Journal of Chromatography B, 869 (2008) 16–19

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of erufosine, the first intravenously applicable alkylphosphocholine, in human plasma by isotope dilution liquid chromatography–tandem mass spectrometry using a deuterated internal standard

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ARTICLE INFO

Article history:
Received 26 December 2007
Accepted 5 May 2008
Available online 10 May 2008

Keywords:
Erufosine
Isotope dilution
Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

ABSTRACT

A sensitive and specific liquid chromatography–tandem mass spectrometry method was developed and validated for the quantification of erucylphosphohomocholine (erufosine, $ErPC_3$) in pharmacokinetic studies. Nine-fold deuterated $ErPC_3$ was used as the internal standard. Following protein precipitation, reversed phase chromatography was performed. For analyte detection, electrospray ionization in the positive mode was applied. The mass transition m/z 504.4 > 139.1 was recorded for $ErPC_3$, and the transition m/z 513.7 > 139.1 for the internal standard, respectively. Good linearity with a correlation coefficient >0.99 was found for the range of 0.48–15 mg/L $ErPC_3$ in plasma (0.93–29.8 μM), the important range for clinical pharmacokinetic analysis. Interassay coefficients (n = 10) of variation between 4.2% and 5.5% were found for $ErPC_3$ pool samples with concentrations between 4.7 mg/L and 44.0 mg/L, respectively. The method has been used for analyses during a phase I clinical trial of $ErPC_3$.

1. Introduction

Alkylphosphocholines represent a novel class of lipid molecules with antiproliferative properties demonstrating strong antitumoral and antiprotozoal activity [1]. After integration into cellular membranes they interfere with the phospholipid metabolism and several signal transduction pathways. The most accepted "mode of action" is their ability to activate (trimerize) FAS-receptor subunits within the plasma membrane, followed by the induction of the apoptotic cascade [2,3]. Hexadecylphosphocholine (miltefosine), the lead compound of this substance class has been approved for the topical treatment of skin metastases in breast cancer (Miltex®, [4,5]) and the oral treatment of leishmaniasis (Impavido®, [6,7]).

Miltefosine as well as its analogue perifosine, which is currently investigated in several phase II studies for cancer treatment, are restricted to oral application for systemic treatment. Given intravenously they cause strong side effects such as haemolysis and thrombophlebitis. However, gastrointestinal side effects prevent from effective oral dosing. Therefore, convincing antitumor activity in clinical studies has not yet been described for this first-generation alkylphosphocholines [8–11]. This important limitation for oral dosing can be overcome by longer alkyl-chains with *cis*-mono-unsaturation. For instance, erucylphosphocholine contains an alkyl-chain with 22 carbon atoms and one *cis*-double bond in the <omega>-C-9 position. This structural variation allows parenteral administration and is even more effective in comparison to the first-generation alkylphosphocholines after oral application [12].

Erufosine (erucylphosphohomocholine, $ErPC_3$), which was further modified in the polar part of the molecule, now represents the first intravenously applicable alkylphosphocholine which has been administered in a clinical phase I trial in tumor patients (BfArM-Nr. 4020889/EL0104, Department of Internal Medicine III, Hospital of the University of Munich, Germany, ongoing). For pharmacokinetic measurements during this dose escalation study a method for sensitive $ErPC_3$ detection in human plasma samples was needed. Since the target analyte molecule does not contain a chromophore or an electroactive group, conventional detection techniques used in HPLC are not useful to monitor the $ErPC_3$ plasma concentration. Light scattering detection can basically be

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doi:10.1016/j.jchromb.2008.05.007

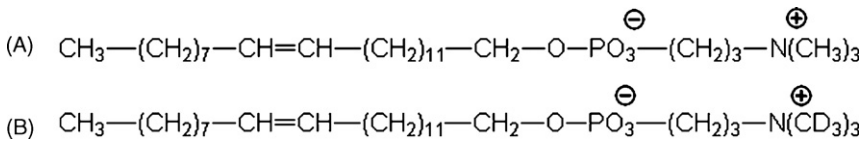

Fig. 1. Chemical structure of (A) $ErPC_3$ and (B) $ErPC_3$-D_9, internal standard.

used for the detection of alkylphosphocholines [13,14]. However, the sensitivity of light scattering detection is inferior compared to emerging mass spectrometry. For perifosine the quantification in plasma by mass spectrometry using hexadecylphosphocholine as an internal standard has been demonstrated [15]. To the best of our knowledge, there is no method reported in the literature for the quantitative determination of $ErPC_3$ applying liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). The establishment and application of a sensitive and reproducible method for determining $ErPC_3$ in human plasma by LC–ESI-MS/MS using a deuterated analogue as an internal standard is presented in this paper. This method will allow to generate pharmacokinetic data of $ErPC_3$ within clinical trials.

