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# Phenylisothiocyanate and dansyl chloride precolumn derivatizations for the high-performance liquid chromatography analysis of the antitumoral agent ES-285 in dog plasma

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**Abstract**

Chromophor and fluorophor addition reactions involving phenylisothiocyanate (PITC) and dansyl chloride (DC) were optimized to adapt two high-performance liquid chromatography (HPLC) procedures designed for the accurate determination of the novel antitumoral agent ES-285 in Beagle dog plasma. ES-285 was reacted with PITC at 60 °C for 15 min in the presence of triethylamine. The dansyl derivative was obtained by reaction of ES-285 with dansyl chloride in a basic medium at 80 °C for 20 min. Both reactions also worked for ES-299, a compound structurally related to ES-285 which was used as internal standard. The treatment of ES-285 and ES-299 spiked plasma samples with a basic phosphate buffer and MeOH permitted the extraction of the drug from the matrix. The purification of the analytes was carried out by solid-phase extraction followed by precolumn derivatization with PITC and DC. The phenylisothiocyanate adducts were analyzed by isocratic HPLC with UV detection at 254 nm. The ES-285 and ES-299 derivatives were eluted from a $C_{18}$ column at ~6.9 and ~8.1 min, respectively. The eluent ACN–water (95:5, v/v) was delivered to the column at a flow-rate of 1 ml/min and the analysis was completed in 15 min. The dansyl derivatives were analysed by a two-HPLC column system with fluorescence detection and gradient elution. The column temperature was maintained at 40 °C. The analysis lasted 55 min with the elution of ES-285 and ES-299 derivatives at ~35.2 and ~37.9 min, respectively. The PITC- and DC-based procedures were characterized by limits of quantification of 20 and 15 ng/ml, respectively. Both procedures were validated according to the ICH and FDA guidelines. They were selective for ES-285 and provided accurate, precise and reproducible results. ES-299 was shown to be a suitable internal standard. The HPLC procedure involving derivatization with DC was more sensitive and permitted to process plasma sample volumes as low as 100 μl. On the other hand, the PITC-based procedure characterised by a quite similar LOQ permitted a higher throughput but implied the processing of a 500-μl plasma volume.
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*Keywords:* Derivatization, LC; ES-285; Phenylisothiocyanate; Dansyl chloride

## 1. Introduction

ES-285 [(2*S*,3*R*)-2-amino-3-hydroxy-octadecane] is a a new investigational drug with antitumoral

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activity and isolated from the marine organism *Spisula polynima* [1,2]. ES-285 demonstrated an in vivo and in vitro selective cytotoxic activity against solid tumors such as colon, gastric, pancreas, pharynx and renal [1,2]. It has been postulated that ES-285 exerts its antiproliferative activity through the modulation of the function of the GTP-binding protein Rho [2]. The inhibition of Rho activity could explain the reduced formation of actin stress fibers observed when cultured cells were incubated with ES-285 [2]. Moreover, the chemical structure of ES-285 (Fig. 1) was shown to be related to lysophosphatidic acid (LPA) and sphingosine which are involved in the regulation of Rho activity and the prevention of the programmed cell death, respectively [2–4]. The antitumoral activity of ES-285 was therefore suggested to be the consequence of an antagonistic inhibition of receptors involved in the regulation of Rho activity [2].

In order to support further pre-clinical studies for the investigation of the pharmacokinetic behaviour of ES-285 in laboratory animals, assays based on chromatographic techniques must be developed to monitor the plasma levels of the drug after parenteral administration. The assay must be fit for purpose, i.e. the accurate quantification of ES-285 levels in plasma samples proceeding from administered animals. Since ES-285 is a new drug under pre-clinical development, little is known about its analytical behaviour in biological samples. As can be seen in Fig. 1, the lack of chromophore by ES-285 prevented the development of a quantitative bioassay involving high-performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection. Nonetheless, ES-285 possesses a primary amino functional group in position 2 of the hydrocarbon skeleton which was considered for chromophore or fluorophore labelling. Precolumn derivatization was therefore explored to render feasible the detection of ES-285 by ultraviolet and fluorescence detection.

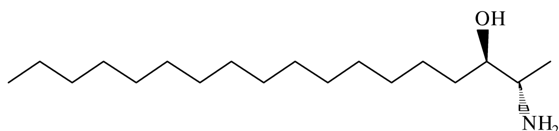


Fig. 1. Chemical structure of ES-285.

This paper describes two HPLC procedures involving precolumn derivatization reactions designed for the analysis of ES-285 in Beagle dog plasma. The derivatizing reagents were phenylisothiocyanate (PITC) and dansyl chloride (DC), two reagents especially used in amino acid analysis [5–10]. Both procedures were optimized and validated according to the compliance criteria laid down in the ICH and FDA guidelines [11–14]. The linearity, accuracy and intra- and inter-assay precision and accuracy of the procedures were assessed. The stability of ES-285 in plasma and of the ES-285 adducts obtained after precolumn derivatization was also evaluated.

## 2. Experimental

### 2.1. Chemicals

ES-285.HCl and ES-299.HCl (internal standard) were provided by PharmaMar (Madrid, Spain). HPLC-grade acetonitrile and methanol were obtained from SDS (Peypin, France). HPLC-grade water was used (CIDA, Barcelona, Spain). Phenylisothiocyanate (PITC) was purchased from Fluka (Switzerland). Analytical-grade $K_2HPO_4$ and 85% orthophosphoric acid were purchased from Panreac (Barcelona, Spain). Analytical grade $Na_2HPO_4.2H_2O$ was obtained from Merck (Darmstadt, Germany). Triethylamine and dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) 95% were purchased from Sigma–Aldrich (Madrid, Spain).

