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Development and validation of a liquid chromatography-ultraviolet absorbance detection assay using derivatisation for the novel marine anticancer agent ES-285·HCl [(2*S*,3*R*)-2-amino-3-octadecanol hydrochloride] and its pharmaceutical dosage form

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Abstract

ES-285·HCl [(2*S*,3*R*)-2-amino-3-octadecanol hydrochloride] is a novel investigational anticancer agent, which has shown in vitro and in vivo cytotoxic activity against various tumor cell lines with selectivity for certain solid tumors. The pharmaceutical development of ES-285·HCl warranted the availability of an assay for the quantification and purity determination of ES-285·HCl active pharmaceutical ingredient (API) and its pharmaceutical dosage form. A liquid chromatographic method (LC) comprising of derivatisation of ES-285·HCl with phenylisothiocyanate and UV-detection was developed. The method was found to be linear, precise and accurate. The assay also proved selectivity as determined by analysing ES-285·HCl in combination with 15 analogues and in combination with hydroxypropyl-β-cyclodextrin, the excipient used in the lyophilised pharmaceutical dosage form. Stress testing showed that the degradation products were separated from the parent compound, confirming its stability indicating capacity. The method was found robust as determined with design of experiments (DoE), which made it possible to predict system suitability responses in worst case experimental conditions and to define criteria for system suitability testing.

Keywords: Derivatisation, LC; Design of experiments; (2*S*,3*R*)-2-amino-3-octadecanol hydrochloride; Phenylisothiocyanate; ES-285·HCl

1. Introduction

ES-285·HCl [(2*S*,3*R*)-2-amino-3-octadecanol hydrochloride] is a novel investigational anticancer agent, which was first isolated from the marine clam *Mactromeris polynoma* (Fig. 1) [1]. The substance

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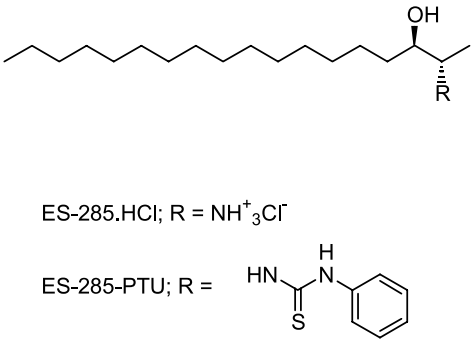

Fig. 1. Chemical structures of ES-285·HCl ($C_{18}H_{40}ClNO$, $M_w = 321$) and ES-285-PTU ($C_{25}H_{44}N_2OS$, $M_w = 420$).

is currently obtained by a total chemical synthesis route. ES-285·HCl has shown in vitro and in vivo cytotoxic activity against various tumor cell lines with selectivity for certain solid tumors (i.e. hepatocellular, prostate and renal). Its mode of action is believed to be exerted by inhibition of Rho activity, a small GTP-binding protein involved in growth signal transduction pathways including stimulation of the appearance of actin stress fibers [1]. The formation of actin stress fibers is believed to be correlated to the cell's capacity for adhesion to the surrounding matrix [2].

The pharmaceutical development of ES-285·HCl necessitated the availability of an assay for the quantification and purity determination of ES-285·HCl active pharmaceutical ingredient (API), and its pharmaceutical dosage form [3,4]. In this paper, a reversed-phase liquid chromatography (RP-LC) method with ultraviolet (UV) detection for ES-285·HCl and its related compounds after derivatisation with phenylisothiocyanate is described. Validation of the analytical method was performed according to international guidelines [5,6].

2. Materials and methods

2.1. Chemicals

ES-285·HCl, ES-285-phenylthiourea (ES-285-PTU) and ES-285·HCl analogues (see Table 1) were provided by PharmaMar Sociedad Unipersonal (Colmenar Viejo, Spain). ES-285·HCl lyophilised product was manufactured in-house (Department of Pharmacy & Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands). 2-Hydroxypropyl-β-cyclodextrin (HPβCD) was purchased from Roquette (Lestrem, France). Phenylisothiocyanate (PITC) was obtained from Sigma Aldrich Chemie (Zwijndrecht, The Netherlands), and acetonitrile (ACN) and methanol (MeOH) from Biosolve Ltd. (Amsterdam, The Netherlands). Triethylamine (TEA) and Water for Chromatography were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade and were used without further purification.

