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Quantification of perifosine, an alkylphosphocholine anti-tumour agent, in plasma by pneumatically assisted electrospray tandem mass spectrometry coupled with high-performance liquid chromatography

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Abstract

An HPLC assay with tandem mass spectrometric detection in the positive-ion Turbo-Ion-Spray™ (TISP) mode for the fast and sensitive determination of perifosine ((**I**), D-21266) in human plasma was developed, utilising the structural analogue, miltefosine ((**II**), D-18506), as internal standard. Automated solid-phase extraction of diluted plasma samples, based on 250-μl plasma aliquots, at pH 6.5, allowed a reliable quantification of perifosine down to 4 ng/ml. Injection of 200 μl of plasma extracts onto a 100×3 mm normal-phase analytical column at a flow-rate of 0.5 ml/min provided retention-times of 2.4 and 2.1 min for perifosine (**I**) and the internal standard (**II**), respectively. The standard curves were linear from 4 to 2000 ng/ml using weighted linear regression analysis ($1/Y^2$). The inter-assay and intra-assay accuracies for the calibration standards were within +0.9% and −0.2%, exhibiting precisions (C.V.) of ±6.5 and ±7.3%, respectively. Up to 100 unknowns may be analysed each 24 h per analyst.

Keywords: Perifosine; Alkylphosphocholine

1. Introduction

Perifosine ((**I**), D-21266), belongs to a novel class of alkylphosphocholine anti-tumour agents and is currently tested in patients with solid tumours in clinical phase-I trials. It is structurally related to miltefosine ((**I**), D-18506) the lead compound of this class of drugs [1,2]. Proof of the broad antineoplastic activity of phospholipid analogue compounds was given in the early 1990s [3]. Based on an increased in vitro potency and selectivity against the growth of breast cancer, prostate, colon and larynx carcinoma cells perifosine (**I**) was intended to be developed as 'follow-up compound' for miltefosine (**II**) [4]. In addition, it was demonstrated that perifosine (**I**) evokes less gastro-intestinal side effects such as acute nausea and vomiting after oral administration to humans [5]. Owing to its structural similarity to physiological membrane components, alkylphosphocholine compounds are thought to exert their cytotoxic action on tumour cells by interactions with vital cell membrane functions. The mode of action is considered to be related to the inhibition of protein

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kinase C, phospholipase C, and the phosphatidylcholine biosynthesis [6].

Conventional quantitative HPLC assays with ultraviolet/fluorescence or electrochemical detection for perifosine were impossible or difficult to develop due to the lack of chromophores and electroactive groups in the molecule. Another analytical technique for the determination of miltefosine (**II**) in human plasma using HPTLC (high-performance thin-layer chromatography) provided insufficient sensitivity (limit of quantification: 400 ng/ml). Additionally, this method comprised long chromatographic run-times and labour-intensive work-up procedures, which might not be favoured in large clinical pharmacokinetic studies [7].

HPLC with tandem mass spectrometry using pneumatically assisted electrospray with a heated nitrogen probe (TISP) in combination with automated solid phase extraction (SPE) should provide the required sensitivity, straightforward sample workup, and fast capability for trace level quantification of perifosine (**I**) in human plasma.

Our aim was to develop a fast mass spectrometric method using HPLC–TISP–MS–MS in order to be able to analyse perifosine (**I**) in human plasma samples in the nanogram per millilitre range during an ongoing pharmacokinetic study in tumour patients. In order to enable us to report plasma concentrations to the clinical pharmacology unit as fast as possible we envisaged having an efficient sample workup procedure without time-consuming sample concentration/redissolving and sample transfer steps.

2. Experimental

2.1. Materials

1,1 - Dimethyl - 4 - [[(octadecyloxy)hydroxyphosphinyl]oxy]-piperidinium inner salt (**I**) (perifosine,

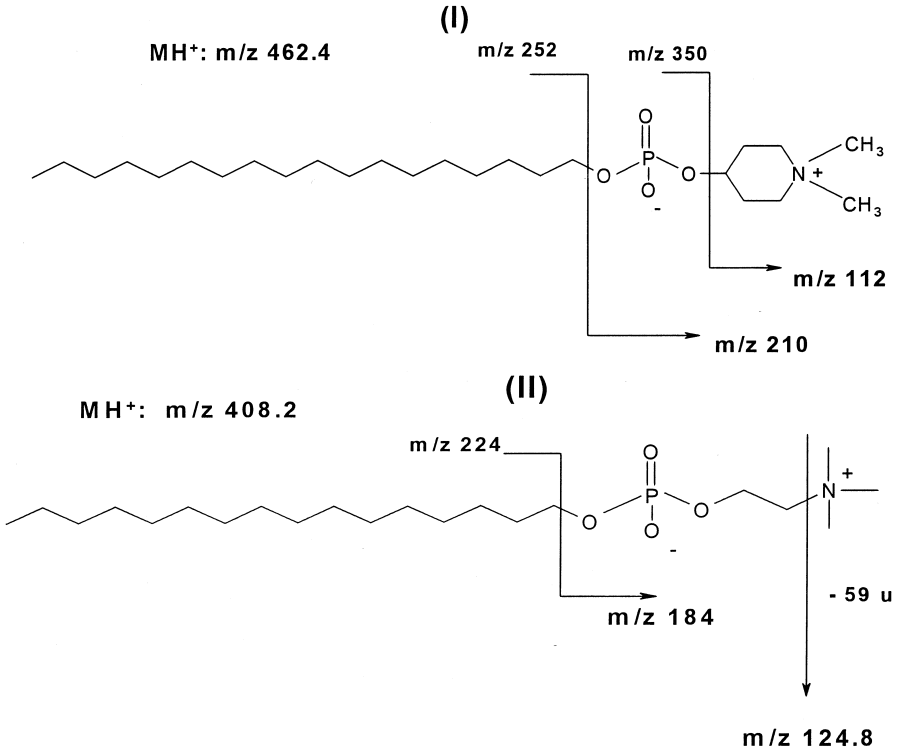


Fig. 1. Structures of perifosine (**I**) and the internal standard (D-18506, (**II**)). The major losses of *m*/*z* 350, 252 (**I**), and the loss of *m*/*z* 224 with the subsequent loss of *m*/*z* 59 (**II**) to yield the major fragments, are indicated.