2. Experimental

2.1. Chemicals and standards

Water, methanol, acetonitrile and ethanol were obtained from Baker (Deventer, The Netherlands) and were of HPLC-grade.

2.2. Synthesis of the internal standard $ErPC_3$-D_9

$ErPC_3$ (MW 503.75) (Fig. 1A) is the (*N*,*N*,*N*-trimethyl)-propylammoniumester of erucyl-phosphoric acid. For the quantitative measurement of $ErPC_3$ in biological samples a deuterium labelled analogue has been prepared: deuterated $ErPC_3$ ($ErPC_3$-D_9) (MW 512.82) (Fig. 1B) and successfully used as internal standard. The introduction of the deuterium label into the propylammonium group has been performed by permethylation with CD_3–O–SO_2–CH_3 at slightly alkaline conditions.

In short, erucanol was phosphorylated with phosphorusoxychloride in the presence of triethylamine. The resulting erucyl phosphorusoxydichloride was reacted with propanolamin to form the respective erucyl-oxaza-phospholan. Ring fusion at slightly acidic conditions at the PN-bond resulted in erucyl-phosphopropylammium, which after permethylation with CD_3-mesylate gave $ErPC_3$-D_9 in a total yield of about 90%. The purity of the compound, as confirmed by high performance thin layer chromatography, was better than 99%.

2.3. Sample preparation

An aliquot of 50 μL plasma was spiked with 20 μL ethanol containing 20 mg/L $ErPC_3$-D_9 used as the internal standard in a 2 mL test tube. After vigorous mixing and equilibration for 20 min at room temperature, 1 mL of methanol/acetonitrile 9:1 (v/v) was added for protein precipitation. After centrifugation for 15 min at 16,000 × g, the clear supernatant was diluted 1:9 (v/v) with methanol/acetonetrile 9:1 (v/v) and transferred into an HPLC vial.

2.4. High performance liquid chromatography

A Waters Alliance 2795 HPLC system (Waters, Milford, USA) equipped with a ReproSil-Pur CN (20 mm × 4 mm I.D., 5 μm particle size) column (Dr. Maisch GmbH, Ammerbuch, Germany) kept at 30 °C was used. Isocratic elution was applied with 70% methanol and 30% 0.1% formic acid delivered at a flow rate of 0.9 mL/min. The eluate was transferred to the MS system via a T-piece and a split ratio of approximately 1:9. The target analyte and the internal standard compound had a retention time of approximately 1.1 min. The total run time was 4 min.

2.5. CID-MS/MS analyses

A Quattro Ultima Pt tandem mass spectrometry system was used (Waters, Milford, USA). It features a mass range of 0–4000 *m*/*z* with unit mass resolution. The instrument was run electrospray ionization in positive ion mode with a capillary voltage of 2.8 kV, a cone voltage of 35 V, a source temperature of 90 °C, a desolvation gas temperature of 350 °C. Low energy collision induced dissociation (CID) ion scans of the protonated molecules ($ErPC_3$, *m*/*z* 504.4; ErPC3-D_9, *m*/*z* 513.7) were recorded (Fig. 2; collision energy