Table 1
ES-285·HCl (compound **9**) and ES-285·HCl analogues

Compound	Structure
1	3-NH_3Cl-4-OH-$C_{16}H_{32}$
2	2-NH_3Cl-3-OH-$C_{16}H_{32}$
3	3-NH_3Cl-4-OH-$C_{17}H_{34}$
4	2-NH_3Cl-3-O-$C_{17}H_{34}$
5	2-NH_3Cl-3-OH-$C_{17}H_{34}$
6	4-NH_3Cl-5-OH-$C_{18}H_{36}$
7	3-NH_3Cl-4-OH-$C_{18}H_{36}$
8	2-NH_3Cl-3-O-$C_{18}H_{36}$
9	2-NH_3Cl-3-OH-$C_{18}H_{36}$
10	4-NH_3Cl-5-OH-$C_{19}H_{38}$
11	3-NH_3Cl-4-OH-$C_{19}H_{38}$
12	2-NH_3Cl-3-O-$C_{19}H_{38}$
13	2-NH_3Cl-3-OH-$C_{19}H_{38}$
14	3-NH_3Cl-4-OH-$C_{20}H_{40}$
15	2-NH_3Cl-3-O-$C_{20}H_{40}$
16	2-NH_3Cl-3-OH-$C_{20}H_{40}$

2.2. Sample preparation

2.2.1. ES-285-PTU standard reference solution

ES-285-PTU standard reference solution was prepared by accurately weighing 3.2 mg reference standard and subsequent dissolution in 10 ml of MeOH. The resulting solution (500 μl) was transferred to an autosampler vial containing 100 μl of MeOH to obtain a final concentration of 267 μg/ml ES-285-PTU, corresponding to 204 μg/ml ES-285·HCl.

2.2.2. Derivatisation

ES-285·HCl was derivatised to form ES-285-PTU using PITC as the derivatising agent and TEA as proton acceptor. The derivatisation was optimised for the concentration level of 200 μg/ml ES-285·HCl. For this, the incubation time and molar ratio ES-285·HCl

to TEA were held constant at 30 min and 1:3, respectively, while the ES-285·HCl to PITC molar ratio was varied between 1:0 and 1:200. The recovery of ES-285-PTU in the samples was determined with the described LC–UV method using ES-285-PTU standard reference solution set as 100% recovery. The stability of the resulting derivative was determined after 24 h of storage at ambient light and temperature. The final molar ratio of ES-285·HCl to PITC required for the complete derivatisation of ES-285·HCl to ES-285-PTU in a 200 μg/ml ES-285·HCl solution was found to be 1:73. Following these results, the final derivatisation method for 500 μl of ES-285·HCl solution comprised of the subsequent addition of 40 μl of MeOH, 20 μl of a 1% (v/v) TEA in MeOH solution and 40 μl of a 10% (v/v) PITC in MeOH solution. This corresponds to molar ratios of ES-285·HCl to TEA and PITC of 1:3 and 1:73, respectively, for a 240 μg/ml ES-285·HCl solution (final concentration of 200 μg/ml ES-285·HCl). Derivatisation was carried out in the autosampler vial for 30 min at 40 °C.

2.2.3. ES-285·HCl calibration and quality control samples

ES-285·HCl stock solution was prepared by accurately weighing 3.0 mg of ES-285·HCl drug substance and subsequent dissolution in 10 ml of MeOH. Calibration samples and quality control samples were prepared from two separately weighed stock solutions. To obtain sample solutions containing 25, 50, 100, 150, 200 and 250 μg/ml ES-285·HCl, subsequently 50, 100, 200, 300, 400 and 500 μl of stock solution was transferred to an autosampler vial. MeOH was added to a volume of 500 μl. The stock solutions were derivatised as described above resulting in a final volume of 600 μl.

