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Development and validation of a simple and versatile method for the quantification of Everolimus loaded in H-ferritin nanocages using UHPLC-MS/MS

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Highlights

- The method was developed to quantify Everolimus in nanocages of H-Ferritin
- HEverolimus is a new naturally occurring delivery system
- Ultra High Performance Liquid Chromatography-tandem mass spectrometry was employed
- Assay was validated according to the Food and Drug Administration Guidance
- Assay for measurements of the Nanoformulated Everolimus in cytoplasmic fractions

Abstract

Everolimus (Eve) is an immunosuppressive macrolide that is being analyzed in various biological matrices and fluids. Its antitumor activity makes this drug suitable not only for organ transplantation but also for breast cancer treatments. In the attempt to reduce the incidence and severity of its side effects, Eve was loaded in H-ferritin (HFn), a natural biomolecule that is involved in specific cellular uptake pathways. Thus, Eve pre-complexed with Cu(II) and encapsulated in HFn resulted in an Eve nanoformulation, named HEve. The quantification of HEve was performed using a tailored pH-induced procedure to precipitate H-ferritin. This sample preparation was effective enough to reduce the ion suppression effect on the mass spectrometric responses of Eve in electrospray ionization (ESI). The ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-ESI-MS/MS) system operating in positive ionization mode showed to be a versatile technique in achieving more than 77% recovery of Eve from the cytoplasmic compartment. This simple, selective and sensitive method enabled the quantification of Eve within the linear range of 2.5-100 ng/mL in matrix spiked with the isotope-labeled internal standard, EveD4. This method was validated according to FDA Guidance. The intracellular distribution of HEve and its accumulation at a cytoplasmic level were studied in breast cancer cell lines. As expected, HEve was more effective than free Eve on sensitive (i.e. BT474) and resistant cell lines, as a result of a better penetration into the target subcellular compartment.

Key words: Everolimus, UHPLC-ESI-MS/MS, H-ferritin (HFn), protein precipitation, nanopharmaceutical, cytoplasm

Development and validation of a simple and versatile method for the quantification of Everolimus loaded in H-ferritin nanocages using UHPLC-MS/MS

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1. Introduction

Everolimus (Eve) is an immunosuppressive macrolide with a molecular mass of 957.6 Da ($C_{53}H_{83}NO_{14}$). This agent is one of the most frequently prescribed immunosuppressant drugs (ISDs) along with cyclosphorine A, tacrolimus and sirolimus. Eve is a derivative of sirolimus (40-*O*-(2-hydroxy) ethyl-rapamycin) with a side chain at position 40, as shown in Figure 1. In the last 20 years, this molecule has been analyzed in whole blood and plasma for Therapeutic Drug Monitoring (TDM) because of its pharmacokinetic behavior. Indeed, this compound is characterized by a narrow therapeutic index, therefore severe adverse effects (e.g. nephrotoxicity) are observed when the drug is overdosed, while an under dosage leads to rejection in organ transplantation [1]. Eve is currently employed not only in organ transplantation but also in breast cancer treatments (BC). In the attempt to reduce the incidence and severity of its side effects, the development of a new protein-based formulation of Eve was described in our previous study [2]. Ferritin protein proved to be a powerful tool for therapeutic purposes because of its protein cage structure, surface properties and high biocompatibility. In physiological conditions, ferritin displays a stable 24-mer cage architecture that encloses a cavity of 8 nm in diameter. This structure is very useful from the nanotechnological point of view to encapsulate many small-molecule drugs. Moreover, it exhibits the self-assembly or disassembly of its quaternary structure in response to different stimuli, such as pH variations and/or the addition of denaturants [3]. Nanocages constituted by omopolymers of H-ferritin (HFn) were internalized in cells thanks to their specific uptake mediated by the Transferrin Receptor-1 (TfR1), so they are very useful in delivering drugs or other molecules to cells with a highly active iron metabolism, such as cancer cells, since they overexpress this receptor [4]. To treat in vitro breast cancer models, in our previous study, Eve was pre-complexed with Cu(II) and encapsulated in HFn, resulting in an Eve nanoformulation, named HEve [2].

In this paper, we describe an accurate, specific and reliable method using an ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-ESI-MS/MS) system to evaluate both the amount of Eve encapsulated in HEve for formulation monitoring and the uptake of HEve in cytoplasm samples. To the best of our knowledge, no study has yet reported the quantification of Eve loaded in HFn nanoparticles and in cytoplasm samples to assess the anticancer efficacy of HEve in comparison with free Eve. In the literature, the most widely reported quantitative analyses of Eve in biological matrices, such as whole blood, plasma, tissues and cells, are HPLC-based techniques coupled with mass spectrometry [5–10]. The vast majority of these studies emphasize that the matrix effect (ME) is one of the most important analytical pitfalls [7–9,11–15]. In particular, high concentrations of organic compounds (e.g. proteins and phospholipids) that may co-elute and compete with Eve ionization have been deemed to interfere with its quantification. Mueller et al., suggested an alternative procedure to enhance the assay specificity and avoid the ion suppression effect [16]. The authors applied atmospheric pressure chemical ionization (APCI) operating in negative ionization in order to increase the sensitivity. Although the tandem mass spectrometry detector is being recognized as the gold standard for detecting Eve in biological samples, in 2018 Vosough et al. used diode array detection (HPLC-DAD) as an alternative analytical technique to avoid ME [11]. Thanks to advantages offered by ChemStation Software used by Vosough et al., the multivariate

