

Quantitative determination of the antitumor alkyl ether phospholipid edelfosine by reversed-phase liquid chromatography-electrospray mass spectrometry: application to cell uptake studies and characterization of drug delivery systems.

MARIA J. BLANCO-PRIETO^{a*}, MIGUEL A. CAMPANERO^b, FAUSTINO MOLLINEDO^c

^aDepartment of Pharmacy and Pharmaceutical Technology, University of Navarra, E-31080 Pamplona, Spain

^bServicio de Farmacología Clínica, Clinica Universitaria, E-31080 Pamplona, Spain

^cCentro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, CSIC-Universidad de Salamanca, E-37007 Salamanca, Spain

***Corresponding author:**

María J. Blanco-Príeto

Departamento de Farmacia y Tecnología Farmacéutica

Facultad de Farmacia

C/ Irunlarrea, 1

31080 Pamplona, Spain

Tel: ++34 948 425600

Fax: ++34 948 425649

e-mail: mjblanco@unav.es

ABSTRACT

Edelfosine is a synthetic alkyl ether phospholipid that represents a promising class of antitumor agents. However, analytical methods to measure these type compounds are scarce. The lack of a reliable methodology to quantify edelfosine is a major problem in ongoing and scheduled preclinical and clinical trials with this drug. We evaluated the applicability of high-performance liquid chromatography–mass spectrometry to determine edelfosine in biological samples and polymeric delivery systems. Sample pre-treatment involved polymer precipitation or cell lysis with methanol. HPLC separation was performed on an Alltima RP18 narrow-bore column and edelfosine quantification was done by electrospray ionization mass spectrometry (ESI-MS) using positive ion mode and selected ion monitoring. Assays were linear in the tested range of 0.3–10 µg/ml. The limit of quantification was 0.3 ng/sample in both matrices, namely biological samples and polymeric delivery systems. The interassay precision ranging from 0.79 to 1.49%, with relative errors of –6.7% and 12.8%. Mean extraction recovery was 95.6%.

HPLC-ESI-MS is a reliable system for edelfosine analysis and quantification in samples from different sources, combining advantages of full automation (rapidity, ease of use, no need of extensive extraction procedures) with high analytical performance and throughput.

Keywords: edelfosine; HPLC-MS; drug delivery systems; antitumoral agents

Abbreviations: ET-18-OCH₃, (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, edelfosine); ESI-MS, Electrospray Ionization Mass Spectrometry; DDS, Drug Delivery Systems

1. Introduction

Synthetic alkyl ether phospholipids represent a promising class of antitumor agents that, unlike most conventional chemotherapeutic drugs, do not target the DNA but act at the level of the cell membrane [1-4]. The prototype of these antineoplastic ether phospholipids is ET-18-OCH₃ (edelfosine, 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine) [1-4] that exerts a selective cytotoxic action against cancer cells [1, 5]. The antitumor effect of edelfosine is based mainly on two different mechanisms that can act synergistically against neoplastic growth. Edelfosine enhances the tumoricidal activity of macrophages [6] and exerts a direct cytotoxic effect on tumor cells through the induction of apoptosis [5, 7-9]. This combination of a stimulatory effect on host defense cells and a direct destructive effect on neoplastic cells in one molecule makes edelfosine a potentially effective antitumor drug [4]. The antitumor activity of edelfosine is mostly due to its ability to induce apoptosis in tumor cells through a Fas/CD95 death receptor-mediated process [10, 11]. In addition, this ether lipid has been reported to inhibit tumor cell proliferation, metastasis and angiogenesis [4]. Encouraging data on purging leukaemic bone marrow prior to analogous bone marrow transplantation have been reported [12, 13].

Although edelfosine has been shown to achieve remarkable *in vitro* effects against a variety of cancer cells, so far the *in vivo* antitumor effect has been rather modest. Several reports have documented the advantages of incorporating anticancer agents into drug delivery systems, such liposomes, micro- and nanoparticles, in order to improve therapeutic efficiency while markedly reducing non specific *in vivo* toxicity and to enhance antitumor efficacy [14]. A liposome-based formulation of edelfosine has been shown to be less hemolytic both *in vitro* and *in vivo* [15]. Nevertheless, liposomes are rapidly cleared from systemic circulation and are relatively quickly degraded in the body. Polymeric materials provide an alternative means for delivering chemotherapeutic agents, offering a number of advantages over liposome carriers. In this work, drug delivery systems (DDS)

loaded with edelfosine have been developed using biodegradable polymers (a copolymer of lactic and glycolic acid, PLGA) [16, 17]. These polymers have been used as surgical sutures or bone-connecting devices, have proven non-toxic and are approved by FDA for their parenteral use in humans.