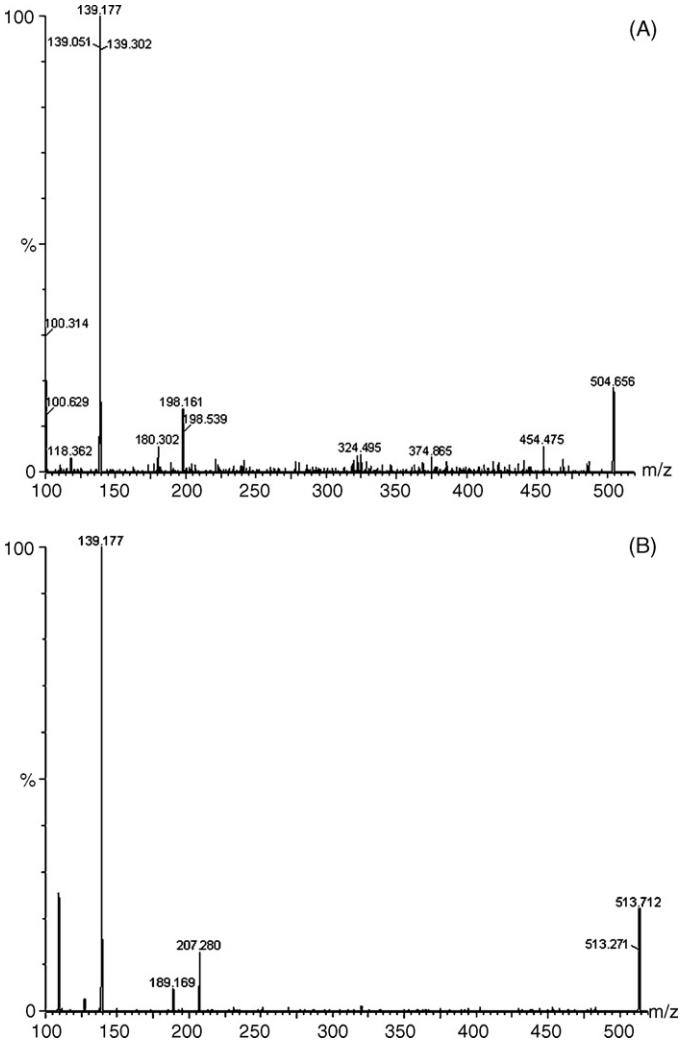

Fig. 2. Collision induced mass spectra of (A) $ErPC_3$ and (B) $ErPC_3$-D_9, internal standard.

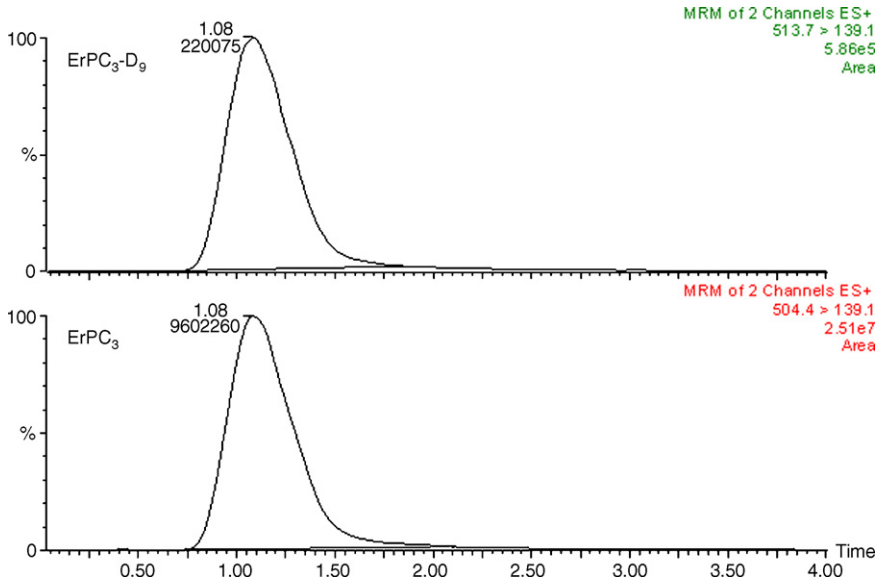

Fig. 3. Quantification of $ErPC_3$ in human plasma samples: representative chromatogram (plasma concentration 2.1 mg/L).

30 V). For quantification of ErPC3 in multiple reaction monitoring (MRM) the mass transition 504.4 > 139.1 was monitored for the target analyte, and the transition 513.7 > 139.1 was monitored for the deuterated internal standard compound, respectively.

2.6. Calibration

Drug free plasma was spiked with a stock solution of $ErPC_3$ in methanol to obtain a concentration of 15 mg/L. This stock calibrator was diluted serially 1:1 (v/v) with drug free plasma in five steps, resulting in a bottom calibrator with a concentration of 0.48 mg/L. The calibrator materials were aliquoted and stored at −80 °C. Six point calibration was performed in all analytical series. No weighting of axis transformation was applied.

2.7. Method validation

Plasma samples obtained from study patients after administration of $ErPC_3$ were used to prepare three plasma pools at different concentration levels. Quantification of $ErPC_3$ concentration was done in 10 independent analytical series to calculate the interassay coefficient of variation.

In order to investigate the recovery of the sample preparation and to test for relevant ion suppression or ion enhancement effects, we submitted 10 plasma samples from not $ErPC_3$ treated patients and a pure solution of 5% methanol, respectively, to the analytical process. The mean areas of the internal standard peaks were compared (the 5% methanol sample was analyzed in five-fold determination).