### 2.2. Preparation of standards stock and working solutions

Stock solutions A and B of 0.5 mg/ml of ES-285 were prepared in ACN/MeOH (4:1, v/v). The working solutions of 25, 5, 2, 0.5, 0.2 and 0.02 μg/ml were prepared from the stock solutions in ACN/MeOH (4:1, v/v). The working and stock solutions were stored at $-25\pm5$ °C. The stock solution A and its derived working solutions were used to prepare the calibration standards while the stock solution B was used to prepared the quality control samples. The stock and working solutions were freshly prepared for each validation assay. A stock solution of 0.5 mg/ml of ES-299 was prepared

in ACN/MeOH (4:1, v/v). The ES-299 working solutions of 25 and 2 μg/ml were prepared from the stock solutions in ACN/MeOH (4:1, v/v).

### 2.3. HPLC procedure with precolumn derivatization with PITC

#### 2.3.1. Sample pretreatment and purification

Two milliliters of $K_2HPO_4$ 30 m*M* pH 9.0 were added to 500 μl of Beagle dog plasma previously transferred to a test-tube. The mixture was vortex-mixed for 30 s. Then 300 μl of MeOH were added. The mixture was vortex-mixed and left to stand at room temperature for 10 min. Solid-phase extraction (SPE) clean-up was automated using the vacuum manifold Vac-Elut SPS 24 (Varian, Harbor City, CA, USA). An SPE cartridge coated with 30 mg of polymeric sorbent (Oasis™ HLB, Waters, Milford, MA, USA) was conditioned with 1 ml of MeOH and 1 ml of water. The extract was applied onto the cartridge. When the extract loading was completed, the cartridge was washed consecutively with 1 ml of water adjusted at pH 2.5 with 85% $H_3PO_4$, 1 ml of water and 2 ml of $H_2O$–ACN (95:5, v/v). The cartridge was subsequently dried by air aspiration. ES-285 was eluted with 1 ml of MeOH. The methanolic fraction was evaporated to dryness at 50 °C in a Zymark® TurboVap® nitrogen evaporator (Zymark, Hopkinton, MA, USA).

#### 2.3.2. Precolumn derivatization

ACN/MeOH (180 μl, 4:1, v/v), 70 μl of ACN, 20 μl of triethylamine (dilution 1:100 in ACN) and 20 μl of the 25 μg/ml solution of ES-299 were added to the test-tube containing the dried methanolic fraction obtained after SPE clean-up. Then 10 μl of phenylisothiocyanate (1:10 dilution in ACN) was finally added. The phenylisothiocyanate solution was freshly prepared just before the derivatization step. The test-tube was vortex-mixed for 30 s. The derivatization was carried out at 60 °C for 15 min in a water-bath. After reaction, the test-tubes were cooled down in a water-bath at 4 °C. The mixture was evaporated to dryness at 50 °C in a Zymark TurboVap nitrogen evaporator (Zymark). The dry residue was redissolved in 100 μl of MeOH–ACN–water (4:4:2, v/v/v). Then 50 μl were injected into the HPLC system.

#### 2.3.3. Determination of the ES-285 derivative by reversed-phase high-performance liquid chromatography

The analysis of ES-285 was carried out using a HPLC system consisting of a Waters Alliance™ 2690 separation module (Waters) equipped with a dual wavelength ultraviolet detector 2487 from Waters. A $C_{18}$ HPLC precolumn from Tracer (Teknokroma, Spain) was coupled to a 250 mm×4.6 mm I.D. Symmetry Shield® $C_{18}$ HPLC column (5 μm) from Waters. The detection wavelength was set at 254 nm. The mobile phase used was ACN–HPLC-grade water (95:5, v/v). The flow-rate was set at 1 ml/min. The injector and column oven temperature were set at 4 and 30 °C, respectively. HPLC analysis of the samples was carried out in 15 min.

### 2.4. HPLC procedure with precolumn derivatization with dansyl chloride

#### 2.4.1. Sample pretreatment and purification

The same sample pretreatment process described in Section 2.3.1 was applied to 100 μl of Beagle dog plasma excepting that 25 μl of the 2 μg/ml solution of ES-299 was added to the plasma prior to buffer addition. The 30 mg Oasis HLB (Waters) SPE cartridge was used for sample clean-up and conditioned as described in Section 2.3.1. When the extract loading was completed, the cartridge was washed consecutively with 3 ml of HPLC-grade water and 2 ml of $H_2O$–ACN (95:5, v/v). The cartridge was subsequently dried by air aspiration. ES-285 and ES-299 were eluted with 1 ml of MeOH. The methanolic fraction was evaporated to dryness at 50 °C in a Zymark TurboVap nitrogen evaporator (Zymark).

#### 2.4.2. Precolumn derivatization

The dry residue was redissolved in 100 μl of MeOH–$Na_2HPO_4.2H_2O$ 30 m*M* (1:1, v/v). Buffer pH was adjusted to 7.0 with 85% $H_3PO_4$. Dansyl chloride (30 μl, 20 m*M*) in ACN was also added. Then 20 μl of ACN was finally added to obtain a final volume of 150 μl. The mixture was vortex-

mixed. The derivatization was carried out at 80 °C for 20 min in a water-bath. After completion of the reaction, the test-tubes were cooled down in a water-bath at 4 °C. Then 25 μl was injected into the HPLC system.