The remarkable antitumor properties of edelfosine have promoted ongoing and scheduled preclinical and clinical trials with this drug. These clinical studies require the availability for some biochemical probes in order to carry out toxicity, pharmacokinetics and pharmacodynamics studies with edelfosine. However, a major problem in pharmacokinetic studies lies in the lack of a reliable method for quantification of non-radiolabeled edelfosine in plasma and other organs. The quantification of edelfosine in cells and organs demands highly selective and sensitive analytical methods able to detect low drug concentration levels and to discriminate from the presence of metabolites and endogenous components. Conventional quantitative high-performance liquid chromatography (HPLC) assays with ultraviolet/fluorescence or electrochemical detection for edelfosine are not feasible due to the lack of chromophores and electroactive groups in the molecule. High-performance thin-layer chromatography with fluorescence detection has been evaluated for the quantification of ether phospholipids, but had several drawbacks such as lack of selectivity on silica gel surfaces and detection problems [18]. HPLC combined with light scattering detection was also investigated for edelfosine and its homologue ET-16-OCH₃, which was used as the internal standard [19]. The retention and elution characteristics of both compounds were studied on silica, poly(ethylene glycol)-coated silica, reversed phase materials and polymeric resins. This method can only be used for studies of relatively high-concentration edelfosine samples [19]. The possibility of using capillary gas chromatography for the analysis of edelfosine was also investigated [20, 21]. This technique appears to be very attractive because of its intrinsically high selectivity and sensitivity. Nevertheless, dephosphorylation is required and this reaction is far from being

quantitative [21]. Radioassays were also used for the measurement of edelfosine concentrations in pre-clinical studies. However, this technique is unsuitable for the specific determination of this cytostatic drug in clinical samples since it involves the use of radioactive-labelled edelfosine. Therefore, there is a great need for fast and sensitive analytical methods to support edelfosine clinical trials.

During the last few years high-throughput techniques have emerged, including fast and automated sample handling as well as data analysis and interpretation. Liquid chromatography-tandem mass spectrometry is one of these efficient analysis tools. The addition of mass spectrometry to liquid chromatographic applications has dramatically improved the analysis of drugs and some endogenous compounds in biological samples. This technique provides low detection limits, reduced influence of interference and the possibility for shorter run times. Here, we describe a sensitive analytical method based on a highly specific and selective HPLC separation coupled to electrospray ionization mass spectrometry (ESI-MS). Two different applications of the developed method have been evaluated in this study: the quantification of edelfosine in polymeric DDS, as a quality control procedure of the manufacture of the formulation, and in uptake studies in cancer cells.

2. Experimental

2.1 Reagents

Edelfosine (ET-18-OCH₃) was from INKEYSA (Barcelona, Spain). Formic acid 99% was purchased from Aldrich (Barcelona, Spain). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). Ammonium acetate RPE-ACS was obtained from CARLO ERBA (Milan, Italy).

2.2 Instrumentation and chromatographic conditions

The apparatus used for the HPLC analysis was a Model 1100 series LC coupled with an atmospheric pressure (AP)-electrospray ionization (ESI) mass spectrometer (HP 1100 with MSD VL, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett-Packard computer using the ChemStation G2171 AA programme.

Separation was carried out at 50 °C on a reversed-phase, 150 x 2.1 mm column packed with C₁₈, 5 µm silica reversed-phase particles (Alltima[®]) obtained from Alltech (Sedriano, Milan, Italy). This column was preceded by a reversed-phase, C₁₈, 5 µm guard column (kromasil[®], 20x4 mm, Symta, Spain). The mobile phase was a mixture of methanol-1% formic acid (95:5, v/v). The aqueous solvent was filtered through a 0.45 µm HV filter (millipore) and degassed using a membrane degasser. Separation was achieved by isocratic solvent elution at a flow-rate of 0.5 ml/min.

