from

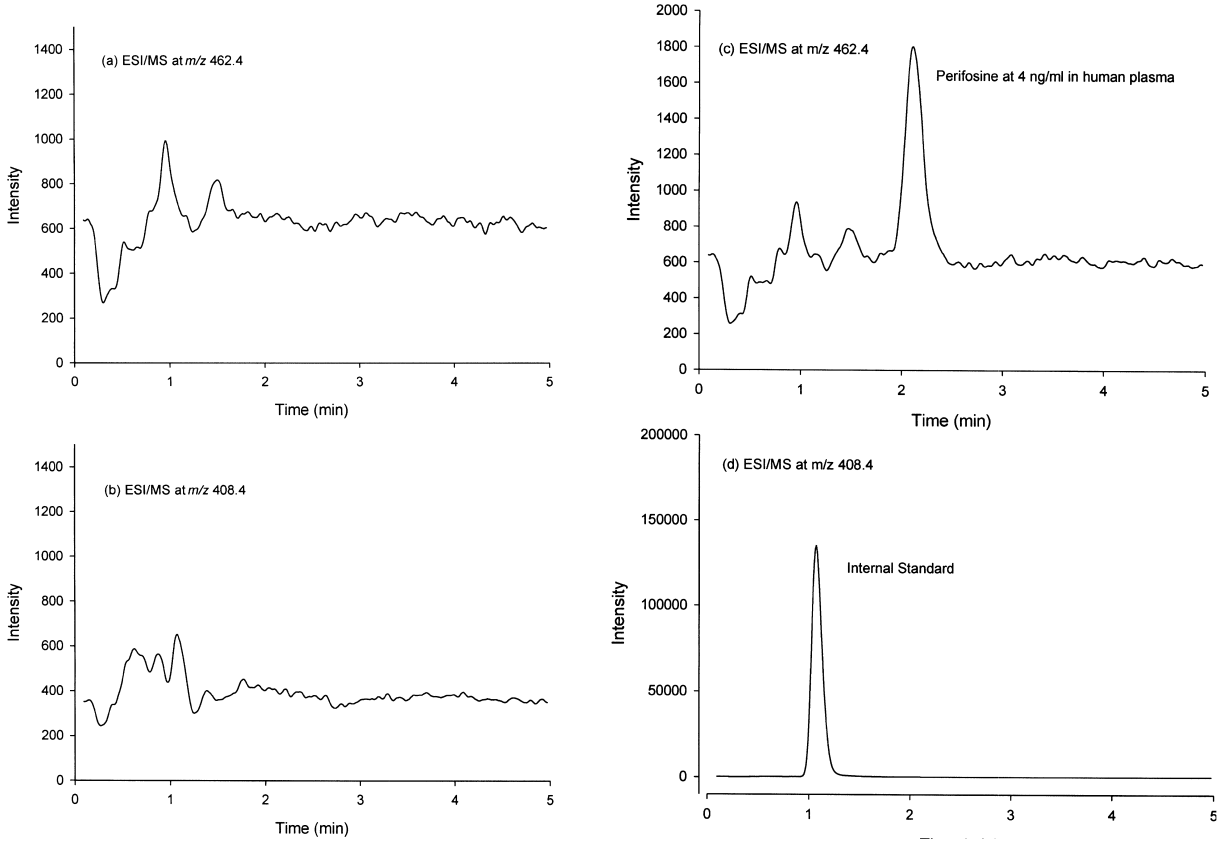


Fig. 3. Chromatograms of the blank plasma sample when monitored by SIM for (a) perifosine and (b) internal standard. SIM chromatograms of plasma standard spiked with (c) 4 ng/ml of perifosine and (d) in the presence of internal standard.