#### 2.4.3. Determination of the ES-285 derivative by reversed-phase high-performance liquid chromatography

The analysis of ES-285 was carried out using a HPLC system consisting of a Merck–Hitachi ternary HPLC pump L-6200A (Darmstadt, Germany), a Kontron autoinjector (Kontron, Italy), a Waters column oven and a Waters 474 fluorescence detector. A 125×4.0 mm I.D. LiChrospher® $100C_{18}$ HPLC column (5 μm) from Merck (Darmstadt, Germany) was connected to a 250 mm×4.6 mm I.D. Terra® RP18 HPLC column (5 μm) from Waters. A Terra® RP18 HPLC precolumn from Waters was coupled to the first HPLC column. The excitation and emission wavelengths were set at 350 and 550 nm, respectively. The column temperature was maintained at 40 °C. The mobile phase solvent A was HPLC-grade water, the solvent B was MeOH and ACN was the solvent C. A gradient elution was used to elute ES-285, ES-299 and late-eluting compounds from the column (0–3 min, 100%A→50%A, 40%B, 10%C; 3–8 min, 50%A, 40%B, 10%C→15%A, 60%B, 25%C; 8–25 min, 15%A, 60%B, 25%C→15%A, 85%B; 25–36 min, 15%A, 85%B; 36.1–38 min, 100%B; 38.1–55.1 min, 100%A). The analysis lasted 55 min. A gradient flow-rate was also used (0–8 min, 1.1 ml/min; 8–25 min, 1.1→1.5 ml/min; 25–36 min, 1.5 ml/min; 36.1–38 min, 1.7 ml/min; 38.1–55, 1.7→1.3 ml/min; 55.1 min, 1.1 ml/min). The mobile phase was initially delivered to the column at a flow-rate of 1.1 ml/min. The flow-rate was increased during the run in order to reduce the gradient time.

### 2.5. Validation

#### 2.5.1. Calibration standards and quality control samples

Plasma samples proceeding from untreated Beagle dogs were spiked with various amounts of ES-285 to obtain the calibration standards and the quality control samples. The calibration standards used for the UV-HPLC procedure were prepared at concentrations of 20, 50, 200, 500, 1000 and 2000 ng/ml. The corresponding quality control samples were prepared at concentrations of 20, 200 and 1000 ng/ml. The calibration standards used for the fluorescence HPLC procedure were prepared at concentrations of 15, 50, 100, 250, 500 and 1000 ng/ml. The corresponding quality control samples were prepared at concentrations of 15, 100 and 1000 ng/ml. Five independent replicates were prepared at each quality control sample concentration for each procedure and for each validation assay. The lowest concentration corresponded to the limit of quantification (LOQ). The LOQ is defined as the lowest concentration of the analyte which can be detected and quantified with a precision (coefficient of variation, RSD) of not more than 20% and a deviation not more than 20% from the nominal value for accuracy [13,14].

#### 2.5.2. Linearity, accuracy, precision and specificity

The validation of each analytical procedure was carried out over 3 days. The linearity of the analytical procedure was evaluated by plotting the detector response (analyte/internal standard height ratio) versus the nominal concentration of ES-285 present in the plasma sample. The processing of chromatograms, the calculation of correlation coefficients ($r$) and of values for the calibration curve slope and intercept were carried out using the Millenium[32] chromatography manager (version 3.2, Waters). The concentration ranges studied were 20–2000 ng/ml for the UV-HPLC procedure, and 15–1000 ng/ml for the fluorescence HPLC procedure. The precision (RSD) of the analytical procedure was evaluated by determining the intra- and inter-day coefficients of variation. The intra-day precision is defined as the repeatability of the assay while the inter-day precision is defined as the intermediate precision of the assay [11–15]. The accuracy of the analytical procedure is the extent to which the test results generated by the procedure and the true value agree [11–15]. The accuracy is expressed as the relative error of measurement (RE%):

$$\mathrm{RE}(\%) = \frac{(\text{Mean calculated concentration} - \text{true concentration})}{\text{True concentration}} \times 100$$

The intra- and inter-day accuracies were evaluated. The intra-day accuracy and precision of the determination should not be above 15% for the quality control sample concentrations other than the LOQ [13,14]. The selectivity of the procedure was evaluated by analyzing daily non-spiked plasma samples from various untreated animals.

#### *2.5.3. Evaluation of the absolute recovery*

Peak heights were used for recovery calculations. The ES-285 amount recovered from the spiked plasma sample was determined by comparing the response (peak height) of the extract with the response of the corresponding external standard dissolved and derivatized as described in Sections 2.3.2 and 2.4.2. For each analyte concentration, duplicate reference samples were prepared and the mean response was used in order to assess the concentration of the analyte in the extract.

#### *2.5.4. Stability*

Plasma samples spiked with ES-285 concentrations of 200, 1000 and 2000 ng/ml were stored at $-25\pm5$ °C for 1 month. Three independent replicates were prepared for each concentration. The same was carried out to assess the stability of ES-285 spiked plasma samples frozen at $-25\pm5$ °C for 1 month and submitted to three freeze/thaw cycles. These stability quality control samples were processed and derivatized as described in Sections 2.3.1 and 2.3.2. ES-285 was analyzed as reported in Section 2.3.3. The ES-285 concentrations and accuracy were assessed as described in Section 2.5.1 and 2.5.2.