ESI-MS conditions were as follows:

Ionization mode:	ESI, positive
	Selected ion monitoring (SIM)
Ions (m/z):	
Edelfosine	524.40
Interface variables:	
Temperature	350 °C
Drying gas	N ₂ (12 l/min)
Nebulizer gas	N ₂ (30 p.s.i.; 1 p.s.i.=6894.76 Pa)
Capillary voltage	4000 V
Fragmentator voltage	140 V

Optimization of the interface variables, such as gas flows and voltages was done manually during direct infusion of 10 $\mu\text{g/ml}$ of the target analyte dissolved in methanol.

2.3 Calibration standards

A stock solution of edelfosine with a concentration of 100 $\mu\text{g/ml}$ was prepared by dissolving 1 mg of edelfosine in 10 ml of methanol. Ten standard solutions of 0.3, 0.6, 1, 1.2, 2, 2.5, 5, 6, 8 and 10 $\mu\text{g/ml}$ of edelfosine were made by further dilution of the stock solution with appropriate volumes of methanol. The concentration range of edelfosine for the standard curve samples was between 0.3 and 10 $\mu\text{g/ml}$.

The stock solutions of edelfosine were kept at 4°C.

2.4 Sample preparation

For the analysis of edelfosine in DDS a known amount of particles was dissolved in 1 ml of chloroform. Then, 3 ml of methanol were added to precipitate the polymer. The samples were vortex-mixed for 1 min and centrifuged for 10 min at 9,400 g. A 500 μl -aliquot of the

supernatants were transferred to limited volume autosampler vials capped and placed on the HPLC autosampler. A 1 μl -aliquot of the supernatant was injected onto HPLC column. The drug loading was expressed as μg of edelfosine per mg of DDS.

Edelfosine was extracted from the cell samples by repeated freeze/thaw in liquid nitrogen. Then, 150 μl of methanol were added, samples were vortexed and centrifuged at 10000 g for 10 min at 4°C (Biofuge Heraeus, Hanau, Germany). Blank cells samples were also extracted with methanol. A 1 μl -aliquot of the supernatant was injected onto HPLC column.

2.5 Validation

We applied an unweighted least-squares linear regression of the responses (chromatographic area of edelfosine) as a function of the nominal concentrations for obtain the equation of each calibration curve. The parameters of each equation were used to compute back-calculated concentrations of calibrators and to obtain concentration values for that day's quality-control samples and unknown samples.

The method was validated by analysis of extracted cells samples without edelfosine. The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of cell compounds in the matrix were compared with those of edelfosine.

LOD was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration which can be determined with an accuracy and precision < 20%. In this work LOD of the assay method was determined by analysis of the peak baseline noise in ten blank samples.

Three samples of each quality control pool and calibration samples were analyzed on three different days. On day 1 the number of samples of quality control was five. Precision and accuracy was determined. We assessed the within-day precision and the recoveries by performing replicate analyses of QC samples (1, 2 and 8 $\mu\text{g}/\text{ml}$). The procedure was repeated on different days with different samples of the same calibrators to determine between-day values. The mean recovery was calculated as (mean measured

concentration/theoretical concentration)x100. Imprecision of a method is expressed as the relative standard deviation (RSD) of the obtained values for each calibrator. Accuracy was measured according to the following equation:

$$\text{Percentage difference from the theoretical value} = \left[\frac{X - C_T}{C_T} \right] \times 100$$

Where X is the determined concentration of a quality control and C_T is the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

For ruggedness and robustness studies, a different phase columns such as Kromasil C₁₈ 25 x 0.4 (Teknokroma, Spain), and Zorbax Stablebound (150x2.1 mm, Agilent, USA) were used. Similarly, the influences of mobile phase (percentage of methanol ranged from 90% to 99%) and column temperature (50°C) on the analytical procedure were also evaluated.

2.6 Application of the method

This analytical HPLC method was applied to determine the edelfosine content in DDS and in human acute myeloid leukemia HL-60 cell line incubated with this cytostatic drug.

Quality control of the formulations

DDS loaded with edelfosine using biodegradable polymers were prepared. Drug loading was calculated from the ratio of the mass of drug in the particles to the mass of particles and expressed as µg of edelfosine per mg of DDS.