Table 1
Deviation from the nominal concentration (DEV) and relative standard deviation (RSD) at calibration standards of perifosine in human plasma calibration curves from the three validation runs

| Nominal concentration (ng/ml) | Mean found concentration (ng/ml) | DEV (%) | RSD (%) |
|---|---|---|---|
| 4 | 4.26 | 6.4 | 3.4 |
| 10 | 9.36 | −6.4 | 1.9 |
| 25 | 23.82 | −4.7 | 2.0 |
| 100 | 96.05 | −4.0 | 3.0 |
| 250 | 247.06 | −1.2 | 2.1 |
| 500 | 496.72 | −0.7 | 1.4 |
| 1000 | 1013.80 | 1.4 | 1.1 |
| 2000 | 1996.52 | −0.2 | 0.9 |

the nominal values, and highly precise with between-run precision less than 1.6% and within-run precision less than 3.6% for each concentration of QC sample tested.

### *3.4. Recovery*

To account for the effect on ionization efficiency in the ion spray by plasma matrix, perifosine and the internal standard were spiked before and after protein precipitations. The recoveries of perifosine and the internal standard were consistently 45.8% and 99.9%, respectively, across the tested range (Table 3).

### *3.5. Stability*

No significant loss of perifosine (≤6.6%) was observed after storage of plasma at room temperature on the bench-top for at least 24 h (Table 4). Plasma samples were stable over at least three freeze–thaw cycles (Table 5), indicating that the plasma samples can be frozen and thawed at least three times prior to analysis. Processed samples were stable up to 72 h in the autosampler tray (Table 6). Plasma samples were stable at −20°C for at least 6 months with no significant loss (≤8.8%, Table 7).

## 4. Discussion

Analysis of perifosine in human plasma using normal-phase LC with MS–MS detection has been described by Knebel et al. [13]. In their study, perifosine and the internal standard (hexadecylphosphocholine) were almost co-eluting. Nevertheless, the poor LC resolution was compensated by the high mass selectivity of the MS–MS instrumentation. Because our study used a single-quadrupole MS system, an efficient LC separation was desired to increase specificity and sensitivity by preventing potential interference from co-eluting compounds.

A reversed-phase HPLC column generally offers enhanced selectivity in the separation of phospholipids where only subtle differences exist in their structure such as two extra carbon atoms or double bond in the alkyl chain [12]. However, in agreement with the Knebel et al. study [13], perifosine was either unretained or subjected to severe peak-tailing with commonly used reversed-phase columns, probably due to the bulky charged piperidinium group in perifosine. In contrast, we found that the Develosil $C_{30}$ reversed-phase column allowed sufficient LC retention and the subsequent separation of perifosine and the internal standard probably due to its strong hydrophobicity of the long alkyl-chain. Nevertheless,

Table 2
Accuracy and precision of the perifosine assay in human plasma

| | Nominal concentration (ng/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 12 | 400 | 800 | 1600 | 4000[a] | 8000[a] |
| Accuracy ($n$=18) | | | | | | |
| Mean found concentration (ng/ml) | 11.73 | 393.19 | 799.70 | 1558.46 | 3853.21 | 7672.63 |
| Accuracy (DEV, %) | −2.22 | −1.70 | −0.04 | −2.60 | −3.67 | −4.09 |
| Precision | | | | | | |
| Between-run (RSD, %, $n$=18) | 1.57 | 1.43 | 1.29 | 1.57 | 0.92 | 1.45 |
| Within-run (RSD, %, $n$=6) | 2.42 | 3.57 | 1.54 | 1.37 | 1.18 | 1.74 |

[a] Dilution QC samples with dilution factor of 10.

Table 3
Recovery of perifosine and hexadecylphosphocholine, the internal standard (I.S.) in human plasma ($n=3$)