D-21266) and 2-[[(hexadecyloxy)hydroxyphosphinyl]oxy] - *N,N,N* - trimethylethylammonium inner salt (**II**), the structural analogue, (miltefosine, D-18506) (Fig. 1), used as internal standard, were synthesised within the Department of Chemical Research at ASTA Medica (Frankfurt, Germany). Acetonitrile and methanol were obtained from Merck (Darmstadt, Germany) and were of HPLC-grade. All other reagents and chemicals were of either HPLC- or analytical-grade and were used without any further purification.

2.2. Method

2.2.1. Sample preparation

Blood samples were collected into heparinised or EDTA pre-treated tubes, cooled in ice and centrifuged for plasma (3000 *g*) as soon as possible (<1 h). Frozen samples were thawed at room temperature.

To 250 μl of plasma, 100 ng internal standard in 25 μl of 50% aqueous methanol (v/v) and 750 μl of ammonium acetate (0.8 *M*, pH 6.5) were added. Ammonium acetate (0.8 *M*, pH 6.5) was added to each vial, followed by brief vortex mixing. The solid-phase extraction (SPE) was carried out by an automatic sample processor (RapidTrace™, Zymark, Hopkinton, MA, USA) running overnight using 1-ml – Phenyl Bond Elut® – SPE cartridges (Varian, Harbor City, CA, USA). Conditioning of the SPE cartridges with 1 ml each of acetonitrile and 0.8 *M* aqueous ammonium acetate (pH 6.5) was followed by loading of 1.0 ml of plasma dilution, washing with 1 ml of 50% aqueous methanol and elution with 1.5 ml of 99.9% methanol–0.1% triethyl amine. The extracted samples were eluted into the appropriate HPLC autosampler vials by the RapidTrace™ robot. Volumes of 200 μl from the eluate were injected onto the HPLC–MS system. The presence of 0.1% of triethyl amine in the eluate had no major effect in terms of sensitivity for the positive electrospray ionisation process.

2.2.2. HPLC–TISP–MS–MS

HPLC was performed on a ChromSpher 5 SI, 100×3 mm analytical glass column with an SS 10×2 mm Chromsep guard column (Chrompack®, Bergen op Zoom, The Netherlands) using a Perkin-Elmer quarternary pump (Perkin-Elmer, Norwalk, CT, USA) and a Merck–Hitachi LaChrom programmable autosampler (Merck). The injection volume was 200 μl onto the column. The mobile phase consisted of 40% acetonitrile–30% methanol and 30% 0.008 *M* ammonium acetate (pH 6.5) (v/v/v) and the flow-rate was 0.5 ml/min.

Mass spectrometric detection was carried out using a PE Sciex API 300 series triple quadrupole instrument upgraded to a 365-version model containing an improved curtain plate (PE Sciex, Concord, Ont., Canada) operating in the positive Turbo-Ion-Spray™ mode. Selected reaction monitoring (SRM) was employed using nitrogen (high purity, 5.0 grade) as collision gas at a pressure setting of 3 units, with a collision energy of 50 eV. The TISP–MS operating parameters can be summarised as follows: turbo heater temperature 475°C, nebulizer gas (nitrogen, high purity, 5.0 grade) at 7000 ml/min (set at 8 units), auxiliary gas (nitrogen, high purity, 5.0 grade) flow set at 10 units (exact flow-rate in litres per minute were not measured), curtain gas set at 3 units, ion spray voltage at 4800 V, orifice voltage (OR) at 30 V. Precursor to product ion transitions were monitored at m/z 462.4 to m/z 112.0 for perifosine (**I**), and at m/z 408.2 to m/z 124.8 for the internal standard (**II**) with a dwell-time of 200 ms per ion. The pause time was set at 2 ms.

Data were acquired by the PE-Sciex software (MassChrom 1.0) and peak areas measured using the PE-Sciex quantification software (MacQuan, version 1.5).

2.2.3. Preparation of calibration curve

Calibration samples and quality control samples (QC) were prepared with each batch of unknown test samples to cover the range of 4–2000 ng/ml. To 250 μl of blank human plasma 0.001, 0.0025, 0.005, 0.0125, 0.025, 0.05, 0.1, 0.25 and 0.5 μg of analyte (perifosine) in standard dilutions of 50% aqueous methanol in volumes of 25 μl were added to give a calibration range from 4 to 2000 ng/ml. The internal standard (100 ng) was added to each tube in volumes of 25 μl. After brief vortex mixing, the samples were submitted to automated SPE as described above.

2.2.4. Preparation of quality control samples

QC samples were prepared by an independent analyst at three concentrations towards the top, the middle, and bottom of the calibration curve (QC1, 20 ng/ml; QC2, 200 ng/ml; and QC3, 1600 ng/ml). QC samples were stored for 6 months at −20°C, thawed before analyses, and analysed together with the unknown test samples in every batch.

2.2.5. Quantification

Calibration curves were constructed by plotting peak area ratios of the analyte and the internal standard against the analyte's concentrations. The results of the raw data were transferred to the PE Sciex quantification software (MacQuan™, version 1.5). MacQuan was used to calculate the weighted linear regression fit of the peak areas of the standards of perifosine relative to the internal standard. The weighted ($1/Y^2$) linear regression line was fitted over the 500-fold concentration range. It was found that the weighting by the response-squared ($1/Y^2$) factor yielded better accuracies for the back-calculated values of the QC samples in comparison with to concentration ($1/X$) or concentration-squared ($1/X^2$) weighting. Drug concentrations in the unknown and quality control samples were calculated from this line.