curve resolution (MCR/ALS) method was developed. By using a proper second-order calibration method, like MCR/ALS, the most important ISDs, including Eve, were fully resolved and accurately quantified in complex whole blood samples [11]. The optimization of the sample preparation procedure together with UHPLC-ESI-MS/MS analysis is crucial in obtaining sensitive signals even in complex matrices such as blood, tissues and cells [17]. As nanotechnology engineering led to the internalization of Eve into the cavity of HFn, in our study, efforts were made in order to improve the efficiency of the clean-up process over the previously reported methodologies [10–13,15]. These pretreatment protocols (such as the use of additives in precipitation solvents or the mixture of these, off-line and on-line SPE) were non-effective in achieving satisfactory results in terms of good assay sensitivity. When we considered H-ferritin from its isoelectric point and its structural change [3,18], during the disassembly phase, a new protein precipitation procedure was developed. This procedure was then applied to quantify Eve in samples that were exposed to quantifiable amounts of Eve and HEve. The analytical method was fully validated according to Food and Drug Administration (FDA) Guidance on Bioanalytical Method [19]. Here, the performances of a UHPLC system coupled with tandem mass spectrometry also served to assess the uptake of HEve in eve-sensitive cell lines (i.e. BT474). Consistent with clinical needs, the feasibility of the present method and its applicability were demonstrated by analyzing actual cytoplasm samples.

2. Material and Methods

2.1. Reagents and Chemicals

Everolimus powder, as reference substance and the labeled internal standard, EveD4 (ISTD), as ethanol solution (10 mg/mL) were obtained from Aurogene S.R.L. HFn nanocages were purchased from Molirom s.r.l. (Roma, RM). The complexed drug (Eve) with Cu(II) loaded in H-ferritin (HEve) was prepared by Nanomedicine Lab according to the procedure reported in our previous paper [2]. For chromatography, acetonitrile, methanol, 2-propanol LC-MS-grade solvents and formic acid/ammonium formate LC-MS-grade water (Merck House, Poole, UK) were used throughout the study. Water was deionized and purified on a Milli-Q system from Millipore (Marlborough, MA, USA). For protein precipitation, Trichloroacetic acid (TCA), the organic solvent, methanol, acetone, and hydrochloric acid (37%) were purchased from Merck (KGa A64271 Darmstadt, Germany). The SPE cartridges were OASIS® hydrophilic–lipophilic balance (HLB) cartridges (10 mg, 1mL) purchased from Waters Associates Milford, MA, USA. Eppendorf tubes (15.0 mL), as well as pipette models (from P20 to P5000) were purchased from Eppendorf (Netheler-Hinz-GmbH, Hamburg, Germany). Disposable pipette tips were obtained from Rainin Instruments, Woburn, MA, US. The Microcentrifuge 5415r was obtained from Merck (KGaA, 64271 Darmstadt, Germany).

2.2. Standard Solutions

Everolimus powder was dissolved in ethanol to prepare the stock standard solution of Eve at the concentration of 10 mg/mL and stored in several 100 μL aliquots at -80°C in dark conditions. Each vial was allowed to thaw at 4°C to prepare the working standard solution in methanol at the concentration of 100 μg/mL. Serial dilutions were prepared to obtain a six-point calibration curve with the following levels: 2.5, 4, 10, 12.5, 40 and 100 ng/mL and 5, 20 and 50 ng/mL for the quality control levels (QCs). Similarly, to prepare EveD4 the stock solution (10 μg/mL methanol) was diluted in mobile phase at the concentration of 200 ng/mL.

2.3. Mass Spectrometry and chromatography conditions

Eve quantification was performed using an UHPLC system equipped with a Triple Quadrupole Mass Spectrometer (6460 Agilent Technologies, CA, USA). Mass Hunter workstation was used for data acquisition and analysis. The instrument, operating in positive mode with ESI, was used to carry out MS/MS analysis (UHPLC-MS/MS). Ion source parameters for positive mode were as follows: vaporizer temperature, 350°C; sheath gas, 11 mL/min with a temperature of 350° C; nozzle voltage, 500 V and capillary voltage, 3000 V. Finally, the instrument was operated using nitrogen as nebulizer gas set at 45 psi with a flow rate of 5 L/min. Selected reaction monitoring (SRM) transitions of Eve and EveD4 (ISTD) were: m/z 975.4 and 979.6→ m/z 908.4 and 916.6, respectively. In order to obtain the best chromatographic performance a mixture of organic solvents (acetonitrile-methanol-2-propanol 40:30:30 v/v/v) was prepared in the presence of ammonium formate buffer (20mM) and prepared in water by using 1 mL/L of formic acid (pH=3.5). For the mobile phase A, the organic/buffer ratio was 20:80 (v/v). Whereas, for the mobile phase B, the ratio between organic solvents and formate buffer was that of 80:20 (v/v). A Zorbax Eclipse plus C18 column (2.1x50 mm), 1.8μm particle size, allowed the detection of Eve at 1.6 min with a flow rate of 300 μL/min. The column oven was set at 40°C and the autosampler tray at 25°C. The mobile phase B started at 80% and it was maintained constant for 1 min, then the mobile phase B was increased linearly to 100%. The total run time was of 4 min.