| Nominal concentration (ng/ml) | Ratio of peak area of perifosine vs. I.S. | | | | | | Recovery (%) | |
|---|---|---|---|---|---|---|---|---|
| | *a* | | *b* | | *c* | | Perifosine (100×*b*/*a*) | I.S. (100×*c*/*b*) |
| | Individual | Mean | Individual | Mean | Individual | Mean | | |
| 6.00 | 0.055 | 0.052 | 0.025 | 0.024 | 0.024 | 0.024 | 46.8 | 100.6 |
| | 0.050 | | 0.023 | | 0.023 | | | |
| | 0.050 | | 0.025 | | 0.026 | | | |
| 12.50 | 0.102 | 0.103 | 0.050 | 0.049 | 0.051 | 0.050 | 48.1 | 101.9 |
| | 0.105 | | 0.050 | | 0.050 | | | |
| | 0.102 | | 0.049 | | 0.050 | | | |
| 200.00 | 2.111 | 2.101 | 0.896 | 0.905 | 0.922 | 0.925 | 43.0 | 102.3 |
| | 2.093 | | 0.919 | | 0.933 | | | |
| | 2.100 | | 0.899 | | 0.921 | | | |
| 400.00 | 4.353 | 4.338 | 1.973 | 1.943 | 1.904 | 1.932 | 44.8 | 99.4 |
| | 4.333 | | 1.936 | | 1.973 | | | |
| | 4.328 | | 1.920 | | 1.919 | | | |
| 800.00 | 8.924 | 8.843 | 3.980 | 4.012 | 3.843 | 3.911 | 45.4 | 97.5 |
| | 8.820 | | 4.025 | | 3.913 | | | |
| | 8.785 | | 4.032 | | 3.976 | | | |
| 1600.00 | 17.332 | 17.192 | 8.059 | 8.065 | 7.944 | 7.893 | 46.9 | 97.9 |
| | 16.985 | | 8.100 | | 7.691 | | | |
| | 17.260 | | 8.036 | | 8.043 | | | |
| Mean±SD | | | | | | | 45.8±1.8 | 99.9±2.0 |

*a*: Post-protein precipitation for perifosine and pre-protein precipitation for I.S. *b*: Pre-protein precipitation for perifosine and pre-protein precipitation for I.S. *c*: Pre-protein precipitation for perifosine and post-protein precipitation for I.S.

some peak tailing was still observed. The phospholipid head group has been known to show extensive peak tailing due to its non-specific interaction with column material. Amine salts such as triethylamine have been used to minimize this unwanted interaction by competing with the head group moieties for binding sites on the stationary phase [12]. However, the addition of 1% (v/v) triethylamine to the eluent did not improve the perifosine peak shape in our experiment, possibly due to the amphoteric nature of perifosine.

The effects of the aqueous mobile phase pH on the peak shape of perifosine were also studied. Mobile phase at low pH has been shown to improve the peak shape of basic compounds by suppressing the ionization of the silanol group of the silica-based LC packing [15]. However, lowering the pH to 3 worsened the peak shape of perifosine. Kim et al. used a high pH mobile phase to separate phosphocholines [16]. Due to the presence of quaternary amine in the perifosine molecule, it is not possible to suppress the charge of perifosine completely with a mobile phase commonly used in LC. Nonetheless, increasing the pH to 8–9.5 improve the peak shape significantly. The pH 8 aqueous mobile phase was finally chosen because a higher value would compromise the stability of the $C_{30}$ column according to the manufacturer.

The use of short HPLC columns (10 to 20 mm) has been growing in popularity in LC–MS analysis [17–19]. Initially, the analytical Develosil C30-UG column (150×4 mm I.D.) was used with the guard column (10×4 mm I.D.) for separation. Later it was found that sufficient separation of perifosine was still

Table 4
Bench-top stability of perifosine in human plasma ($n=3$)

| Time (h) | Nominal concentration (ng/ml) | Found concentration mean±SD (ng/ml) | DEV (%) |
|---|---|---|---|
| 0 | 12 | 12.18±0.60 | 1.49 |
| | 200 | 204.39±2.02 | 2.19 |
| | 800 | 839.09±5.52 | 4.89 |
| | 1600 | 1685.22±22.79 | 5.33 |
| 5 | 12 | 12.03±0.33 | 0.33 |
| | 200 | 203.24±1.62 | 1.62 |
| | 800 | 821.47±2.68 | 2.68 |
| | 1600 | 1664.70±4.04 | 4.04 |
| 12 | 12 | 11.60±0.51 | −3.33 |
| | 200 | 204.66±1.15 | 2.33 |
| | 800 | 843.53±2.07 | 5.69 |
| | 1600 | 1695.00±20.12 | 5.94 |
| 24 | 12 | 11.89±0.37 | −0.92 |
| | 200 | 207.36±2.60 | 3.68 |
| | 800 | 846.53±2.24 | 5.82 |
| | 1600 | 1705.48±6.82 | 6.59 |