3. Results and discussion

3.1. Mass spectrometry

Triple-quadrupole mass spectrometers with pneumatically assisted electrospray sources in combination with heated nitrogen as auxiliary gas (TISP) for HPLC–MS–MS are now routinely used for quantitative and qualitative determinations of compounds and drugs (and metabolites) in the biopharmaceutical area [8, 9] as well as in environmental analysis [10]. Electrospray is more likely to be utilised for the analyses of thermally labile compounds of biological origin, because of its extremely mild ionisation [9]. Conventional electrospray ionisation is usually used for determinations of various compounds at low flow-rates (i.e. <0.3 ml/min). Moreover, electrospray at flow-rates in the low μl/min or nl/min range are well established for the qualitative determination of peptides [11], proteins [12] and other macromolecules [13, 14].

However, the TISP mode appears to be favoured in biopharmaceutical analysis if thermal stability of analytes is assured, and high sample throughput assays with relatively crude sample work-up procedures are required. Short retention times of analytes can also easily be established by using high flow-rates. In particular, the TISP interface provides increased sensitivity over the conventional electrospray interface (ESI) through the use of the heated nitrogen probe (turbo gas) which enables improved desolvation of analyte molecules by turbulent mixing of the solvent spray at temperatures up to 500°C [15]. Meanwhile, it has been reported by both the manufacturer and several other users of the TISP interface that operating this device at temperatures above 400°C for bioanalytical applications with long run-times might not be advisable due to the increased likelihood of premature blockages and degradation of PEEK® tubing and Teflon sleeves. Nevertheless, we did not observe premature blocking of the TISP interface when operating at 475°C.

The lack of sufficient sensitivity of traditional HPLC-ultraviolet/fluorescence detections in combination with labour intensive work-up procedures was the driving force for the development of an HPLC–MS–MS assay with a simple, straightforward, and automated solid-phase extraction.

The deficiency of sufficient selectivity in single MS instruments made it necessary to use a triple-quadrupole mass spectrometric approach. We also envisaged by the application of tandem mass spectrometric detection to minimise the requirements for sample pre-treatment. In order to obtain an extracted plasma sample that is clean enough to enter the mass spectrometer's interface, solid-phase extraction has already proved its suitability in combination with tandem mass spectrometric detection in many reported pharmaceutical and bioanalytical applications [16,17].

Fig. 2a shows the TISP product ion mass spectrum of perifosine (**I**) using the protonated molecular ion (m/z 462) as the precursor ion. Two main fragments can be observed by alpha-cleavages (Fig. 1). The first fragment ion at m/z 210 is produced by a loss of 252 u from the protonated molecular ion representing the C_{18}-alkyl side chain. The most abundant frag-