2.2.4. API

ES-285·HCl drug substance sample was prepared by accurately weighing 2.4 mg of drug substance and subsequent dissolution in 10 ml of MeOH. 500 μl of the resulting solution was transferred to an autosampler vial and derivatised as described above resulting in a final volume of 600 μl, corresponding to an ES-285·HCl concentration of 200 μg/ml.

2.2.5. Pharmaceutical dosage form

ES-285·HCl 50 mg/vial lyophilised powder for intravenous use was dissolved in 100 ml of MeOH. 250 μl of the resulting solution was transferred to an autosampler vial. MeOH was added to a volume of 500 μl. The solutions were derivatised as described above resulting in a final volume of 600 μl, corresponding to an ES-285·HCl concentration of 208 μg/ml.

2.3. Liquid chromatography (LC) and LC–mass spectrometry (MS)

The chromatographic system consisted of a Model SpectraSYSTEM P1000 isocratic pump (Thermo Separation Products (TSP), Fremont, CA, USA), a Model SpectraSERIES AS3000 automatic sample injection device, equipped with a column oven and 100 μl sample loop (TSP), and a Model UV1000 UV-VIS detector (TSP). Chromatograms were processed using ChromQuest software (Thermo Finnigan, San Jose, CA, USA). Separation was achieved using a Zorbax SB-C_{18} analytical column (150 mm × 4.6 mm i.d., particle size 3.5 μm, Rockland Technologies Inc., Newport, DE, USA), which was protected by a guard column packed with reversed-phase material (3 mm × 10 mm) (Chrompack, Middelburg, The Netherlands). The column temperature was kept at +40 °C. The mobile phase consisted of 88% (v/v) ACN and 12% (v/v) water. The flow rate was 0.8 ml/min and UV-detection was performed at 254 nm. The injection volume was 10 μl. A run time of 20 min was employed for the standard samples (ES-285-PTU standard reference solution, ES-285·HCl calibration and quality control samples), and a run time of 60 min to determine impurities and degradation products in the API or pharmaceutical dosage form.

The LC/MS system consisted of an Agilent 1100 series LC system with LC/MSD quadrupole detector equipped with an electrospray interface (ESI) ionisation source (Agilent Technologies, Waldbronn, Germany). LC conditions were as described above. MS conditions for ESI, drying gas flow, nebuliser gas pressure, drying gas temperature, capillary voltage, fragmentor and mass range were: positive ion mode, 10.0 l/min, 35 psi, 350 °C, 4000 and 250 V, and 500–1000 *m/z*, respectively. Spectra were processed

using Agilent Chemstation for LC/MS quadrupole (Agilent Technologies, Waldbronn, Germany).

2.4. Validation procedure

The LC method was validated with respect to the following parameters: linearity, accuracy, precision, selectivity, stability-indicating capability and robustness.

2.4.1. Linearity

Calibration curves at six concentration levels (25, 50, 100, 150, 200 and 250 μg/ml ES-285·HCl in MeOH) were analysed in duplicate in three separate runs. Least-squares analysis of concentration, weighted by (1/concentration), versus the area of the ES-285·HCl peak was applied. The linearity of the calibration curves was evaluated by means of back-calculated values of the calibration standards, the response factor at different concentration levels and the observed correlation coefficients of the calibration curves.

2.4.2. Accuracy and precision

Accuracy, within-run and between-run precision of the method were determined by assaying quality control samples at three concentration levels (50, 100 and 200 μg/ml ES-285·HCl in MeOH) in triplicate in three separate analytical runs. Accuracy was measured as the percent deviation from the nominal concentration. The within-run and between-run precisions were calculated by analysis of variance (ANOVA) for each test concentration using the analytical run as grouping variable. Form the ANOVA analysis the day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained. Within-run and between-run precision were defined using Eqs. (1) and (2), respectively, where n is the number of replicates.

$$\text{Within-run precision (\%)} = 100 \times \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \quad (1)$$

$$\text{Between-run precision (\%)} = 100 \times \frac{\sqrt{(\text{DayMS} - \text{ErrMS}/n)}}{\text{GM}} \quad (2)$$

2.4.3. Selectivity and stability indicating capability

Selectivity was tested by pooling ES-285·HCl with 15 analogues at approximately 1% of the ES-285·HCl concentration. The analogues are tabulated in Table 1. Selectivity was also tested in the presence of HPβCD, the excipient present in the pharmaceutical dosage form.