achieved when using the guard column alone, in addition to having a significantly shortened analysis time and improved peak shape. Therefore, the guard column was used as the analytical column throughout the experiments. To minimize dynamic elution and improve peak shape when using a short column [20], the acetonitrile content in the reconstituted solution was kept below 50%. The column could be used for at least 400–500 samples without significant peak tailing or losing the LLQ. After that, the column tended to lose the LLQ. To maximize lifetime, the column was washed with 100% acetonitrile followed by 60% acetonitrile in water at the end of batch sample analysis, and kept in 60% acetonitrile in water when not in use.

Table 5
Freeze–thaw stability of perifosine in human plasma ($n=3$)

| Cycle | Nominal concentration (ng/ml) | Found concentration mean±SD (ng/ml) | DEV (%) |
|---|---|---|---|
| 1 | 12 | 11.50±0.12 | −4.20 |
| | 200 | 200.74±0.98 | 0.37 |
| | 800 | 838.39±19.72 | 4.80 |
| | 1600 | 1685.71±6.12 | 5.36 |
| 2 | 12 | 11.39±0.18 | −5.08 |
| | 200 | 200.93±2.11 | 0.46 |
| | 800 | 846.37±5.80 | 5.80 |
| | 1600 | 1677.77±13.80 | 4.80 |
| 3 | 12 | 11.10±0.38 | −7.50 |
| | 200 | 203.23±1.39 | 1.62 |
| | 800 | 836.09±14.98 | 4.51 |
| | 1600 | 1680.29±23.63 | 5.02 |

Table 6
Stability of processed sample in autosampler tray ($n=3$)

| Time (h) | Nominal concentration (ng/ml) | Found concentration mean±SD (ng/ml) | DEV (%) |
|---|---|---|---|
| 24 | 12 | 12.18±0.05 | 1.50 |
| | 400 | 405.70±6.14 | 1.42 |
| | 800 | 821.12±22.74 | 2.64 |
| | 2000 | 1903.00±125.11 | −4.83 |
| 72 | 12 | 10.70±0.30 | −10.81 |
| | 400 | 342.79±7.22 | −14.30 |
| | 800 | 720.97±21.55 | −9.88 |
| | 2000 | 2011.19±19.72 | 0.56 |

For sample preparation, solid-phase extraction, liquid–liquid extraction, and acetonitrile precipitation with or without evaporation were investigated. A simple acetonitrile precipitation with no evaporation step was chosen as the sample preparation method because it allowed good sensitivity and less variability. Furthermore, plasma samples with small volume such as 20 μl or less can be easily processed.

The reason for the relatively low perifosine recovery of 46% from plasma might be due to co-precipitation of perifosine along with protein upon acetonitrile precipitation or interaction with glass or plastic surfaces [13]. However, the recoveries of perifosine and the internal standard were consistent across the tested range and did not affect the assay adversely.

While the internal standard showed no carry-over, perifosine was found to bind on the rubber-based needle seat and needle assembly of the HP-1100 LC system, resulting in significant and persistent carry-

Table 7
Freezer storage stability of perifosine in human plasma ($n=3$)

| Sample stored (weeks) | Nominal concentration (ng/ml) | Found concentration mean±SD (ng/ml) | DEV (%) |
|---|---|---|---|
| 8 | 12 | 12.79±0.20 | 6.6 |
| | 400 | 435.23±9.22 | 8.81 |
| | 800 | 858.63±9.69 | 7.33 |
| | 1600 | 1695.85±51.11 | 5.99 |
| 16 | 12 | 12.17±0.08 | 1.42 |
| | 400 | 422.97±1.95 | 5.74 |
| | 800 | 845.7±2.58 | 5.71 |
| | 1600 | 1694.46±28.53 | 5.9 |
| 24 | 12 | 12.69±0.28 | 5.72 |
| | 400 | 418.25±4.23 | 4.56 |
| | 800 | 842.17±1.82 | 5.27 |
| | 1600 | 1652.72±13.99 | 3.29 |