Cell culture

The human acute myeloid leukemia HL-60 cell line was grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 24 µg/ml gentamicin. Cells (1×10^6) were incubated with 3 and 5 µg/ml edelfosine for 2 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After exhaustive washing (six times) with phosphate-buffered saline-2% bovine serum albumin to

eliminate loosely cell surface-bound ether lipid due to the strong binding of edelfosine to albumin, the drug uptake into the cells was determined.

3. Results and discussion

A HPLC-MS assay to allow the quantitation of edelfosine levels in cells has been developed and validated. Before the 1990s, determination of drug uptake in cells usually required gas chromatographic and radioactive protocols. These traditional analytical methodologies were not amenable to high-throughput analysis because they are slow, costly, and suffer from poor selectivity. Moreover, both approaches required extensive sample preparation procedures and were time-consuming so that their application to clinical analysis is too difficult. The use of the tandem mass spectrometry-liquid chromatography to detect drugs and biomolecules in clinical chemistry has supposed an important breakthrough in clinical chemistry. Actually, the impact of liquid chromatography/tandem mass spectrometry in the development of new clinical chemistry applications is considerable. Liquid-chromatography-mass spectrometry is specific, extremely rapid (the chromatographic process can occur in less than 5 min), has tremendous cycle samples (hundreds of samples can be analyzed sequentially, one after another without pause), and can achieve sensitivities in the picogram range. The electrospray ionization interface on the mass spectrometer is well suited to tolerate high eluent flow rates and “dirty” matrices. Moreover, in some cases, the enhanced selectivity and sensitivity provided by the quadrupole MS systems eliminate the need for extensive sample preparation procedures and, indeed, for chromatographic separation.

3.1. Optimization of mass spectrometer

The MS analysis of edelfosine was first investigated by direct introduction of the reference compound dissolved in a methanol-1% formic acid (95:5) mixture using electrospray interface in both positive and negative ion mode of ionization. The positive ion mode was chosen because greater signal-to-noise ratios were obtained when compared to negative

ion mode. The presence of a quaternary ammonium group in the molecule structure is responsible of the good MS response obtained with this ionization mode. The mass spectra of edelfosine are shown in Fig 1. It was recorded in full scan mode (m/z 100-600). Two main peaks were observed corresponding respectively to the $[M+H]^+$, $[M+Na]^+$, pseudomolecular ions that it is common to see when the electrospray ionization interface was employed (Fig. 1A). When a fragmentation voltage of around 30 V was applied other structurally significant fragmentation ion was observed (Fig. 1B). It is interesting to note that a characteristic product ion of m/z 487.32 was generated under these fragmentation conditions. This primary fragment resulted from the loss of the quaternary ammonium group (Fig. 2) and can be monitoring within the pseudomolecular ions $[M+H]^+$ and $[M+Na]^+$ for structural confirmation purposes.

Parameters such as the capillary temperature and the capillary voltage, the fragmentation voltage as well as the desolvation gas flow were optimized in order to reach a maximum intensity for the fragment ions. In a first step the effect of fragmentator voltage on both the fragmentation pattern and the signal intensity of tested edelfosine was studied by varying the probe voltage between 0 and 280 V in full scan mode (m/z 100-600). Any significant effect was observed in the mass spectra of edelfosine. The pseudo-molecular ions $[M+H]^+$ and $[M+Na]^+$ and the main fragment of $[(M+Na)-59]^+$ were stable even at higher fragmentator voltage. The high signal-to-noise ratios for the pseudo-molecular ions was achieved between 120 and 140 V as can be seen in Figure 3. Above this voltage a decrease of signal intensity of the pseudo-molecular ions $[M+H]^+$ and $[M+Na]^+$ was observed for edelfosine, whereas the intensity of the main primary fragment of $[(M+Na)-59]^+$ was slightly increased to reach a fragmentator voltage of around 180 V.

The effect of the capillary temperature on signal intensity was investigated by varying the temperature between 250°C to 350°C and keeping all other parameters constant. An improvement of the signal response could be achieved by increasing the capillary