3. Method validation and application

The LC-MS/MS method was validated according to Food and Drug Administration (FDA) Guidance on Bioanalytical Method Validation [19]. The precision and accuracy of the method were assessed by intra- and inter-day validation over four non-consecutive days. The intraday accuracy and precision were evaluated by processing QCs in four replicates (n=4) at three concentration levels. The concentration of the QCs was calculated *vs.* the daily calibration curves. The interday accuracy and precision were determined by analyzing QCs in sixteen replicates of each concentration. Accuracy was determined as the ratio between the back-calculated concentration and the actual value and expressed as a percentage. The coefficient of variation (CV%) was used as a measure of precision. The lower limit of detection (LOD) level was obtained by using the peak-to-peak area measurement

method at the lower level of quantification (LLOQ). The linearity of the standard curves was checked by regression analysis using the Pearson's determination coefficient (R^2) and by comparison of the nominal and back-calculated concentrations of the calibration standards.

3.1. Recovery and Matrix Effect

The approach proposed by Matuszewski et al. was followed to assess both extraction recovery (RE) and matrix effect [20]. To prepare calibration curves in set B, working standards of Eve with its ISTD were spiked in dried cytoplasm samples after PP procedure. To obtain calibration curves in set C, 100 μL aliquots of cytoplasm were spiked with working standards of Eve. Then, the cytoplasm mixture underwent sample cleanup via PP precipitation. For set A, Eve standard solutions were prepared in the mobile phase. RE was calculated by following the equation: [(C/B)*100]. Whereas, ME was given by the formula [100-(B/A)*100] to assess a possible occurring ion suppression effect. RE and ME were obtained by using the mean integration ratio of Eve/ISTD peak areas. The percentages of RE and ME were obtained at the LLOQ and at three QC concentrations.

3.2. Stability

The stability of HEve in cytoplasm was assessed by analyzing QC samples at two concentration levels (5.0 and 50.0 ng/mL) during storage and handling. Bench-top stability was determined after 4h at room temperature. Stability in the autosampler was also assessed at room temperature by reanalyzing the processed QC samples 72h after the first injection. Freeze-thaw stability was studied by analyzing the low and high QCs that were frozen overnight, at normal storage temperature (-80°C) and thawed at 4°C. When completely thawed, the samples were frozen again at the same temperature for 24h and thawed. This freeze-thaw cycle was repeated two more times. After the third cycle, the samples were analyzed. To check freeze–thaw stability, an aliquot of each QC sample concentration was freshly prepared, processed and analyzed. The analytes were considered stable at each concentration when the differences between the freshly prepared samples and the stability testing samples did not deviate by more than ±15% from the nominal concentrations.

3.3. Application on real samples

Eve incorporation into the HFn nanocages was optimized in outsourcing [2]. Briefly, Eve was complexed with Cu(II) by incubation with 10 mM $CuSO_4$ because the HFn affinity towards metal ions allowed the uptake of Eve. Then, the complexed drug was added to HFn and incubated to allow Cu(II)-driven incorporation of Eve in HFn, resulting in the development of HEve. Subsequently, 1×10^6 cells were seeded on a 6 multi-well plate and incubated at 37°C with HEve or Eve (1, 10, 50 and 100 nM) for 24h [2]. The amount of Eve encapsulated inside the HFn nanocage resulted in approximately 1 ng/mg HFn. Data were generated from 20 samples of HEve. Protein precipitation (PP) solution was obtained by mixing 120.0 mL of acetone with 10 μL of HCl (37%) in order to

achieve the final concentration of 1mM. The protein precipitation solution had a pH value of 5.0. Afterwards, PP solution was stored at -20°C. Eight times the sample volume of cold (-20°C) PP solution were added to 100 µL aliquots of cytoplasm. The mixture was vortexed and then incubated in an ice bath for 60 min at -20°C. In order to separate a solid precipitate from the rest of the mixture, it was necessary to centrifuge the mixture for 15 min at 4°C. Subsequently, the supernatant was carefully disposed so as not to dislodge the protein pellet. This solution was dried under a weak flow of nitrogen (N_2). Following this, 100 µL of mobile phase (aqueous phase) was added to the dried samples of cytoplasm for their subsequent analysis by means of UHPLC-MS/MS.

4. Results and Discussion

4.1. Analytical method for Eve

The ammoniated adduct ions ($[M+NH_4]^+$) of Eve were generated in positive ESI mode at m/z 975. Consistent with previous findings of Brignol et al., [5] neutral losses of both methanol and water molecules from the proton adduct ions of Eve resulted in the most abundant fragment ion at m/z 908 (panel A, Figure 1). Similarly, EveD4 ammoniated adducts at m/z 979 were fragmented in the collision cell (Q2) to obtain daughter ions at m/z 912 (panel B, Figure 1). To achieve ammoniated rather than sodium adducts, the buffer was prepared at pH 5 with 0.1% formic acid in water. The buffer solution was studied using different concentrations and finally it was set at 20 mM in order to trigger ammonium adduct formation. The choice of the mobile phase composition and the ammonium buffer concentration were suitable to limit in-source fragmentation and trigger intense fragment ions of Eve in the collision cell.