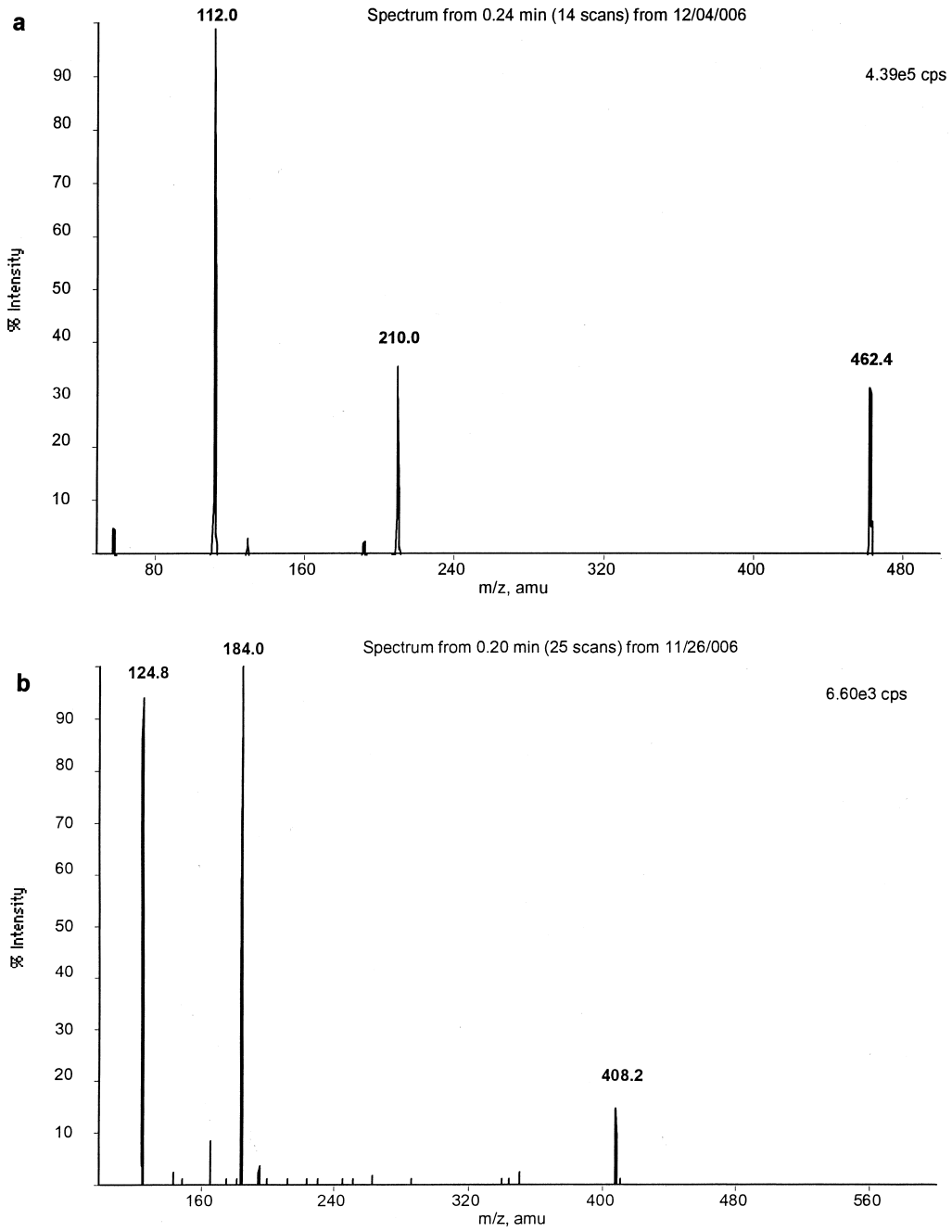


Fig. 2. (a) Positive product ion mass spectrum of perifosine (**I**) precursor ion at m/z 462.4; ~40 pg presented to the source (HPLC–TISP–MS–MS mode). (b) Positive product ion mass spectrum of the internal standard (**II**) precursor ion at m/z 408.2; ~40 pg presented to the source (HPLC–TISP–MS–MS mode).

ment is formed by the cleavage of the phosphonated alkyl side chain yielding the piperidinyl product ion, at m/z 112, used for quantification. The mass spectrometer's parameters (e.g. lens voltages given in the 'tune-file') were set such to optimise the abundance of the major fragment ion at m/z 112 of perifosine (**I**). In particular, the orifice voltage (OR), which can cause general and 'less controlable' fragmentation was set at a relatively low value (OR=30 V). Thus, an optimum yield of the pseudo-molecular ion (m/z 462.4) of perifosine was allowed to enter the region of the first mass analyser (Q1). Applying these for perifosine optimised mass spectrometric conditions on the internal standard (**II**), we made the following observations: Analogous to perifosine the internal standard (**II**) shows two major fragment ions derived from the protonated molecular ion at m/z 408.2 (Figs. 1 and 2b). After cleavage of the C_{16}-alkyl side chain (loss of 224 u) a fragment ion at m/z 184 can be observed. This fragment ion can be correlated to the formula of the remaining quarternary trimethyl amine moiety linked to the phosphonate group via an ethylene group ($C_5H_{15}NPO_4$). The product ion used for the quantification (m/z 124.8) is subsequently formed from the above mentioned precursor ion ($C_5H_{15}NPO_4$) by a cleavage of trimethyl amine (59 u).

Our approach in optimising the analytical product ions was to tune the mass spectrometer's parameters for relatively lower mass resolution in the first mass analyser Q1 (Δm_{Q1} of the protonated molecular ion at half peak height was 2 u), and for higher mass resolution in the second mass analyser Q3 (Δm_{Q3} of the product ion at half peak height was 0.7 u). Hence, a gain in sensitivity was achieved by two factors, first, by setting the OR voltage at a relatively low value of 30 V, and thus increasing the yield of protonated molecular ions and, second, by adjusting the resolution of the first mass analyser to approximately 2 Da. The required selectivity that enabled bioanalysis of perifosine (**I**) in a complex matrix like plasma was achieved by the collisionally induced dissociation of the protonated molecular ions with nitrogen as collision gas in the collision cell (Q2), and subsequently, the setting to unit mass resolution in the second mass analyser (Q3). Optimisation of the mass spectrometer's parameters regarding the optimum fragmentation and formation of the analytical product ions in order to gain sensitivity was performed for perifosine (**I**) only. The such obtained MS settings were then subsequently accepted for the mass spectrometric analysis of perifosine (**I**) in combination with the internal standard (**II**) in the selected reaction monitoring (SRM) mode. This approach in tuning the mass spectrometer has proved to be suitable for the quantification of perifosine since negligible mass 'carry-over' from the plasma matrix (i.e. proteins and salts) or the internal standard was observed, operating in the selected reaction monitoring (SRM) mode. In Fig. 3 a typical product ion chromatogram for a blank plasma sample ('spiked' with internal standard only) is depicted, illustrating the mass contribution(s) for both, the matrix and the internal standard to the ion-trace of perifosine (**I**) (m/z 462.4 $\rightarrow$ 112.0). However, this signal appearing already in the blank plasma sample containing the internal standard (**II**) only was considered to be still acceptable for bioanalytical quantification of perifosine (**I**) in plasma down to 4 ng/ml .

3.2. Chromatography

In order to develop an assay with high sample throughput and to obtain maximum response for the analytes we required as short a retention time as possible. The great advantage of having analytes with different molecular masses and similar retention times in combination with tandem mass spectrometric detection, enabled us to aim at a minimum separation that might remove some salts or matrix components that can suppress or interfere with the analyses from the target components, while maintaining good sample throughput. The 'chromatographic aim' was to localise the analyte and the internal standard at relatively short retention times without the need to implement long chromatographic run-times accompanied with the drawback of decreased sample turnover. Previous experiments have shown that it is paramount to implement at least some crude, minimal chromatographic separation of perifosine for adequate sensitivity and to prevent blockages of the TISP interface at the front end of the mass spectrometer, in particular at the orifice and the skimmer region. Another important reason why the sample should be separated from some con-