The stability indicating capability of the LC method was tested by subjecting ES-285·HCl stock solutions to several stress conditions (acid, alkaline, heat, oxidation and high intensity light). Test solutions were prepared from a 3.02 mg/ml stock of ES-285·HCl in MeOH and were sampled after 4 h (oxidation) or 1 week (other conditions). *Heat*: 1 ml of ES-285·HCl stock solution was exposed to 100 °C. *Oxidation*: to 1 ml of ES-285·HCl stock solution, 1 ml of a 3% hydrogen peroxide solution was added. *Acidic*: to 1 ml ES-285·HCl solution 0.5 ml 2 M hydrochloric acid (HCl) was added. Samples were neutralised using 2 M sodium hydroxide (NaOH). Alkaline: similar to the method described under "acidic", using 2 M NaOH as alkaliser and 2 M HCl to neutralise the sample solution. *High intensity light*: 1 ml of ES-285·HCl in MeOH stock solution was exposed to white light with a total intensity of 3600 lx as determined with a Digital Lux Meter LX-101 (Lutron, Cole-Palmer). All samples were diluted with MeOH to a theoretical concentration of 604 μg/ml ES-285·HCl before derivatisation to ES-285-PTU and subsequent analysis.

2.4.4. Robustness

For the robustness study, six parameters were selected: percentage ACN in the mobile phase, pH of the water component of the mobile phase, detector wavelength, column temperature, flow rate and column types (two different lots from the same material supplied by the same manufacturer) [6]. The parameters included were varied between their expected outer limits as derived from the performance specifications of the apparatus and volumetric glassware (variation of percentage ACN in the mobile phase) used. To enable efficient data collection and data interpretation, a design of experiment (DoE) [7] in combination with statistical evaluation using Fusion Pro software (version 7.0.1; S-Matrix, Eureka, CA, USA) was employed. A Plackett–Burman design was generated using this software. Each run in the design consisted of six replicate injections of ES-285-PTU standard reference solution. The effects of variations in chromatographic parameters were evaluated using %R.S.D. in area between replicate injections, mean retention factor calculated with respect to the visually determined hold up time

(k'), mean theoretical plates (N) and mean tailing factor (T). Recommendations described for these parameters are %R.S.D. $< 1.5\%$, $k' > 2$, $N > 4000$, $T \leq 2$ [8].

3. Results and discussion

3.1. LC–UV method development

The development of an analytical LC assay for ES-285·HCl was hindered by the lack of a chromophoric group in the molecule, which excluded the use of UV or fluorescence detection. LC with refractive index (RI) detection or evaporative light scattering detection (ELSD) displayed insufficient sensitivity for ES-285·HCl and gas chromatography (GC) analysis insufficient accuracy and precision.

To make the molecule suitable for UV detection, an attempt was made to introduce a chromophore in the molecule via derivatisation. The primary amino function of ES-285·HCl seemed a good substrate for derivatisation using the chromophoric compound PITC, which is widely used for the characterisation of peptides via the Edman degradation route [9]. The proton acceptor TEA was added to the reaction medium to generate the basic compound, ES-285, which reacts with PITC to form ES-285-PTU (Fig. 1). An excess of both reagent and proton acceptor was employed to complete and accelerate the derivatisation reaction. Optimisation resulted in a final ratio ES-285·HCl to PITC of 1:73 found to be sufficient to complete the reaction in an acceptable time frame. The resulting ES-285-PTU derivative was stable for 24 h of storage at ambient light and temperature. The absorption spectrum of ES-285-PTU showed absorption maxima at approximately 203 and 250 nm. UV-detection at a wavelength of 254 nm was selected for the analysis of ES-285-PTU. Using an eluent composition of ACN/water (88:12), ES-285-PTU eluted as a single, sharp peak with a retention time of approximately 11 min in the chromatogram (Fig. 2). The excess reagent and proton acceptor eluted within 4 min. Peak identity was confirmed by LC–MS analysis.