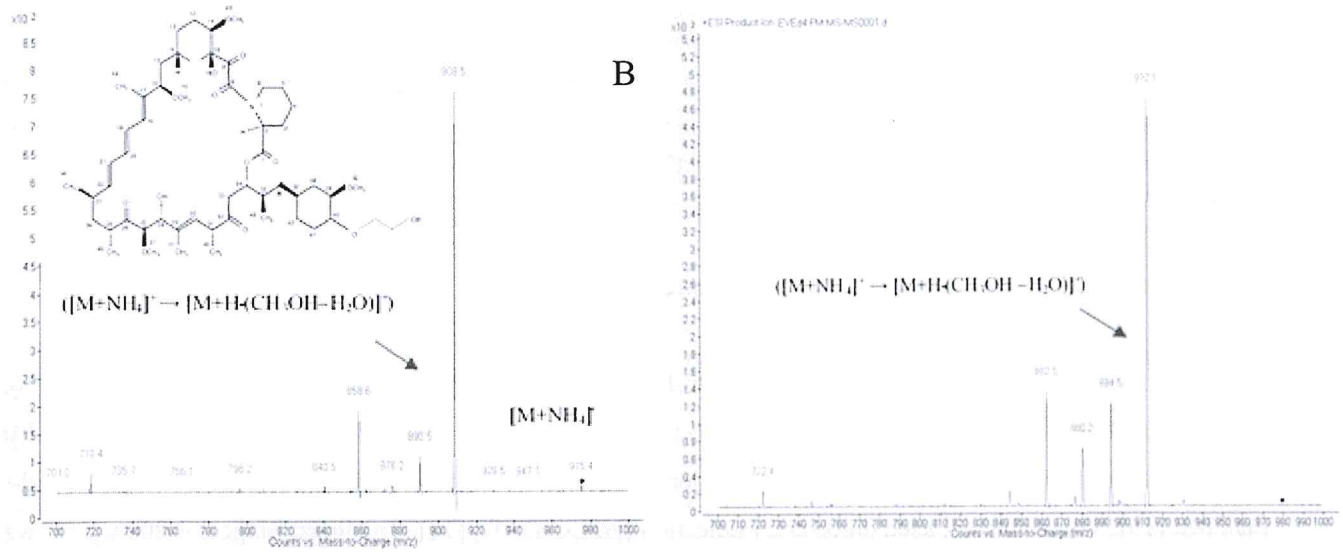

Figure 1. Chemical structure of everolimus [40-O-(2-hydroxy) ethyl-rapamycin] (panel A) and internal standard, EveD4 (panel B).

Representative SRM chromatograms for Eve and ISTD in cytoplasm samples are shown in Figure 2. In panels A and A1, SRM chromatograms of a blank cytoplasm sample and ISTD are reported to confirm that no other interferences were detected at the retention time of Eve.

For Eve analysis in the cytoplasmic compartment, a signal-to-noise ratio (SNR) value of 15.1 was obtained by using peak-to-peak area measurement method, as shown in Figure 2, Panel B. The ESI profile of Eve was obtained by injecting the lowest calibrator prepared in matrix samples at the concentration of 2.5 ng/mL. The signal-to noise ratio (>10) allowed us to set the value of 0.5 ng/mL as the lower limit of detection. Moreover, the LOQ was fixed at 2.5 ng/mL in view of the levels of the analyte expected in the cytoplasm samples.