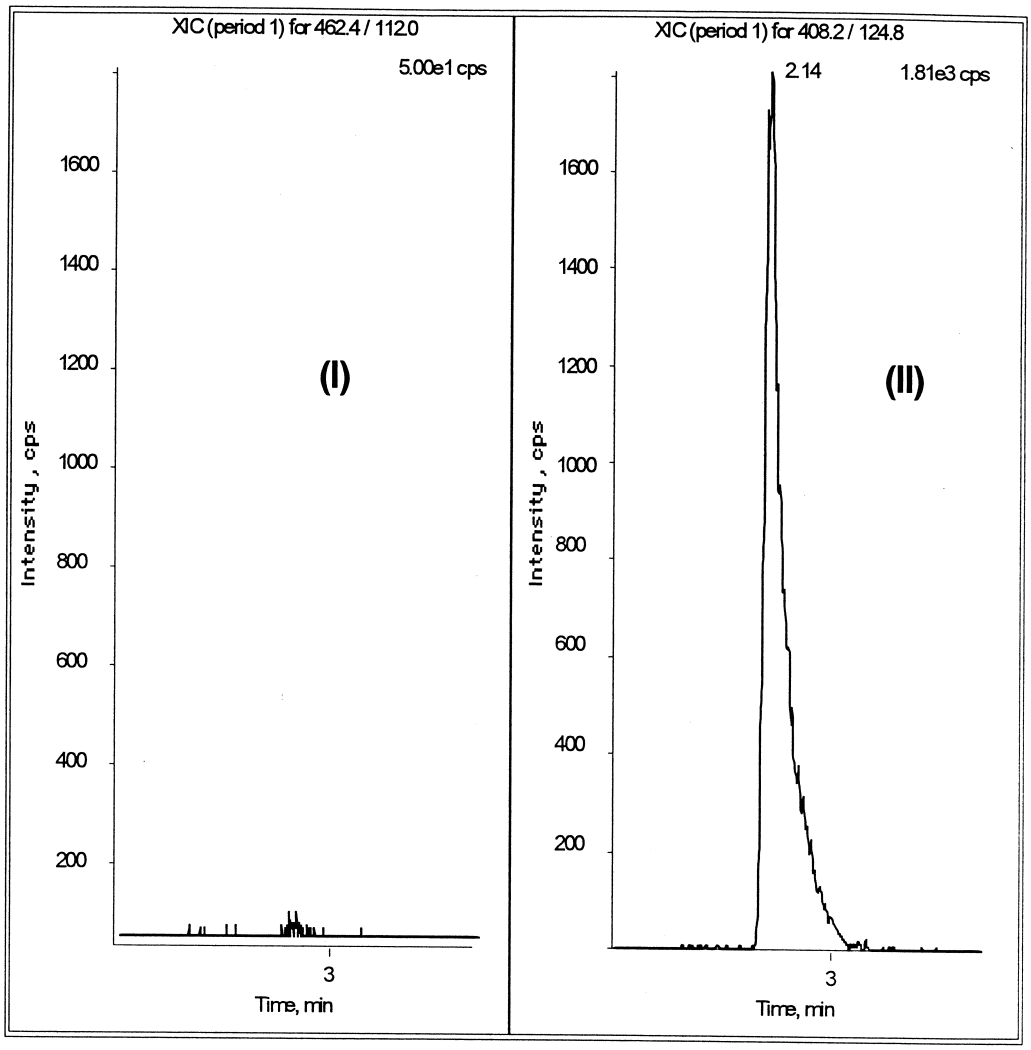


Fig. 3. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of perifosine (**I**) and the internal standard (**II**) in plasma extracts of a blank plasma sample 'spiked' with (**II**) at a concentration of 400 ng/ml. Intensity: cps (counts per scan).

taminants is that the ions formed in the electrospray interface may be depleted when sample and large excess of other components coelute without any guard columns. As a consequence, proper formation of protonated sample and internal standard molecules could no longer take place, and quantification with reproducible precision and accuracy might not be possible [18]. Moreover, matrix effects in quantitative LC–MS–MS analyses reported by various authors appear to affect the ionisation in the TISP mode to a higher extent compared to the atmospheric pressure chemical ionisation [19].

After testing several of the immense variety of chromatographic columns available on the market, we obtained the best results regarding relatively short retention times, and sufficient separation from plasma matrix-derived components with the Chromsep 5 μm SI 100×3 mm (I.D.) glass column

(Chrompack®). This 'normal-phase' silica column provided us with the best compromise in terms of reproducibility of chromatographic peak shape, flow-rate, high sample throughput, and cost effectiveness. All experiments to achieve acceptable chromatographic peaks on commonly applied 'reversed-phase' silica columns failed or revealed unsatisfactory results. This observation might be due to a poor retention of the quarternary ammonium molecules on commonly used 'reversed-phase' packing material. In order to achieve optimum mass spectrometric sensitivity for the analytes we decided not to modify the LC-eluent composition. Hence, this compromise of sensitivity and chromatographic peak shape was accepted and no further attempts were made to reduce the observed 'peak-tailing' (Figs. 3–5). Furthermore, it turned out during routine analysis that one column could be used for only approximately