The stability of the ES-285 derivatives obtained after PITC and dansyl chloride derivatization was evaluated in processed ES-285 spiked plasma sample. Calibration standards and quality control samples were left in the autoinjector and were analyzed after 24 h of incubation. The accuracy values must comply with the criteria defined in Sections 2.5.1 and 2.5.2.

## 3. Results and discussion

### *3.1. Precolumn derivatization with PITC and dansyl chloride*

The chromatographic analysis of the new antitumoral agent ES-285 was a major challenge due to the drug chemical structure. As can be observed in Fig. 1, the marine compound lacks a chromophore precluding the use of the traditional ultraviolet detection for HPLC analysis. Precolumn derivatization was therefore envisaged to adapt a HPLC method for ES-285 analysis. Chromophore and fluorophore additions were considered to enable HPLC analysis by ultraviolet and fluorescence detection. The Edman's reagent or phenylisothiocyanate (PITC) was tested for chromophore addition. The derivatisation reaction must be carried out in a basic medium to ensure a complete yield [16–19]. Phenylisothiocyanate reacted with ES-285 to form an adduct which was easily detected at 254 nm. The reaction was complete when ES-285 was mixed with PITC in the presence of triethylamine at 60 °C for 30 min. At high pH values, ES-285 is under its non-ionised form, which favours the electrophilic attack of the phenylisothiocyanate on the primary amino functional group of the drug.

Dansyl chloride was the reagent which gave the best results for fluorophore addition to ES-285. The primary amino functional group of ES-285 reacted with dansyl chloride under alkaline conditions to give a highly fluorescent derivative. The fluorogenic reaction was found to give maximum yield when carried out at 80 °C for 30 min. Dansyl chloride is hydrolyzed during the reaction to produce a 1-dimethylaminonaphthalene-5-sulphonic acid [20]. Fluorescamine, 9-fluorenylmethylchloroformate (FMOC-Cl) and fluorescein isothiocyanate (FITC) were also tested but gave no satisfactory results. It was not possible to detect a derivative under isocratic conditions nor under gradient elution.

ES-299, an ES-285 structurally related compound (Fig. 2), was also effectively derivatized by PITC and dansyl chloride. ES-299 was therefore expected to correct the possible variability due to the derivatization processes and was used as an internal standard.

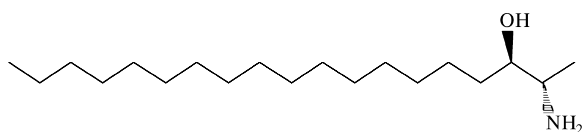


Fig. 2. Chemical structure of ES-299.

### 3.2. Sample pretreatment, drug purification and chromatographic conditions

The physicochemical properties of ES-285 complicated the extraction and purification of the drug from the plasma. The structural similarity with lipid constituents of the cell membrane (e.g. LPA, sphingosine) suggested complex interactions with amphiphilic constituents of the plasma, therefore hindering the extraction and purification of the drug from the matrix. Liquid–liquid extraction (LLE) with chloroform was characterized by poor reproducibility, lack of selectivity and low recovery (e.g. 20% using $CHCl_3$). Moreover, ES-285 was poorly soluble in organic solvents like hexane and ethyl acetate, therefore limiting the application of the LLE. We found that the pretreatment of the sample with a basic phosphate buffer and MeOH permitted to extract the drug from the plasma. The alkaline conditions likely disrupted interactions between ES-285 and matrix components, which favoured drug recovery in the order of or higher than 50%. Solid-phase extraction with the hydrophilic–lipophilic balanced sorbent Oasis permitted to purify ES-285 and provided an extract free of major interferences. The major part of the plasmatic interferences and contaminants were removed by washing the cartridge with acidified $H_2O$ and a mixture of $H_2O$ and ACN. The washing step with acidified water was not necessary in the case of the procedure involving the derivatization with dansyl chloride. ES-285 was not recovered from the plasma when the sample was treated with acidic buffers ($pH<4$), suggesting that ES-285 must be under its neutral form to be efficiently extracted and purified from the matrix.

HPLC in the isocratic mode with a mobile phase of ACN and $H_2O$ (95:5, v/v) permitted to elute ES-285 and ES-299 PITC derivatives as sharp peaks at ~6.9 and ~8.1 min, respectively (Fig. 2). The latter mobile phase was found to provide the best selectivity when the purified extract was chromatographed on the Symmetry Shield reversed-phase HPLC column. No major interferences coeluted with the analytes of interest (Fig. 3). The plasma samples were analyzed in 15 min (Fig. 3). The PITC-based HPLC procedure permitted to achieve a LOQ of 20 ng/ml for ES-285 and for an initial plasma sample volume of 500 μl.

The chromatography of the ES-285 and ES-299 dansyl derivatives was rather complicated. It was impossible to detect the dansyl derivatives in the isocratic mode. The peak of unreacted dansyl chloride overlapped the derivative. A liquid–liquid extraction step to remove the excess reagent was discarded due to the lipid-like structure of the antitumour agent which caused the derivative to be recovered in the organic phase along with unreacted dansyl chloride. Dansyl-ES-285 was finally analyzed by gradient elution. Optimum resolution from interfering peaks was achieved by performing the gradient elution in a two-HPLC column system (Fig. 4). Best results were obtained by coupling a 125 mm × 4.0 mm Lichrospher® column to a 250 × 4.6 mm Terra® RP18 column. The strength of the mobile phase increased with time to reach 100% MeOH after 35 min of analysis (see Section 2.4.3). The type of solvents used and the column temperature (40 °C) allowed a maximum pressure of 170 bars over the entire run. Under these conditions, ES-285 and ES-299 derivatives were separated from unreacted dansyl chloride and interferences and eluted as sharp peaks at ~35.2 and ~37.9 min, respectively (Fig. 4). Elution of strongly adsorbed compounds and column re-equilibration was carried out during the remaining 20 min of the gradient. A limit of quantification of 15 ng/ml was achieved for the analysis of 100 μl of ES-285 spiked plasma samples. The LOQ corresponded to 250 pg of derivatized ES-285 injected onto the two-column system which is twenty-fold the quantity of PITC-derivative injected onto the Symmetry Shield column. The procedure involving derivatization with dansyl chloride was therefore more sensitive than the PITC procedure. Although a much lower amount of derivatized ES-285 injected onto the column was detected using the fluorescence procedure, the limit of quantification characterizing both procedures was quite similar. This latter fact is explained using a smaller plasma sample volume in the case of the analysis of the dansyl derivative.