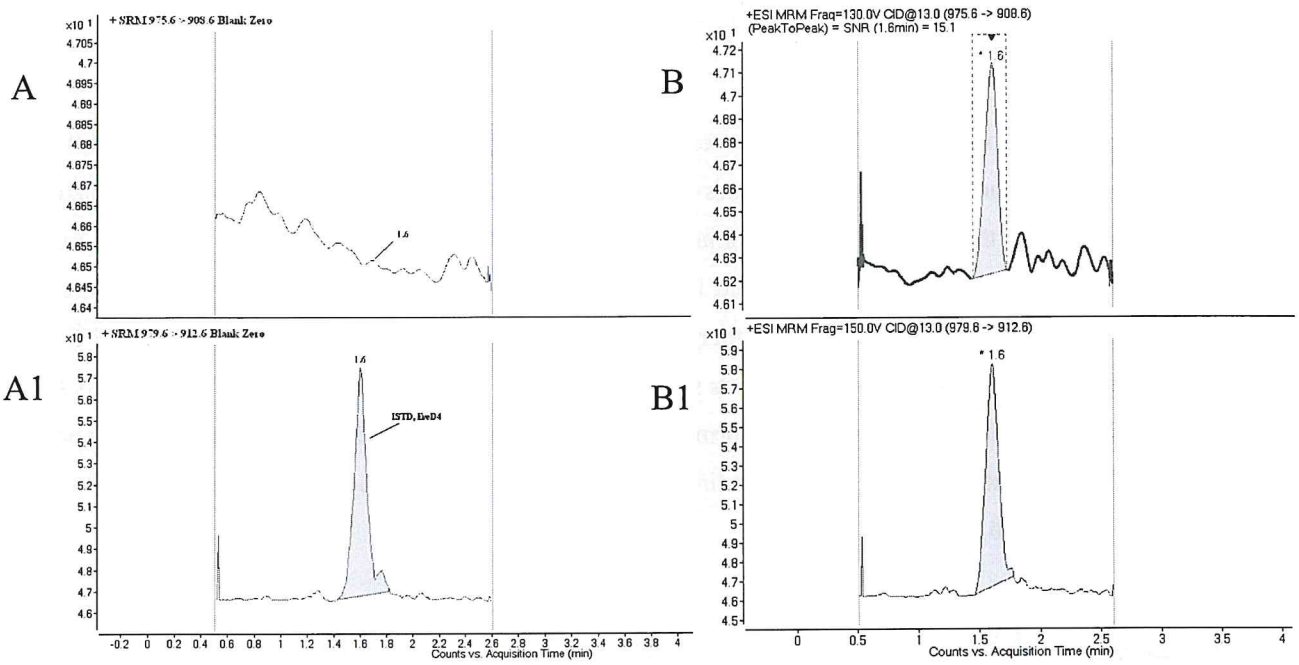

Figure 2. Representative extracted ion chromatograms (XICs) for Eve and ISTD in cytoplasm samples: blank cytoplasmic fractions with and without ISTD (A and A1 panels); cytoplasmic fractions spiked at LLOQ level (B and B1 panels).

4.2. Protein precipitation solution and sample preparation

For the whole range of the studies reported in the literature, method development was aimed at reducing ion suppression effect from the analyzed matrices [10–13,15,16]. Different types of chemical ionization rather than detectors were offered as alternatives to ESI. Mueller et al., used APCI, this technique being less matrix sensitive than ESI [16]. Vosough et al. used diode array detection (HPLC-DAD) [11]. The linear range 2.4-200 ng/mL was achieved by using a multivariate curve resolution (MCR/ALS) method with HPLC-DAD [16]. Eve sodium adducts $[M+Na]^+$ were monitored by Ansermont et al. [12]. A single stage LC-MS procedure showed good sensitivity because the linear range was between 2.5-30 ng/mL. Other approaches to reduce the matrix effect on Eve

ionization regarded sample pretreatment procedures. They ranged from solvent protein precipitation, PP, [10,11,15] to off-line [13] and on-line solid phase extractions directly before LC–MS/MS [12]. Tszyrsznic et al., and Tang et al., [10,15] used PP with methanol and Vosough et al. used methanol with $ZnSO_4$ to facilitate both erythrocyte lysis and protein precipitation [11]. In our study, the extraction of HEve from the cytoplasm samples was studied by investigating four different protocols. They were *i*) the use of ZnSO4; *ii*) TCA 20%; *iii*) cold acetone and methanol (1:1 v/v) followed by SPE; *iv*) cold acetone at pH 5. The pH of the cytoplasm was adjusted to obtain the highest recovery of HEve from the matrix. A duplicate experiment was carried out to study the influence of sample pH on the extraction efficiency, over a pH range from 3.0 to 7.0. The best result was achieved when the cytoplasm sample was at pH 5.0 in accordance with its basal isoelectric point (pI 5.31). By using cold acidified acetone prepared at pH 5, we carried out a denaturation of H-ferritin and an isoelectric precipitation of HFn was achieved. The best recovery percentages for Eve ranged from 71.3% to 86.4% over the LLOQ and the QC values (Table 1). In contrast, the recovery of Eve dropped below 50% by using zinc sulfate, TCA 20% solution in water and cold acetone/methanol followed by SPE with percentage values of 35, 40 and 45, respectively. The pH-induced denaturation was found to be efficient enough to reduce ME determined by the presence of protein cages in a cytoplasmic environment. Moreover, ion suppression effect was reduced by the compensating effect of the isotope labeled internal standard (Eved4). The method was deemed effective because the influence of the matrix on the isotope-labeled internal standard pattern showed to have the same order of magnitude as Eve. We experimentally observed that the responses of this internal standard were comparable to those of the drug over the four days of the validation study. Consequently, CVs of the analyte/IS ratios for samples spiked after PP procedure and the analogous CVs for samples prepared in the mobile phase A are shown to be similar (Table 1). In fact, the ME percentage values ranged between 15.3 at QC3 level and 19.5 at LLOQ level. In this table, the mean IntRatio values for the sets A, B and C of Eve samples at LLOQ and QC levels are also reported.

Mean Int.Ratio±SD and Precision values (%)						
	set A[a]	**CV%**	**set B**	**CV%**	**set C**	**CV%**
LLOQ	0.0153±0.0014	9.4	0.0123±0.0014	11.1	0.0088±0.0006	6.8
QC1	0.0307±0.0018	5.9	0.0251±0.0025	9.9	0.0192±0.0018	9.6
QC2	0.1273±0.0068	5.3	0.1035±0.0066	6.4	0.0894±0.0096	10.7
QC3	0.3239±0.0153	4.7	0.2745±0.0212	7.7	0.2266±0.0198	8.7
		RE%	**ME%**	**PE%**		
	LLOQ	71.3	19.5	57.4		
	QC1	76.6	18.4	62.5		
	QC2	86.4	18.7	70.2		
	QC3	82.5	15.3	69.9		

Table 1 Integration Ratio of everolimus standard solutions in mobile phase (set A); in dried cytoplasm samples after PP procedure (set B); in cytoplasm mixture before protein precipitation procedure.