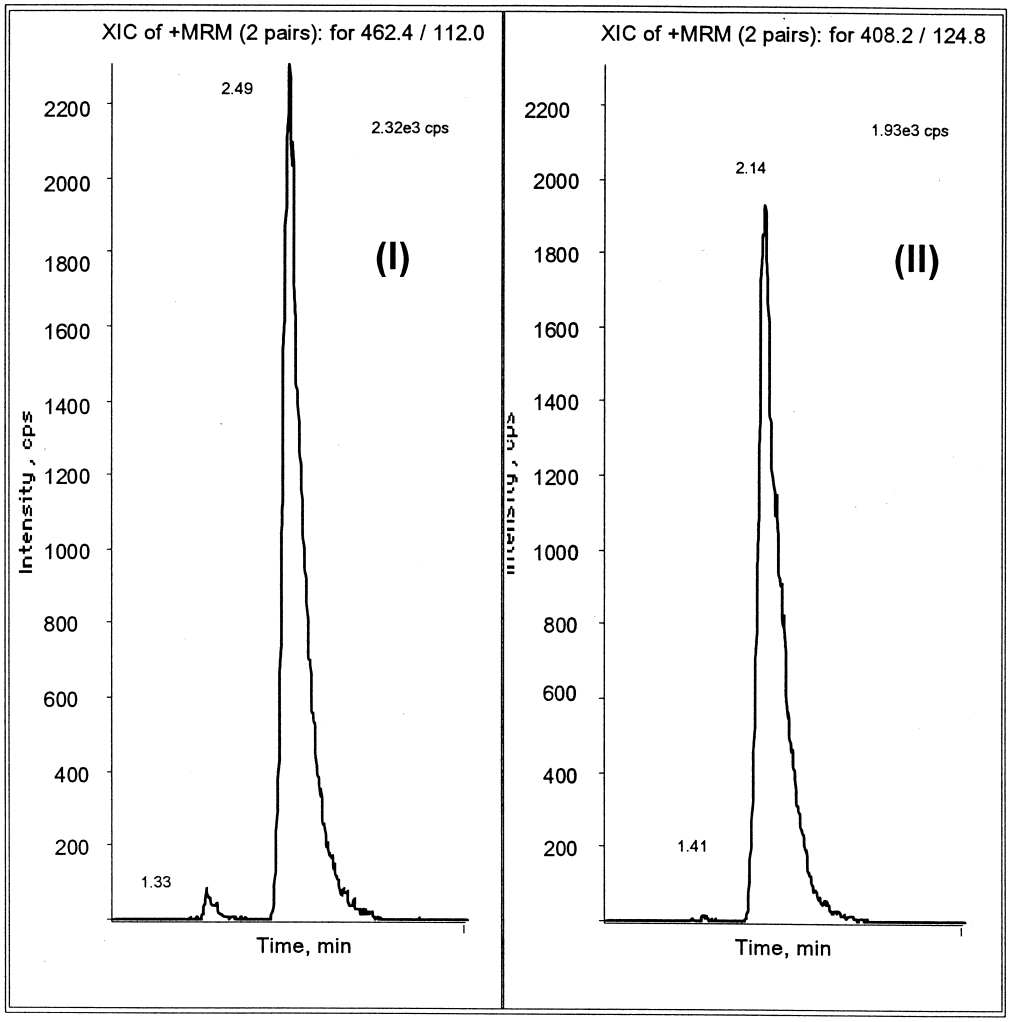
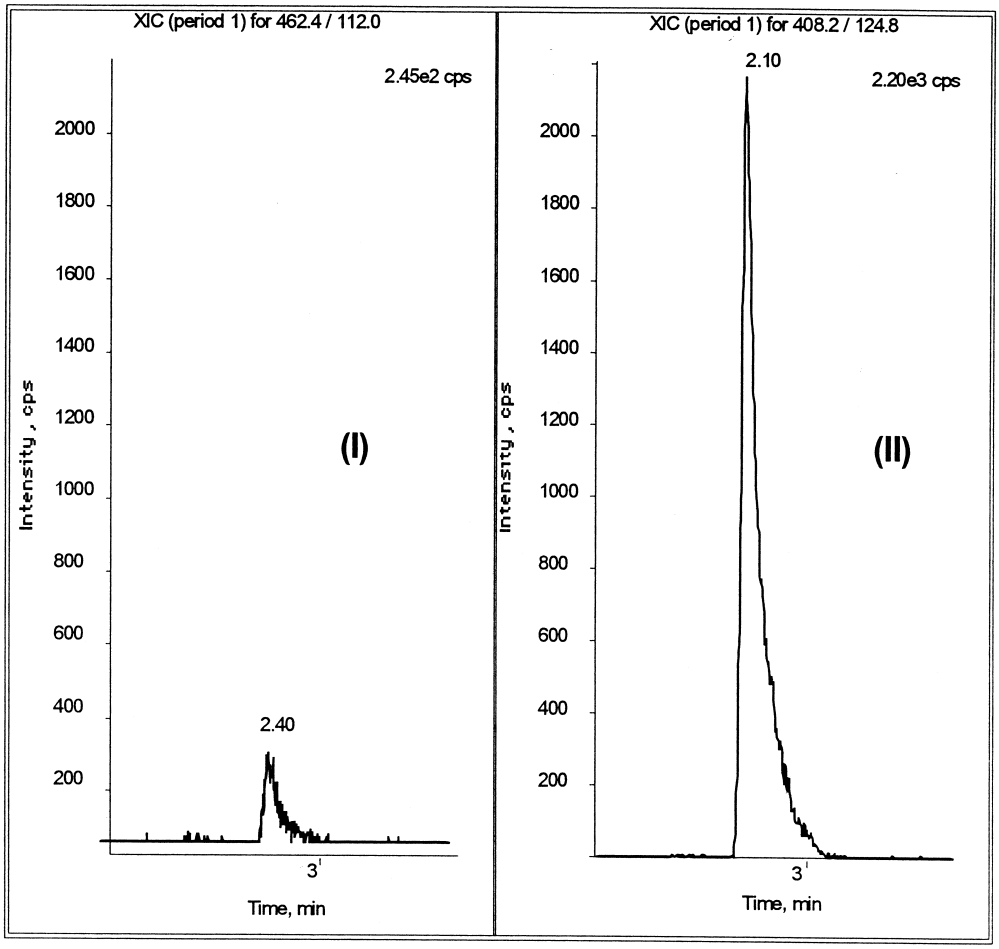


Fig. 4. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of perifosine (**I**) and the internal standard (**II**) in plasma extracts of a blood sample taken from a patient approx. 8 h after oral administration of 100 mg perifosine. The derived concentration of perifosine was back-calculated at ~657 ng/ml plasma. The concentration of (**II**) was 400 ng/ml. Intensity: cps (counts per scan).



Intensity, cps: counts per scan

Fig. 5. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of perifosine (**I**) and the internal standard (**II**) in plasma extracts from a standard of the calibration curve at the limit of quantification (LOQ, 4 ng/ml) and the internal standard at a concentration of 400 ng/ml. Intensity: cps (counts per scan).