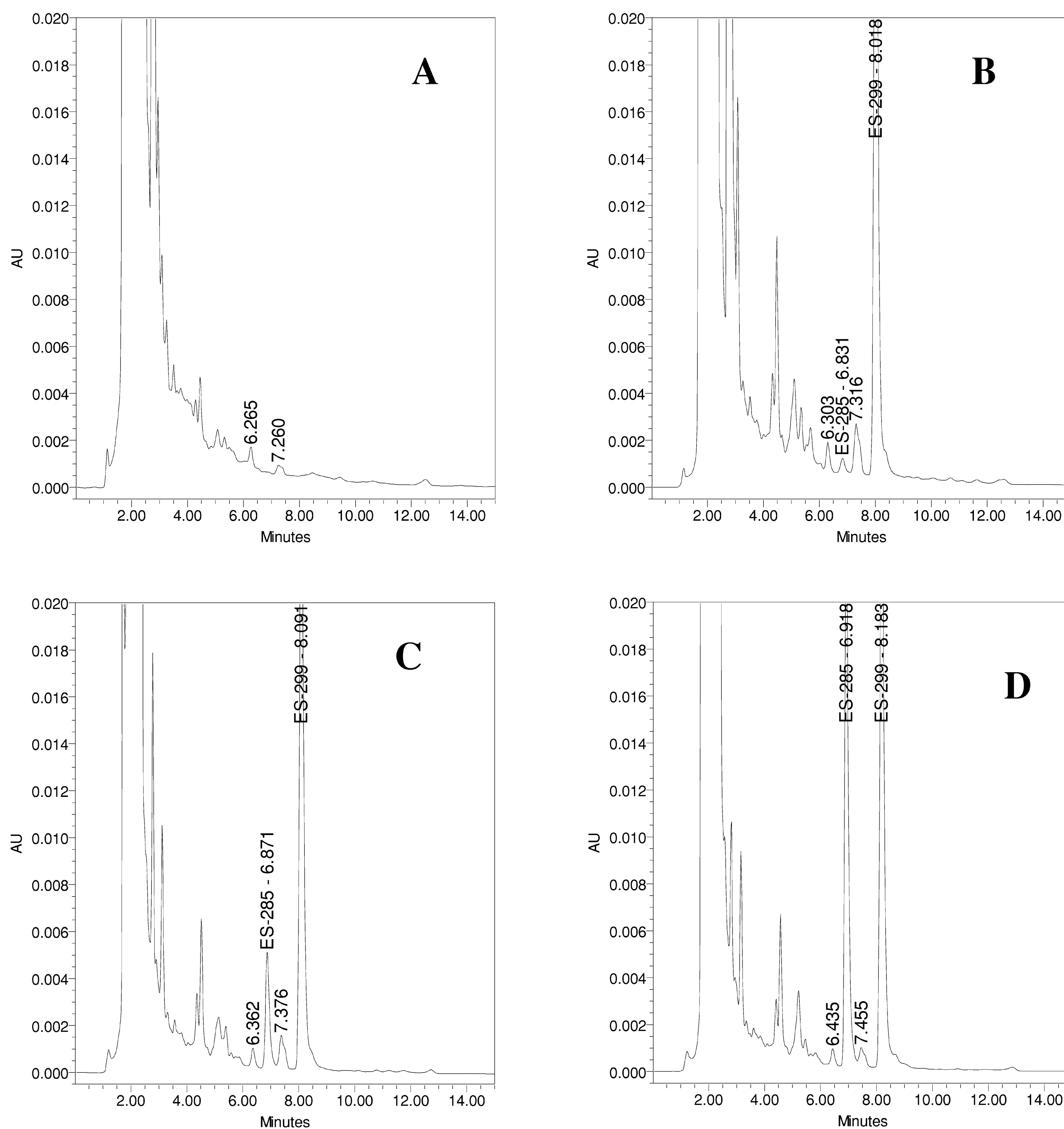


Fig. 3. HPLC–UV analysis of ES-285 in spiked plasma samples. ES-285 and ES-299 (I.S.) were detected as PITC derivatives.

Therefore, dansyl chloride derivatization achieved a similar limit of quantification by processing only 100 μl of plasma sample instead of the 500 μl used in the PITC-based HPLC procedure. Nonetheless, the HPLC analysis of the ES-285 derivative in the isocratic mode was shorter, i.e. the analysis was completed in 15 min while the HPLC analysis with gradient elution lasted 55 min. Higher throughput would therefore be achieved using the UV-HPLC procedure.