temperature. The optimal value of temperature was found 350 °C. No changes in fragmentation pattern of edelfosine could be observed over the applied temperature range. This fact indicates that the fragmentation is not the result of a thermal degradation. Sensitivity also was further improved by lowering the capillary voltage from 4.5 kV to 4 kV. For the quantitative measurements, the fragment ion with the highest signal response at fragmentation voltage of 140 (m/z 524.4) was selected for quantitative MS detection. We operated in the selected-ion monitoring (SIM) mode with an exact mass of m/z 524.4, a peakwidth of 0,25 min and a dwell time of 1000 msec. The use of the SIM detection mode assures better sensitivity and selectivity to this analysis. Increasing dwell time led to a loss in linearity without a significant increase in sensitivity. We added edelfosine to the matrices investigated and never detected interferences with MS detection. Therefore, the tedious and time-consuming sample clean-up procedures (solid-phase extraction, liquid-liquid extraction), performed when this drug is analyzed in biological samples using other techniques, can be avoided. We have also verified that under the chromatographic conditions described in the paper the baseline mass spectrum showed ions with lower m/z ratio (below m/z 275) after analysis of a plasma blank samples. Hence, as the selected mass fragment of edelfosine is the m/z 524 SIM ion, the developed method could be applied to the measure of edelfosine plasmatic concentrations.

3.2. Optimization of chromatographic process

To improve the sample throughput a column of 2.1 mm internal diameter with highly apolar eluent was used. The narrow bore of these columns led to a higher detector response than standard columns due to the decreased diffusion of the sample as it passes through the column. The packing selected (Alltima C₁₈) was found to be durable and provided good retention behaviour at higher organic rates in mobile phase. This column is a polymerically bonded C₁₈ reversed-phase narrow-bore column packed with double-encapped spherical modified silica gel particles synthesised from pure silica gel. The use of this column

enabled us to determine edelfosine in small sample volumes, because of silanol groups have been mostly end-capped and the residual metals have been completely removed. These silanols are highly acidic functional groups and are very reactive with the quaternary ammonium group of edelfosine. The effect of such secondary interaction on the chromatographic behaviour is clear. Edelfosine is eluted from non-deactivated columns as tailing and band-broadening chromatographic peaks, with insufficient chromatographic efficiency to measure this drug in cellular samples.

Figure 4 and 5 show representative blank chromatograms and chromatograms obtained by analysis of edelfosine in cell extracts and in DDS, respectively. The retention time of edelfosine was of 2.5 min. Under the chromatographic conditions used the number of theoretical plates was near to 8500. We evaluated peak skew using the asymmetry coefficient $A_s = b/a$, where b is the distance after the peak maximum and a is the distance before the peak maximum, both a and b being measured at 10% of the total peak height. The asymmetry coefficients ranged between 1.12 and 1.27. No interfering peaks were visible in the chromatogram of cell extracts. On the other hand, a chromatographic peak with retention time of 0.7 min was observed when DDS were analyzed. Nevertheless, edelfosine exhibited a well-separated peak ($\alpha=4.9$, $R_s=4.9$) under the chromatographic conditions described.

3.3. Quantitative analysis

Examination of linearity over the concentration range 0.3–10 $\mu\text{g/ml}$ yielded a linear correlation of > 0.9996 from five separate assays. The relative standard deviation of the slope calculated with calibration curve data was 2.28%, indicating good repeatability (Table 1). For each calibration point, the concentrations were back-calculated from the equation of the linear regression curves. Linear regression of the back-calculated concentrations versus the nominal concentrations provided a unit slope and an intercept equal to 0 (Student t -test). The distribution of the residuals (difference between nominal

and back-calculated concentrations) showed random variations, the number of positive and negative values being approximately equal. The limit of detection for the assay was 0.312 $\mu\text{g/ml}$ (300 pg of edelfosine). Compared with other methods for edelfosine quantification, this limit of detection was lower than those previously published (20 ng of edelfosine) [10]. The mean extraction efficiencies for edelfosine was 95.6 (4.5)% (n=9). The extraction efficiency was independent of concentration over the range studied. Measurement imprecision is shown in Table II. Precision studies showed RSD values ranging from 0.79 % to 1.49 % with relative errors of -6.7 and 12.8%.

The reported method was used for the determination of the drug content in DDS. The drug content was $1.25 \pm 0.07 \mu\text{g}$ of edelfosine per mg of particles with the polymer used in the formulation. These controlled delivery systems are promising systems for the delivery of anticancer drugs such as edelfosine. The method was also applied to the quantitation of edelfosine amount taken up by human acute myeloid leukemia HL-60 cells. As can be observed in Figure 6, about 38-47% of the edelfosine added to the culture medium is taken up by the leukemic cells. This amount of cell-incorporated ether lipid is enough to promote cell death in tumor cells as evidenced by subsequent triggering of apoptosis following 3-6 h of incubation under the same experimental conditions used for drug quantification (data not shown). Thus, the mass spectroscopy method reported here to determine edelfosine levels in biological systems gives more reliable, and accurate figures than other previous methods and therefore can be the best choice for drug measurement in future pharmacokinetic studies with this antitumor drug.