The reduced ion suppression effect (below 19% at LOQ level) allowed the recovery of Eve to be more than 70% in the cytoplasmic fractions. In Figure 3, Panel 3A, the SRM profile of everolimus analyzed in a cytoplasmic sample of BT474 cell lines is depicted. The obtained concentration value is 2.93 ng/mL. This sample is reported as an example of a set of 10 cytoplasmic fractions whose mean value is (3.12±0.51 ng/mL).

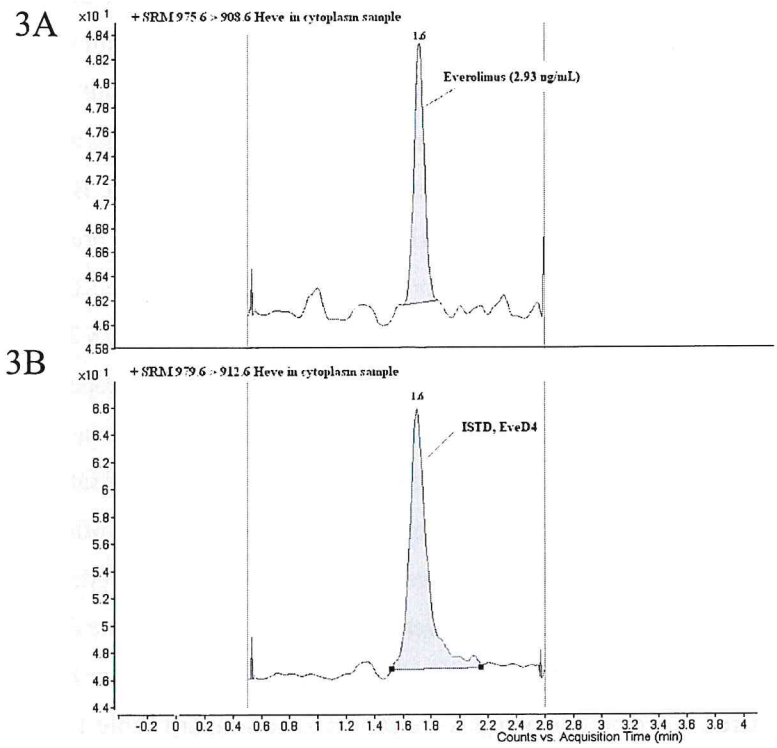

Figure 3. Representative extracted ion chromatograms (XICs) in cytoplasm samples of Everolimus (panel 3A) and ISTD (panel 3B). The UHPLC profile of BT474 cell samples treated at 100 mM for 24h with HEve indicates Everolimus at the concentration of 2.93 ng/mL.

4.3. Method validation

The ratio between the peak-area of Eve and its internal standard was linear along the dynamic range of the calibration curve. The peak-area ratios of the analyte/IS compared with the nominal concentrations were plotted, and a weighted regression function (1/x) was applied to generate the calibration curves that were prepared on four different days. The calibration curves showed excellent linearity and back-calculated concentrations were obtained within a validated range of 2.5-100.0 ng/mL. The mean Pearson's coefficient of determination R^2 was 0.997±0.0004. The mean weighted regression function was y=0.0047± (0.0006) x - 0.0041±(0.0014).
Accuracy and precision (CV%) are shown in Table 2. The method was precise, with interday CV values of less than 5.7%. The LLOQ was fixed at 2.5 ng/mL and was validated through analysis of four replicates. The CVs% were again less than 5.1%. All the other details are reported in Table 2.

Interday	LLOQ	QC1	QC2	QC3
Nominal concentrations (ng/mL)	2.50	5.00	20.00	50.00
Measured concentrations (ng/mL)				
Day1	2.71	5.38	19.29	51.03
	2.77	5.12	20.43	55.81
	2.74	4.42	19.61	50.26
	2.51	4.60	19.23	52.30
Day2	2.77	4.74	21.44	45.11
	2.69	4.54	19.00	50.23
	2.52	5.06	18.96	46.05
	2.45	4.74	20.52	49.32
Day3	2.71	4.75	21.07	48.19
	2.65	4.83	20.76	47.63
	2.51	4.86	20.62	45.89
	2.81	5.06	19.44	48.14
Day4	2.79	4.89	18.80	47.68
	2.56	4.91	18.55	52.94
	2.48	5.30	19.56	50.24
	2.55	4.96	19.70	45.40
Mean±SD (*n*=16)	2.64±0.09	4.88±0.2	19.81±0.91	49.14±2.82
CV (%)	3.2	5.7	4.6	5.7
Accuracy (%)	105.8	97.7	99.1	98.3
Intraday (*Day 1*)				
Mean±SD (*n*=4)	2.69±0.12	4.88±0.4	19.64±0.55	52.35±2.45
CV (%)	4.4	9.2	2.8	2.5
Accuracy (%)	107.5	97.6	98.2	104.7
Intraday (*Day 4*)				
Mean±SD (*n*=4)	2.60±0.13	5.01±0.1	19.15±0.56	49.07±3.25
CV (%)	5.1	3.8	2.9	6.6
Accuracy (%)	103.8	100.3	95.8	98.1

Table 2. Intra- and interday validation of the method for quantitative determination of Eve in cytoplasm

As Eve molecule was functionalized with a polar side chain (Figure 1), its solubility in alcohols increased. Therefore, the stock solutions of Eve were prepared in methanol. These stock solutions were shown to be stable at -80°C for at least 6 months in dark conditions. HEve cytoplasmic fractions were stable at least for 4h at room temperature, and only 24h in the autosampler after protein precipitation in dark conditions. HEve concentration decreased by 45% at the two levels of the quality controls (5.0 and 50.0 ng/mL) in comparison with the control sample when the QCs were analyzed 72h after their processing protocol. HEve was stable in cytoplasm over three freeze–thaw cycles because the concentration left was more than 95% of the nominal concentration.