over that made quantitation impossible. Several measures were then taken to prevent the carry-over: the autosampler seat and needle sets were replaced with a PEEK-based needle seat and needle assembly and Tefzel rotar seal (Agilent Technology) was used in the injector valve. Finally, a needle wash with methylene chloride–isopropanol (1:1, v/v) in an uncapped glass autosampler vial was used to wash the needle tip just prior to the injection. With these measures, we found no carry-over even after injecting high concentration samples directly followed by blank samples. In Knebel et al.'s study, the authors reported that there was minor endogenous interference from blank plasma, but we believe that the interference was likely originated from the carry-over from previously injected or bound perifosine on the instrument rather than from the endogenous source.

A previous study indicated that perifosine has high interaction with surfaces such as glass and stainless steel [13]. However, we found no significant loss of signal intensity when processed perifosine samples were stored either in glass or polyethylene autosampler vials. In addition, no difference was observed when blank plasma spiked with perifosine at four different concentrations between 200 and 2000 ng/ml were stored in either glass or plastic blood collecting tubes. Nonetheless we used polypropylene based materials whenever possible based on the previous study.

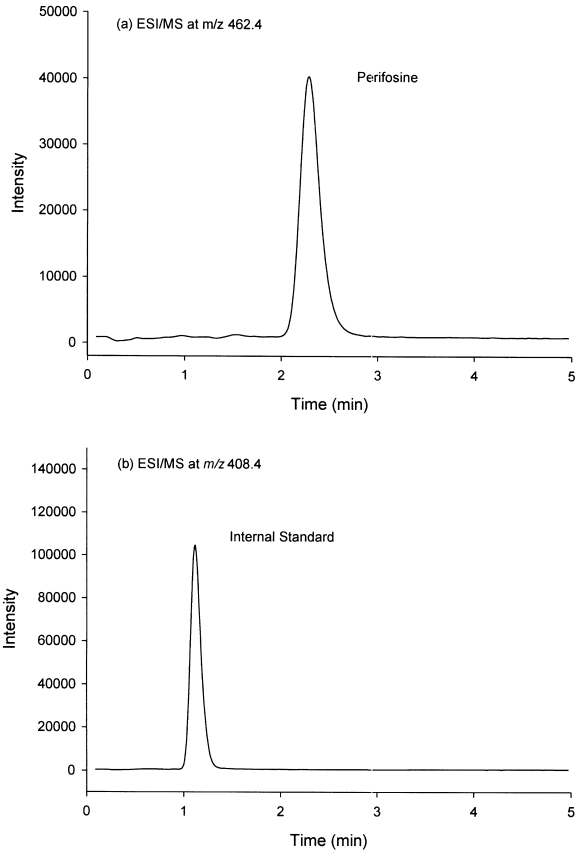


Fig. 4. Typical SIM chromatogram of a patient sample after perifosine oral dosing. The plasma concentration of perifosine was found to be 1585 ng/ml.

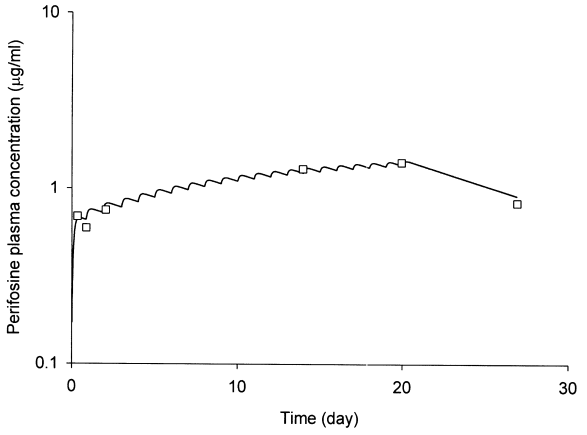


Fig. 5. Typical perifosine plasma concentration–time curve fitted by the ADAPT II model after loading dose of 300 mg followed by daily maintenance dose of 50 mg×21 days.