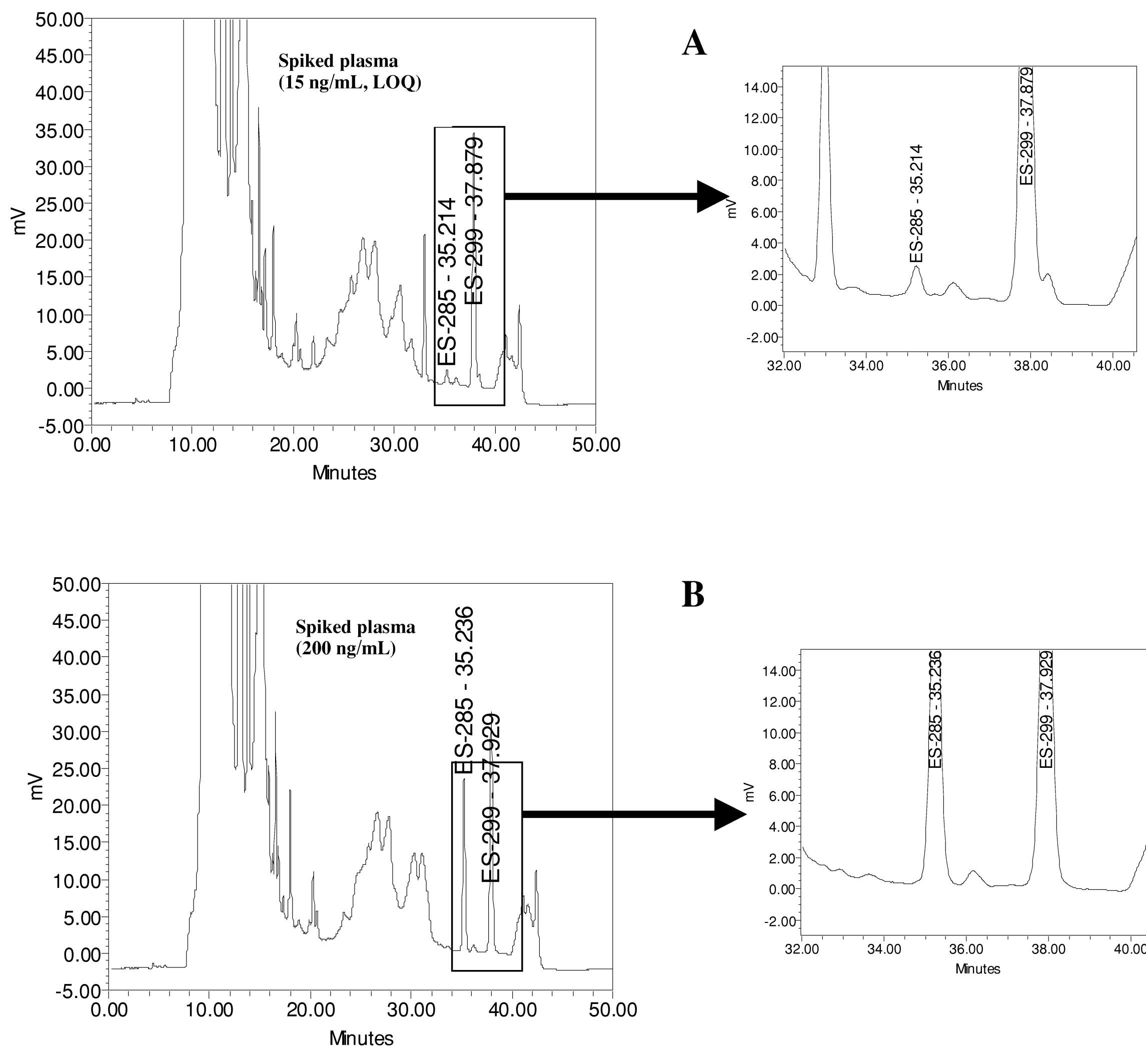


Fig. 4. Determination of ES-285 by HPLC with fluorescence detection. ES-285 and ES-299 (I.S.) were detected as fluorescent derivatives obtained after derivatization with dansyl chloride.

### *3.3. Validation*

Tables 1 and 2 show the results obtained for the evaluation of the linearity, the accuracy and the precision of the analytical procedures developed for the quantification of ES-285 in Beagle dog plasma. The calibration curves were characterized by a coefficient of correlation higher than 0.99. The intra- and inter-day accuracy and precision for the quality control samples spiked with the LOQ were not higher than 20%. The accuracy and RSD values obtained for the other quality control samples were below 15%. These results complied with the criteria defined by the FDA [13,14]. Both HPLC procedures were selective for ES-285. Figs. 3 and 4 illustrate that ES-285 was separated from interfering peaks. Tables 3 and 4 show the absolute recovery values obtained for ES-285. Lower mean recoveries were obtained for the HPLC procedure with UV detection probably due to the cartridge wash with acidified water. The acidic conditions could have enhanced the loss of adsorbed drug when the cartridge was

Table 1
Evaluation of the linearity, accuracy and precision of the HPLC procedure with phenylisothiocyanate precolumn derivatization

| | Calibration curve | 20 ng/ml [LOQ] | | | 200 ng/ml | | | 1000 ng/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean (ng/ml) | Accuracy (%) | RSD (%) | Mean (ng/ml) | Accuracy (%) | RSD (%) | Mean (ng/ml) | Accuracy (%) | RSD (%) |
| Day 1 | $y=1.040x-0.149$ ($r=0.9998$) | 20.73 | 3.65 | 5.47 | 190.95 | −4.52 | 7.17 | 950.97 | −4.90 | 2.48 |
| Day 2 | $y=1.123x-0.430$ ($r=0.9996$) | 20.43 | 2.15 | 5.18 | 186.86 | −6.57 | 2.29 | 902.14 | −9.79 | 13.32 |
| Day 3 | $y=1.078x-0.242$ ($r=0.9995$) | 21.49 | 7.48 | 1.77 | 193.30 | −3.35 | 1.84 | 987.08 | −1.29 | 1.64 |
| Inter-day precision and accuracy | | 20.99 | 4.93 | 4.40 | 190.37 | −4.82 | 4.40 | 949.91 | −5.01 | 7.31 |