3.2. Validation

3.2.1. Linearity, accuracy and precision

Linearity, accuracy, within-run and between-run precision of the LC–UV method over the concentration range of 25–250 μg/ml were examined. This range corresponds to 12–120% of the intended test concentration of 208 μg/ml for the pharmaceutical quality control of ES-285·HCl API and the pharmaceutical dosage form. The assay showed linearity with a relative standard deviation of 1.7% for the relative responses (area divided by concentration) obtained in the tested concentration range and correlation coefficients >0.999 found for all three calibration curves.

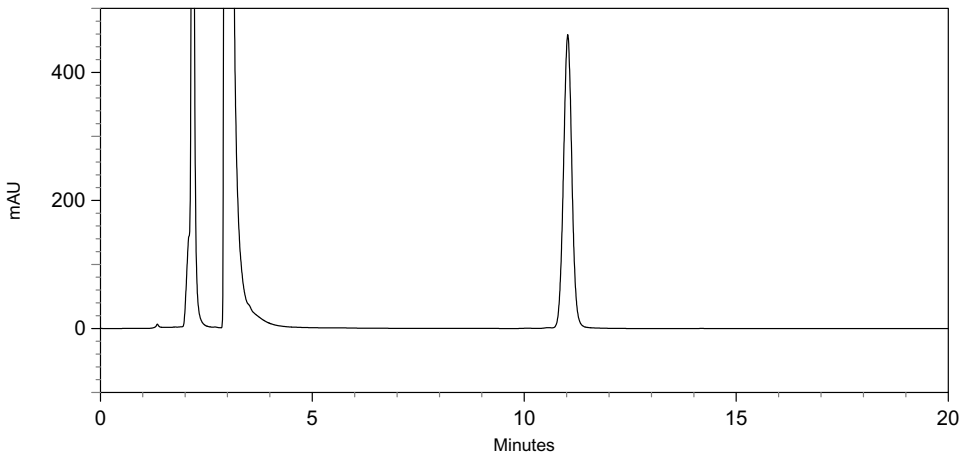

Fig. 2. Representative chromatogram of 200 μg/ml ES-285·HCl raw drug substance solution. The retention time of ES-285-PTU is approximately 11 min.

Table 2
Results of ES-285·HCl quality control samples ($n = 9$)

Concentration (nominal, μg/ml)	Within-run precision (%)	Between-run precision (%)	Accuracy (%)
49.8	1.1	1.3	98.3
99.7	0.6	0.7	97.8
199.4	0.7	0.8	99.7

The average back-calculated concentration for the different calibration concentration levels in the six analytical runs varied between 99.1 and 101.2% of the theoretical concentration for the tested concentration range. The maximal deviation from the nominal concentration observed was 2.4%. Within- and between-run precisions were ≤1.3% with accuracies between 97.8 and 99.7% (Table 2).

3.2.2. Selectivity

Fig. 3 shows a chromatogram of a pooled solution of ES-285·HCl and its analogues. All compounds form PTU derivatives after derivatisation with PITC and elute with sufficient resolution from the ES-285-PTU peak. The compounds **5**, **6**, **8**, and **13** were identified as impurities in different batches of ES-285·HCl drug substance using the developed LC–UV method. Identity of the peaks was confirmed using LC–MS analysis.

ES-285·HCl is pharmaceutically formulated as a lyophilised dosage form containing the excipient HPβCD. HPβCD added to an ES-285·HCl solution was not recovered in the resulting chromatogram and did not affect the response of ES-285·HCl. These results confirm the selectivity of the analytical method for use in the quality control of API and pharmaceutical dosage form.