150 samples without deterioration, such as further increased 'peak-tailing' and decreasing signals, which we observed after the injection of approximately 200 extracted plasma samples. In order to prolong the life-time of these columns, efforts were made by washing these with acetonitrile, acetonitrile–water (1:1, v/v), methanol, and methanol–water (1:1, v/v). Even replacements of the guard columns offered only marginal improvements of the chromatographic peaks for the analytes. As a result, the analytical columns could not be used for further analyses even after washing with acetonitrile by reversed column flow. These observations might also indicate that the plasma sample extracts analysed after SPE were still relatively crude and caused such a fast deterioration of the stationary phase.

Pneumatically assisted electrospray (ionspray) ionisation interfaces behave as concentration- or mass-sensitive detectors [20]. Based on that fact and for the purpose of maximum sensitivity for the analytes, a chromatographic column with a length of 100 mm and 3 mm I.D. was chosen in combination

with a flow-rate of 0.5 ml/min. At flow-rates >0.5 ml/min shorter retention times of approximately 1.5 min were achieved, but accompanied with a drop in sensitivity. However, in agreement with the literature on 'concentration/mass-sensitive detection' of the ionspray process we observed a significantly decline in sensitivity of up to 5-fold at flow-rates ranging from 0.6 to 1 ml/min for perifosine (**I**) and the internal standard (**II**). Thus, implementing a flow-rate of 0.5 ml/min using a 100×3-mm (length×I.D.) column and a mobile phase with 70% of organic contents (40% acetonitrile–30% methanol in 0.008 *M* ammonium acetate) in combination with the TISP interface provided the best compromise regarding mass spectrometric detectability (i.e. sensitivity), chromatographic performance, and analytical ruggedness for the perifosine analysis.

The lack of a suitable stable labelled form of perifosine (**I**), forced us to implement another, structurally related derivative as internal standard. The reasons for choosing (**II**) as the internal standard were its similarities in chromatographic and mass spectrometric behaviour to perifosine in terms of the lipophilicity and recovery from biological matrices after solid-phase extraction from Phenyl-SPE cartridges (recovery of perifosine and (**II**) are approximately 60%). The internal standard (**II**) carries, instead of the C_{18}- just a C_{16}-side-chain linked as an ester to the phosphate moiety. Additionally, (**II**) contains in comparison to perifosine (**I**) a quarternary trimethyl-amino function linked to the phosphate via an ethylene bridge. Whereas perifosine possesses a quarternary piperidinyl group in the analogous position (Fig. 1).

The relatively poor recovery of approximately 60% from the SPE cartridges might be due to structural nature of the phosphocholine structure of the analytes, (**I**) and (**II**). Especially, high interactions with glass surfaces, and to a lower extent with plastic surfaces of diluted plasma samples containing perifosine (**I**) and the internal standard (**II**) could be observed during method development. Hence, polypropylene vials and Peek and polyethylene tubing were used for sample preparation and chromatography. However, this recovery was considered to be satisfactory when simultaneously keeping the advantage of an unattended and automated sample preparation.

Selected ion chromatograms of a plasma extract from a blood sample taken 8 h after administration of 100 mg of perifosine to a patient, are shown in Fig. 4. At these high concentrations of perifosine in study samples, calibration samples and QC samples, we observed small matrix-related peaks at retention times of 1.3–1.4 min at the traces of perifosine (**I**) (m/z 462.4 → 112) and the internal standard (**II**) (m/z 408.2. → 124.8) Nevertheless, these peaks did not interfere with the peaks used for the quantification. In order to illustrate the high selectivity of mass spectrometric detection, selected ion chromatograms of a blank plasma sample 'spiked' only with (**II**) are given in Fig. 3. As a result, negligible contribution occurs at the retention time of perifosine (**I**). Typical chromatograms of a standard at the limit of quantification (LOQ, 4 ng/ml) are presented, revealing a reproducible peak for the analyte (**I**) (Fig. 5) with acceptable assay performances for routine analyses in biological matrices (see Tables 1 and 2.

The analyte (**I**) and the internal standard (**II**) elute at retention times of approximately 2.4 and 2.1 min, respectively. The cycle time per sample was 5 min. The detector response for perifosine was linear over the range from 4 to 2000 ng/ml. The limit of detection was assessed after injection of 10 standard samples at concentrations near the lower limit of quantification (LOQ, 4 ng/ml) and subsequent extrapolation to a signal to noise ratio of $S/N=3$. Accordingly, a limit of detection of approximately 2 ng/ml was calculated.

Typical calibration curves for perifosine comprised a mean slope of 0.008 ($n=5$; relative standard deviation (R.S.D.=0.002) with a mean intercept of 0.016 (R.S.D.=0.006) and a correlation coefficient (r^2) of 0.997.

In order to evaluate the inter-assay performance of the analytical method we prepared and analysed standard curves on five subsequent days with at least six QC samples with each standard curve (duplicate QC samples towards the lower quartile, the middle, and towards the upper quartile of the calibration range). The intra-assay performance (analysed on one day of analysis) was assessed to ensure that the results were acceptable, and could be used for pharmacokinetic analysis. The mean inter-assay precision (C.V.) for the standards of perifosine in plasma was ±6.5% with a mean error of +0.9%, and for the

Table 1
Inter-assay performance for the determination of perifosine (**I**)

True value (ng/ml)	*n*	Calc. conc. (ng/ml)	Accuracy (%)	C.V. (%)
(a) Inter-assay (between-day) performance of perifosine standards				
4	5	3.84	−4.10	5.22
10	5	11.06	+10.62	5.30
20	5	20.01	+0.07	7.47
50	5	49.34	−1.32	4.99
100	5	97.75	−2.25	6.00
200	5	200.92	+0.46	5.63
400	5	408.42	+2.10	9.98
1000	5	1044.85	+4.49	7.27
2000	5	1961.64	−1.92	6.59
Mean			+0.9	±6.5
(b) Inter-assay (between-day) performance of QCs:				
20	17	21.44	+7.19	14.66
200	17	179.39	−10.31	10.01
1600	17	1426.24	−10.86	10.13
Mean			−4.7	±11.6

QC samples ±11.6% with a mean error of −4.7% (Table 1). The mean intra-assay precision (C.V.) for the standards of perifosine in plasma was ±7.3% with a mean error of −0.2%, and for the QC samples ±7.3% with a mean error of −6.6% (Table 2). Therefore, the overall inter- and intra-assay performances for the standards and QC samples were satisfactory for routine on-line HPLC–MS–MS analysis.