Beagle dog plasma sample volume was 500 μl; LOQ, limit of quantification; RSD, relative standard deviation. Five quality control samples were evaluated at each concentration. The assay was repeated on 3 days to obtain inter-day precision and accuracy values. ES-299 was used as the internal standard.

Table 2
Evaluation of the linearity, accuracy and precision of the HPLC procedure with dansyl chloride precolumn derivatization

| | Calibration curve | 15 ng/ml [LOQ] | | | 100 ng/ml | | | 1000 ng/ml | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean (ng/ml) | Accuracy (%) | RSD (%) | Mean (ng/ml) | Accuracy (%) | RSD (%) | Mean (ng/ml) | Accuracy (%) | RSD (%) |
| Day 1 | $y=0.961x+0.277$ ($r=0.9996$) | 16.56 | 10.38 | 7.20 | 94.19 | −5.81 | 4.21 | 1072.29 | 7.23 | 4.89 |
| Day 2 | $y=0.898x+0.490$ ($r=0.9996$) | 17.03 | 13.53 | 7.48 | 107.23 | 7.23 | 11.81 | 1065.12 | 6.51 | 10.33 |
| Day 3 | $y=0.908x+0.473$ ($r=0.9993$) | 17.42 | 16.13 | 11.07 | 94.95 | −5.05 | 4.31 | 1118.90 | 11.89 | 8.40 |
| Inter-day precision and accuracy | | 16.99 | 13.23 | 8.81 | 98.79 | −1.21 | 9.78 | 1085.44 | 8.54 | 7.91 |

Beagle dog plasma sample volume was 100 μl; LOQ, limit of quantification; RSD, relative standard deviation. Five quality control samples were evaluated at each concentration. The assay was repeated on 3 days to obtain inter-day precision and accuracy values. ES-299 was used as the internal standard.

further washed with the mixture of $H_2O$ and ACN (95:5, v/v). A higher variability was observed for the absolute recovery values when compared with the precision of the corresponding concentrations (Tables 1–4). This latter fact demonstrated that ES-299 was a suitable internal standard for the accurate quantification of ES-285 in Beagle dog plasma, essentially contributing to improve the precision of both HPLC assays. The mean ES-299 absolute recoveries were in the order of 65 and 90% for the DC- and PITC-based HPLC procedures, respectively.

Table 5 reports the results obtained for the stability of the derivatized samples. The dansyl derivatized samples were stable for a least 24 h when left in the autosampler at ambient temperature (Table 5). The accuracy and the precision of the assay were not

Table 3
Absolute recovery of ES-285 from spiked Beagle dog plasma samples using the HPLC procedure with phenylisothiocyanate precolumn derivatization

| | Absolute recovery[a] | | | | | |
|---|---|---|---|---|---|---|
| | 20 ng/ml (LOQ) | | 200 ng/ml | | 1000 ng/ml | |
| | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) |
| Day 1 | 60.49 | 10.38 | 71.89 | 7.53 | 71.45 | 9.24 |
| Day 2 | 55.24 | 13.00 | 49.10 | 5.81 | 57.69 | 16.01 |
| Day 3 | 67.27 | 8.49 | 72.22 | 6.47 | 55.10 | 13.68 |

LOQ, limit of quantification; RSD, relative standard deviation.
[a] The mean absolute recovery for each concentration was calculated from five ES-285 spiked replicates.

Table 4
Absolute recovery of ES-285 from spiked Beagle dog plasma samples using the HPLC procedure with dansyl chloride precolumn derivatization

| | Absolute recovery[a] | | | | | |
|---|---|---|---|---|---|---|
| | 15 ng/ml (LOQ) | | 100 ng/ml | | 1000 ng/ml | |
| | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) |
| Day 1 | 97.36 | 11.26 | 79.43 | 4.75 | 69.10 | 3.43 |
| Day 2 | 71.87 | 15.71 | 71.06 | 2.57 | 71.52 | 14.99 |
| Day 3 | 97.99 | 13.64 | 70.64 | 6.27 | 76.00 | 10.48 |

LOQ, limit of quantification; RSD, relative standard deviation.
[a] The mean absolute recovery for each concentration was calculated from five ES-285 spiked replicates.