3.2.3. Stability indicating capability

The stability indicating capability of the assay was examined by accelerated stress testing. No significant degradation was observed in the ES-285·HCl stock solutions subjected to alkaline, acid, heat and high intensity light. No additional peaks were observed after exposure to heat and high intensity light under the tested conditions. Only small additional peaks (≤0.4% of ES-285-PTU peak area) were observed after exposure to acid and alkaline conditions. Exposure to oxidising conditions, however, resulted in the complete disappearance of the ES-285-PTU peak and the appearance of several degradation peaks well separated from the ES-285-PTU peak.

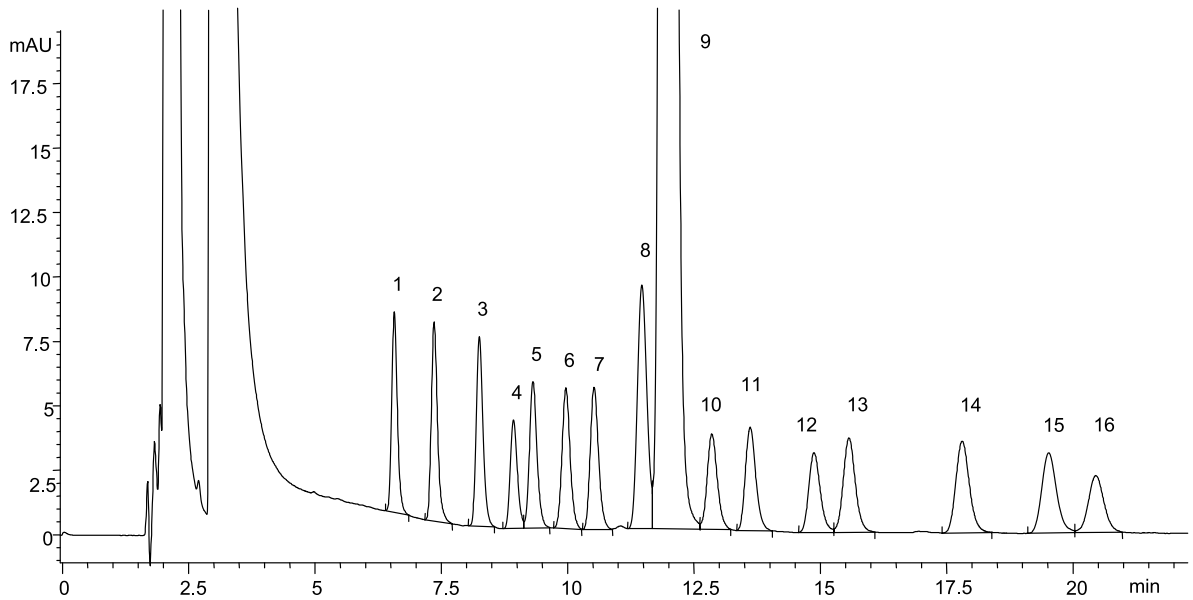

Fig. 3. Chromatogram of ES-285·HCl and its analogues. See Table 1 for corresponding compound structures.

For all conditions, ES-285-PTU peak identity was confirmed with LC–MS, showing no significant additional ions. These observations confirm the stability indicating capability of the analytical method.

3.2.4. Robustness

In order to study the robustness of the analytical method DoE was applied. Using this statistical tool, the individual and interaction effects of multiple parameters on a selected outcome can be assessed in a single experiment requiring a limited number of runs. An eight run Plackett–Burman design was selected to test the six parameters included in the robustness test. A dummy factor was assigned to the remaining potential parameter in the design. Additionally, the final design contained four replicate runs at the nominal value of each parameter to determine the experimental error in the analytical method and to correct for possible drift.