## 5. Application

After the validation study, the assay was applied to determine perifosine plasma concentrations from patient samples in a phase I clinical trial at the NCI. Fig. 4 shows the SIM chromatograms obtained from a patient sample after oral administration of perifosine. Fig. 5 shows a typical perifosine plasma concentration–time curve fitted by the ADAPT II model after a loading dose of 300 mg followed by daily maintenance dose of 50 mg×21 days.

## 6. Conclusion

The validated reversed-phase LC–MS assay was simple, rapid, reliable, and reproducible. The assay used simple sample preparation procedure with acetonitrile precipitation without evaporation step. Using this method, we have analyzed more than 200 patient samples and performed pharmacokinetic studies from a phase I clinical trial of perifosine.

## Acknowledgements

This work has been funded by the US Government.

## References

[1] J. Kotting, M.R. Berger, C. Unger, H. Eibl, Cancer Chemother. Pharmacol. 30 (1992) 105.
[2] N. Marschner, J. Kotting, H. Eibl, C. Unger, Cancer Chemother. Pharmacol. 31 (1992) 18.
[3] D. Berkovic, Gen. Pharmacol. 31 (1998) 511.
[4] S.M. Konstantinov, M.R. Berger, Cancer Lett. 144 (1999) 153.
[5] G.A. Ruiter, S.F. Zerp, H. Bartelink, W.J. van Blitterswijk, M. Verheij, Cancer Res. 59 (1999) 2457.
[6] H.H. Grunicke, K. Maly, F. Uberall, C. Schubert, E. Kindler, J. Stekar, H. Brachwitz, Adv. Enzyme Regul. 36 (1996) 385.
[7] S.M. Konstantinov, H. Eibl, M.R. Berger, Cancer Chemother. Pharmacol. 41 (1998) 210.
[8] K. Maly, F. Uberall, C. Schubert, E. Kindler, J. Stekar, H. Brachwitz, H.H. Grunicke, Anticancer Drug Des. 10 (1995) 411.
[9] R. Becher, O. Kloke, A. Fuger, K. Bremer, A. Drozd, U.R. Kleeberg, D. Fritze, K. Rieche, H. Sindermann, Onkologie 16 (1993) 11.
[10] C. Unger, R. Becher, K. Rieche, H.E. Wandler, G. Friedlich, L. Edler, Onkologie 16 (1993) 260.
[11] W.F. Steelant, E.A. Bruyneel, M.M. Mareel, E.G. Van den Eeckhout, Anal. Biochem. 227 (1995) 246.
[12] J.F. Brouwers, B.M. Gadella, L.M. van Golde, A.G. Tielens, J. Lipid Res. 39 (1998) 344.
[13] N.G. Knebel, S. Grieb, M. Winkler, M. Locher, E. van der Vlis, E.R. Verheij, J. Chromatogr. B 721 (1999) 257.
[14] M. Jemal, S. Rao, I. Salahudeen, B.C. Chen, R. Kates, J. Chromatogr. B 736 (1999) 19.
[15] D.V. McCalley, J. Chromatogr. A 794 (1993) 31.
[16] H.Y. Kim, T.C. Wang, Y.C. Ma, Anal. Chem. 66 (1994) 3977.
[17] A.C. Hogenboom, P. Speksnijder, R.J. Vreeken, W.M.A. Niessen, U.A.Th. Brinkman, J. Chromatogr. A 777 (1997) 81.
[18] K. Heinig, J. Henion, J. Chromatogr. B 732 (1999) 445.
[19] J. Zweigenbaum, K. Heinig, S. Steinborner, T. Wachs, J. Heinion, Anal. Chem. 71 (1999) 2294.
[20] M. Yao, V.R. Shah, W.C. Shyu, N.R. Srinivas, J. Chromatogr. B 718 (1998) 77.