Table 5
Stability of ES-285 derivatives after precolumn derivatization of extracted plasma samples

| | Parameter |
|---|---|
| *Phenylisothiocyanate precolumn derivatization*[a] | |
| Calibration curve | $y=1.063x-0.563$ ($r=0.9997$) |
| | |
| % deviation of the back-calculated concentrations | |
| 20 ng/ml | −0.98 |
| 50 ng/ml | 0.40 |
| 200 ng/ml | 3.73 |
| 500 ng/ml | 13.99 |
| 1000 ng/ml | −7.39 |
| 2000 ng/ml | 4.73 |
| | |
| Accuracy of the quality control samples | |
| 20 ng/ml | −9.35 |
| 200 ng/ml | −13.54 |
| 1000 ng/ml | −12.42 |
| | |
| *Dansyl chloride precolumn derivatization*[b] | |
| Calibration curve | $y=0.9685x+0.290$ ($r=0.9980$) |
| | |
| % deviation of the back-calculated concentrations | |
| 15 ng/ml | 14.02 |
| 50 ng/ml | −13.29 |
| 100 ng/ml | −8.48 |
| 250 ng/ml | 4.42 |
| 500 ng/ml | 2.92 |
| 1000 ng/ml | 2.82 |
| | |
| Accuracy of the quality control samples | |
| 15 ng/ml | 9.20 |
| 100 ng/ml | −8.38 |

[a] Processed ES-285 spiked plasma samples (quality control samples and calibration standards) were derivatized with PITC and left at 4 °C for 24 h.
[b] Processed ES-285 spiked plasma samples (quality control samples and calibration standards) were derivatized with dansyl chloride and left at room temperature for 48 h.

above 15%. The dansyl derivatives were also found to be stable for a period of at least 1 month when stored at −25 °C (data not shown) as previously reported in the literature for dansyl chloride derivatized eflornithine [21]. On the other hand, the samples must be left at 4 °C during the UV-HPLC analysis due to the lower stability of the PITC derivatives at ambient temperature as also reported by Guitart et al. [22]. Nonetheless, the PITC derivatives were found to be stable when stored at −25 °C immediately after derivatization as also reported in the literature [18,23]. ES-285 concentrations of 193.6, 948.4 and 2108.8 ng/ml were, respectively obtained for the 200, 1000 and 2000 ng/ml quality control samples left at −25 °C for 1 month. Regarding the quality control samples submitted to three freeze/thaw cycles, the concentrations obtained were 174.0, 900.6 and 2171.3 ng/ml. The frozen storage and the freeze/thaw cycles did not have any negative effect on the stability of ES-285, since the quantified ES-285 concentrations for each stability quality control sample did not deviate by more than 15% from its nominal value.

## 4. Conclusions

The evaluation of the parameters used to understand the pharmacokinetic behaviour of a drug relies on the accuracy and sensitivity of the bioanalytical assay to analyze the plasma samples obtained from treated animals. In this paper, two precolumn derivatization reactions were optimized to make feasible the quantification of the novel antitumoral agent ES-285 in Beagle dog plasma by HPLC with fluorescence and ultraviolet detection. The drug was successfully derivatized with the Edman's reagent phenylisothiocyanate and dansyl chloride. Both procedures were suitable for the quantification of ES-285 in Beagle dog plasma and were fully validated according to ICH and FDA guidelines. The procedures were selective for ES-285 and provided accurate, precise and reproducible results.

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## References

[1] J.M. Jimeno, D. Garcia-Gravalos, J. Avila, B. Smith, W. Grant, G.T. Faircloth, Proc. Am. Assoc. Cancer Res. 5S (1999) 3792s.
[2] R. Cuadros, E. Montejo de Garcini, F. Wandosell, G. Faircloth, J.M. Fernández-Sousa, J. Avila, Cancer Lett. 152 (2000) 23.
[3] D.J. Mackay, A. Hall, J. Biol. Chem. 273 (1998) 20685.
[4] O. Cuvillier, G. Pirianov, B. Kleuser, P.G. Vanek, O.A. Coso, J.S. Gutkind, S. Spiegel, Nature 381 (1996) 800.
[5] K.A. Cohen, J.W. Dolan, S.A. Grillo, J. Chromatogr. A 316 (1984) 359.
[6] S.A. Cohen, B.A. Bidlingmeyer, T.L. Tarvin, Nature 320 (1986) 769.
[7] S.R. Hagen, J. Augustin, E. Grings, P. Tassinari, Food Chem. 46 (1993) 319.
[8] C. De Jong, G.J. Hughes, E. Van Wieringen, K.J. Wilson, J. Chromatogr. A 241 (1982) 345.
[9] S. Weinstein, S. Weiner, J. Chromatogr. A 303 (1984) 244.
[10] M. Simmaco, D. De Biase, D. Barra, F. Bossa, J. Chromatogr. A 504 (1990) 129.
[11] International Conference on Harmonisation (ICH) (1996) Topic Q2A: Validation of Analytical Procedures: Definitions and Terminology.
[12] International Conference on Harmonisation (ICH) (1996) Topic Q2B: Validation of Analytical Procedures: Methodology.
[13] P.V. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[14] Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM): Guidance for Industry: Bioanalytical Method Validation, May 2001.
[15] EURACHEM Working Group, The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics, 1998.
[16] P. Edman, Acta Chem. Scand. 4 (1950) 277.
[17] P. Edman, G. Begg, Eur. J. Biochem. 1 (1967) 80.
[18] R.L. Heinrikson, S.C. Meredith, Anal. Biochem. 136 (1984) 65.
[19] R.W. Beaver, D.M. Wilson, H.M. Jones, K.D. Haydon, J. Assoc. Off. Anal. Chem. 70 (3) (1987) 425.
[20] Y. Ohkura, M. Kai, H. Nohta, J. Chromatogr. B 659 (1994) 85.
[21] J.L. Cohen, R.J. Ko, A.T.L. Lo, M.D. Shields, T.M. Gilman, J. Pharm. Sci. 78 (2) (1989) 115.
[22] A. Guitart, P. Hernández, J. Cacho, Analyst 116 (1991) 399.
[23] S.R. Hagen, B. Frost, J. Augustin, J. Assoc. Off. Anal. Chem. 72 (6) (1989) 912.