To verify the specificity of the method, residual plasma samples from 30 intensive care patients not receiving $ErPC_3$ were analyzed in absence of internal standard compound in the precipitation solution; the chromatograms were inspected for the presence of any peak signal in the MRM trace of the target analyte and the internal standard compound, respectively.

To rule out carry over effects in the method, a plasma sample spiked to an analyte concentration of 300 mg/L was injected three times followed by the injection of methanol/water 1:1 (v/v) as a sample; the chromatogram of this sample was inspected for peak signals.

The bench-top stability within 24 h at room temperature as well as the freeze–thaw stability of $ErPC_3$ in human plasma up to three cycles was acceptable with less than 10% loss of $ErPC_3$. Also the processed samples were stable at 4 °C for at least 96 h with no significant loss of $ErPC_3$ ($\leq$10.0%).

3. Results and discussion

First experiments with the analogous compound oleoylphosphocholine used as the internal standard were disappointing since the drift in the results observed for longer analytical series was more than 10% (data not shown). We therefore synthesized a deuterated molecule of the target analyte applicable as internal standard for the quantification of $ErPC_3$.

Applying a nine-fold deuterated $ErPC_3$ we were able to develop a robust and convenient method for the quantification of $ErPC_3$ in human plasma samples. A representative chromatogram obtained from a clinical sample is given in Fig. 3. Correlation of determination was >0.99 in the calibration of all 10 validation series, demonstrating a linear response of the method within a concentration range from 0.48 mg/L to 15 mg/L. This analytical window has been chosen as this is typically the effective serum concentration of $ErPC_3$ in preclinical models [16,17]. In human pharmacokinetic studies of perifosine, maximum plasma levels of less than 10 mg/L have been observed [18,19]. For the measurement of the perifosine plasma concentration, a LC–MS/MS method with hexadecylphosphocholine as the internal standard was used [15].

For the three pool samples investigated in the precision study the following mean concentrations and coefficients of variation (CV) were found: pool A, 44.0 mg/L, CV 4.2%; pool B, 10.0 mg/L, CV 4.8%; pool C, 4.7 mg/L, CV 5.5%.

The signal-to-noise ratio of the lowest calibrator (0.48 mg/L) was found between 97:1 and 142:1 within these series.

The mean peak area of the internal standard compound found in pure 5% methanol solution analyzed as a sample was 122.172; in 10 drug free plasma samples a mean area of 124.963 was found, thus ruling out relevant ion suppression effects and substantial loss of analyte during protein precipitation which is performed for sample preparation.

The specificity of the method could be demonstrated with plasma samples of 30 patients receiving multiple medications but

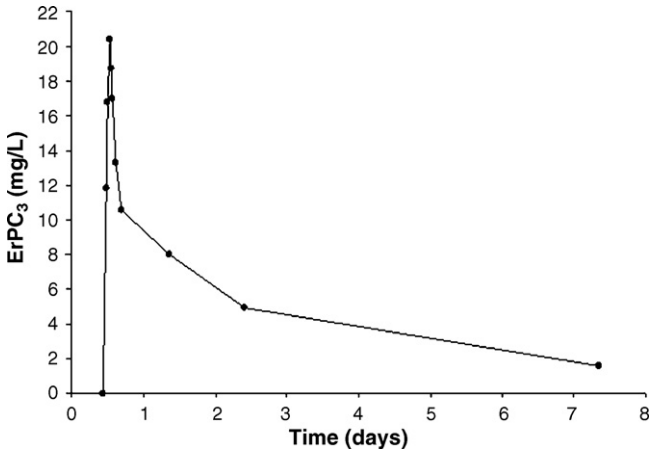

Fig. 4. Representative plasma concentration–time curve after intravenous administration of $ErPC_3$ 3.74 mg/kg b.w. $ErPC_3$ was dissolved in 1000 mL NaCl 0.9% and given as continuous infusion over 2 h.

not $ErPC_3$. After sample processing without the internal standard, no peak signals have been found in the two MRM traces recorded. Furthermore, no peak signal was found in the carry over investigation.

In summary we conclude that the method described herein is applicable in pharmacokinetic studies of the novel anticancer compound $ErPC_3$.

4. Application

After the validation study, the assay was applied to determine $ErPC_3$ plasma concentrations from patient samples in a phase I clinical trial at the Hospital of the University of Munich. Fig. 4 shows a typical $ErPC_3$ plasma concentration–time curve after a single intravenous administration of 3.74 mg $ErPC_3$ per kg body weight using continuous infusion over 2 h.

Acknowledgement

The authors would like to thank Mrs. K. Wachholz for the excellent technical assistance.

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