4.4. Application of the method

To date, we should consider that one of the main issues in Eve treatment is its poor uptake. This point is controversial and still under investigation [2]. In fact, the inclusion of Eve in HFn cages such as Cu(II) complex was studied to promote the drug uptake, since HFn's affinity towards metal ions plays an important role in this pathway. For this reason, in the present paper, a comparative study between cell penetration of Eve and HEve was

undertaken. BT474 cells were treated for 24h with 10, 50 or 100 nM with free Eve or HEve and the drug was quantified in the cytoplasmic compartment by UHPLC-MS/MS. The analysis of HEve samples showed the presence of the active drug inside the cytoplasm fraction only at the highest cell exposure dose (100 nM). In contrast to HEve, the analysis of free Eve samples revealed no detectable concentration of this drug. The SRM profile of HEve sample prepared at the concentration of 100 nM is reported in Figure 3 (panel 3A) as an example of cytoplasmic fractions. The analysis of these specimens confirmed the outcomes achieved from the study on the anti-proliferative activity of Eve [2]. In Eve sensitive tumor cells, the drug activity was not detected. Moreover, the application of the present study demonstrated that the inclusion of Eve in cages of HFn plays a crucial role concerning its uptake. In fact, the LOQ of the method was considered suitable for the present application since it was previously demonstrated that the lower HEve dosage (10 and 50 nM HEve) do not significantly affect cell death.

Conclusions

An analytical method to measure the concentrations of everolimus in cytoplasmic samples of breast cancer cells was developed and fully validated. In this methodology, analytical samples were prepared by precipitating HFn protein nanocages, and then analyzed by UHPLC-MS/MS. The use of an isoelectric protein precipitation solution was a very effective way to obtain protein pellets and clear supernatants over the more conventional denaturation approaches with organic solvents (e.g. methanol and/or acetonitrile) or other additives (e.g. zinc sulfate). The developed UHPLC-MS/MS method was fully validated. The LOQ of the method allowed the establishment of HEve uptake in BC cell lines and the quantification of Eve in the cytoplasmic fractions.

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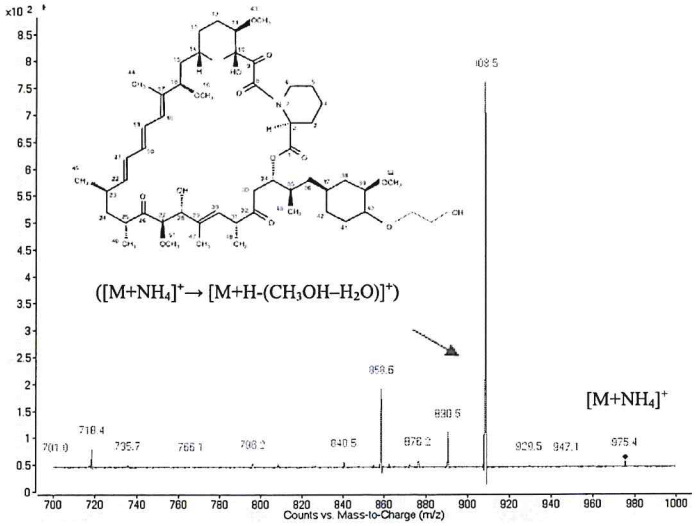
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Figure 1

A



B

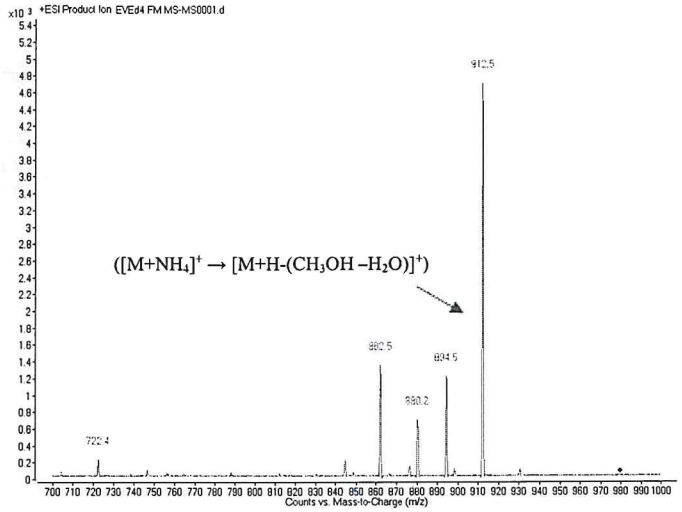


Figure 1. Chemical structure of everolimus [40-O-(2-hydroxy) ethyl-rapamycin] (panel A) and internal standard, EveD4 (panel B).