The resulted ranges observed for mean retention factor, mean theoretical plates, mean tailing factor, and %R.S.D. in area were $4.73 \leq k' \leq 13.29$ min, $1.24 \times 10^4 \leq N \leq 1.77 \times 10^4$, $1.03 \leq T \leq 1.22$, and 0.16–1.16%, respectively. The effects of the individual parameters on the different responses were determined using Fusion Pro software. Table 3 gives the statistically significant effects expressed in percentage of the response at nominal value when changing the parameter from the lower to the upper limit. The responses obtained in the replicate runs were defined as the nominal values. No significant effects were found for the %R.S.D. in area for the replicated injections due to the observed large experimental error. The quadratic term in Table 3 was introduced to describe the observed non-linear responses. For the mean retention factor, the dummy parameter was found significant. This may be due to an uncontrolled factor, which was not adequately corrected for in the replicated runs, or to one of the interactions perfectly correlated with this dummy parameter.

In Table 4, the 95% confidence intervals of the predicted lowest and highest responses for the retention factor, number of theoretical plates and tailing factor are given. All responses are well within the recommended criteria. However, the broad response range observed for the retention factor was not desirable in view of the detection of possible impurities and degradation products. Therefore, the control limits for percentage ACN were confined to 88 ± 0.4 ml by preparing the eluent on weight rather than volume. This resulted in a response range of 10.19–1.16 min for the retention factor. The capability of separating impurities under these conditions was tested using a representative ES-285·HCl drug substance sam-

Table 4
95% confidence intervals of the predicted lowest and highest responses for the retention factor, number of theoretical plates and tailing factor

Response	Lowest response 95% prediction interval	Highest response 95% prediction interval
Retention factor	3.72–4.46	12.96–13.71
Theoretical plates	1.03×10^4	1.58×10^4
	-1.34×10^4	-1.91×10^4
Tailing factor	1.00–1.04	1.25–1.29

Table 3
The statistically significant normalised effects of the main parameters and quadratic parameter when changed from the lower to upper limit on the retention factor, number of theoretical plates and tailing factor

	Mean retention factor (%)	Mean theoretical plates (%)	Mean tailing factor (%)	%R.S.D. in area
ACN	−62.3**	−17.9**	12.6**	n.s.
Flow rate (ml/min)	n.s.	n.s.	0.9*	n.s.
Column	+2.8**	12.0**	−6.3**	n.s.
Wavelength (nm)	n.s.	n.s.	n.s.	n.s.
Temperature (°C)	−8.2**	n.s.	n.s.	n.s.
pH	+9.7**	n.s.	−2.7**	n.s.
Dummy	−4.2**	n.s.	n.s.	n.s.
Quadratic term	−19.1**	−6.4*	5.4**	n.s.

* $P < 0.05$.
** $P < 0.01$.

ple. The separation of the impurities **5**, **6**, **8**, and **13** present in the ES-285·HCl drug substance sample in percentages of 0.02% (impurity **5**) to 0.2% (impurity **8**) of ES-285-PTU peak area was not affected in this response range.

No prediction profile could be determined for the %R.S.D. in area between the replicate injections. However, no problem for the robustness is expected as the values for %R.S.D. in area between the replicate injections obtained in the tested runs were low ($\leq$1.2%).

Data and analyses obtained using DoE confirm the robustness of the analytical method for the experimental variations in the parameters included in the study.

The results of the robustness study were translated in criteria for system suitability testing. Criteria for retention factor, theoretical plates, tailing factor, and %R.S.D. in area (six injections) were defined at: %R.S.D. $< 1.5\%$, $10.2 \leq k' \leq 11.2$, $N > 8000$, $0.7 \leq T \leq 1.3$, respectively.

4. Conclusion

In conclusion, a stability indicating analytical method comprising of derivatisation of ES-285·HCl with PITC and subsequent separation and detection with LC–UV was developed for the novel investigational anticancer agent ES-285·HCl. The method was found to be linear in the range of 25–250 μg/ml, precise and accurate. The assay also proved selectivity as determined by analysing ES-285·HCl in combination with 15 analogues and in combination with HPβCD, the excipient used in the lyophilised pharmaceutical dosage form. Stress testing showed that degradation products were well separated from the parent compound, confirming its stability indicating capacity. The method was found robust as determined with DoE, which made it possible to predict chromatographic responses in worst case experimental conditions and to define criteria for system suitability testing. This stability-indicating LC method will be used in the pharmaceutical quality control of ES-285·HCl API and final product.

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