4. Conclusion

The experiments have demonstrated that HPLC with ESI (electrospray ionization) tandem mass spectrometric detection, together with the simplest extraction procedure, is a good alternative to other methods traditionally employed for the determination of edelfosine in biological fluids. The simplicity of the technique, the shorter analysis time and its high

sensitivity, together with the lack of need for using radiolabeled compounds, makes this technique particularly attractive for edelfosine pharmacokinetic studies. This methodology provides a useful and critical tool in the biomedical and clinical research of edelfosine.

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References

- [1] P.G. Munder, O. Westphal, *Chem. Immunol.* 49 (1990) 206.
- [2] H. Brachwitz, C. Vollgraf, *Pharmacol. Ther.* 66 (1995) 39-82.
- [3] W.J. Houlihan, M. Lohmeyer, P. Workman, S.H. Cheon, *Med. Res. Rev.* 15 (1995) 157.
- [4] C. Gajate, F. Mollinedo, *Curr. Drug Metab.* 3 (2002) 491.
- [5] F. Mollinedo, J.L. Fernandez-Luna, C. Gajate, *Cancer Res.* 57 (1997) 1320.
- [6] R. Andreesen, J. Osterholz, G.A. Luckenbach, U. Costabel, A. Schulz, V. Speth, *et al.* *J. Natl. Cancer Inst.* 72 (1984) 53.
- [7] F. Mollinedo, R. Martinez-Dalmau, M. Modolell, *Biochem. Biophys. Res. Commun.* 192 (1993) 603.
- [8] L. Diomedea, F. Colotta, B. Piovani, F. Re, E.J. Modest, M. Salmona, *Int. J. Cancer* 53 (1993) 124.
- [9] L. Diomedea, B. Piovani, F. Re, P. Principe, F. Colotta, E.J. Modest, M. Salmona, *Int. J. Cancer* 57 (1994) 645.
- [10] C. Gajate, R.I. Fonteriz, C. Cabaner, G. Alvarez-Noves, Y. Alvarez-Rodriguez, M. Modolell, F. Mollinedo, *Int. J. Cancer* 85 (2000) 674.
- [11] C. Gajate, F. Mollinedo, *Blood* 98 (2001) 3860-3863.
- [12] W.R. Vogler, W.E. Berdel, *J. Hematotherapy* 2 (1993) 93.
- [13] M.P. Königsmann, M. Notter, C.A. Papadimitrou, D. Oberberg, B. Reufi, C. Mücke, *et al.* *Bone Marrow Transplant.* 18 (1996) 549.
- [14] K. Lawrence, W. Fung, M. Saltzman, *Adv. Drug Del. Rev.* 26 (1997) 209.
- [15] I. Ahmad, J.J. Filep, J.C. Franklin, A.S. Janoff, G.R. Masters, J. Pattassery, *et al.*, *Cancer Res.* 57 (1997) 1915.
- [16] M.J. Blanco-Príeto, E. Fattal, A. Gulik, J.C. Delieu, B.P. Roques, P. Couvreur, *J. Control. Release* 43 (1997) 81.

- [17] M.J. Blanco-Príeto, K. Besseghir, O. Zerbe, D. Andris, P. Orsolini, F. Heimgartner, H.P. Merkle, B. Gander, *J. Control. Release* 67 (2000) 19.
- [18] J. Coene, M.E. Ramos, E. Van den Eeckhout, P. Sandra, W. Van den Bossche, *Chromatographia* 30 (1990) 414.
- [19] J. Coene, M. Ghis, E. Van den Eeckhout, W. Van den Bossche, P. Sandra, *J. Chromatogr.* 553 (1991) 285.
- [20] J. Coene, E. Van den Eeckhout, P. Herdewijn, P. Sandra, *J. Chromatogr.* 612 (1993) 21.
- [21] W.F. Steelant, E.A. Bruyneel, M.M. Mareel, E. Van den Eeckhout, *Anal. Biochem.* 227 (1995) 246.