Figure 2

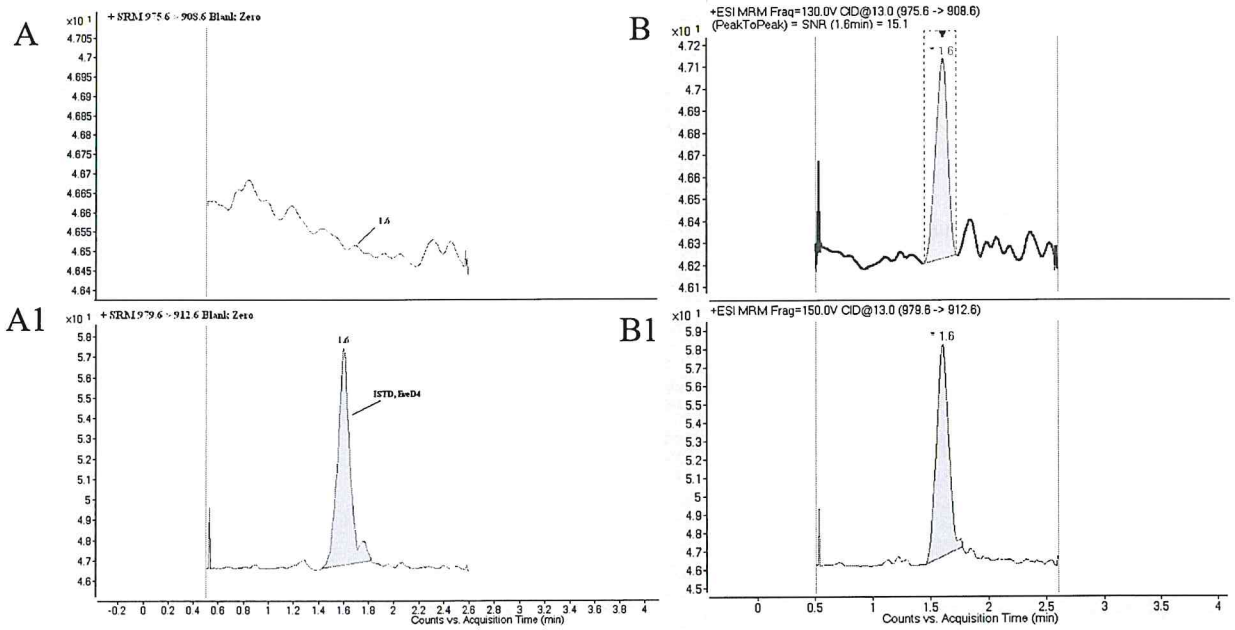


Figure 2. Representative extracted ion chromatograms (XICs) for Eve and ISTD in cytoplasm samples: blank cytoplasmic fractions with and without ISTD (A and A1 panels); cytoplasmic fractions spiked at LLOQ level (B and B1 panels).

Figure 3

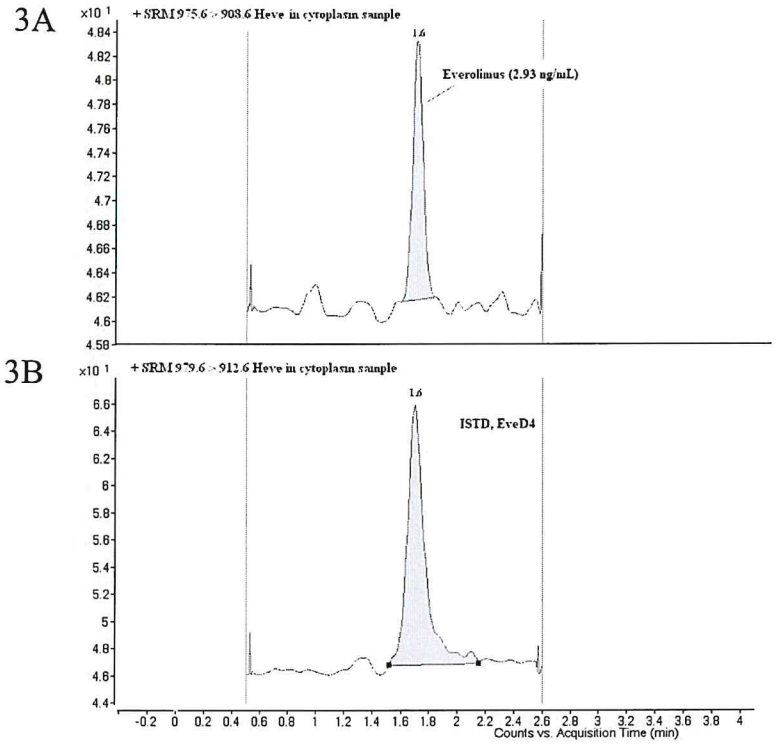


Figure 3. Representative extracted ion chromatograms (XICs) in cytoplasm samples of Everolimus (panel 3A) and ISTD (panel 3B). The UHPLC profile of BT474 cell samples treated at 100 mM for 24h with HEve indicates Everolimus at the concentration of 2.93 ng/mL.