The reported method can also be applied for both, the analysis of perifosine (**I**) in plasma from other species (i.e., mouse, rat, dog) and in urine samples.

Table 2
Intra-assay performance for the determination of perifosine (**I**)

True value (ng/ml)	*n*	Calc. conc. (ng/ml)	Accuracy (%)	CV (%)
(a) Intra-assay (within-day) performance of perifosine standards:				
4	4	3.80	−5.00	6.88
10	5	10.32	+3.16	7.83
20	5	19.79	−1.05	8.08
50	5	48.84	−2.32	6.01
100	5	98.98	−1.02	5.88
200	5	209.86	+4.93	9.27
400	5	421.44	+5.36	6.09
1000	5	960.26	−3.97	10.92
2000	5	2085.63	+4.28	5.17
Mean			−0.2	±7.3
(b) Intra-assay (within-day) performance of QCs:				
20	6	20.97	+4.84	11.81
200	6	172.51	−13.75	2.91
1600	6	1424.53	−10.97	7.29
Mean			−6.6	±7.3

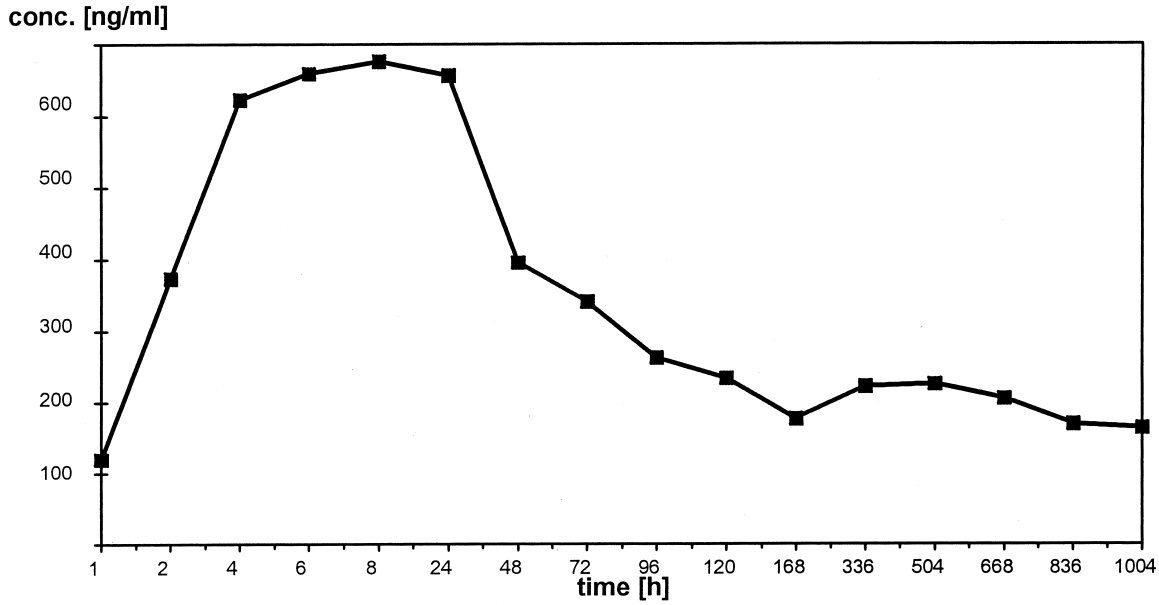


Fig. 6. Plasma concentration versus time profile after oral administration of a single dose of 100 mg of perifosine (**I**) to a patient.

Furthermore, the anti-tumour compound, miltefosine (**II**) can be analysed in a similar fashion using perifosine (**I**) as the internal standard. Achieved assay performances for the analysis of miltefosine (**II**) are comparable to perifosine (**I**) assay performances. All mass spectrometric and chromatographic settings as well as the sample work-up procedure are identical to the perifosine analysis (assay performance results for the miltefosine analysis not given).

4. Conclusion

In conclusion, a fast, sensitive, selective and robust assay with tandem mass spectrometric detection has been developed. The results of the assay performances clearly show that an assay for perifosine (**I**) in plasma with a limit of quantification at 4 ng/ml could easily be established utilising a relatively rough and automated sample clean-up procedure by SPE. This method has been employed successfully for the determination of the pharmacokinetics of the phosphocholine anti-tumour agent, perifosine (**I**), in preclinical and clinical pharmacokinetic studies, in which patients received single and multiple doses of 100, 200, 350, 450 and 600 mg via oral administration. An example of a concentration versus time profile, in which drug concentrations were measured up to 1000 h (42 days) for perifosine after oral administration of a single dose of 100 mg of perifosine (**I**) is outlined in Fig. 6. It could be demonstrated that maximum plasma levels (C_{max}) of up to approximately 700 ng/ml were achieved 8 h after single oral administration of 100 mg of perifosine.

Once more, HPLC–MS–MS in combination with a TISP interface has demonstrated itself to be an excellent tool for fast analysis regarding the support of pharmacokinetic studies of non-volatile, low-molecular weight compounds especially when lacking a chromophor.

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