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Development and validation of an anion-exchange LC-UV method for the quantification and purity determination of the DNA plasmid pDERMATT

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ABSTRACT

The pDERMATT (plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c) plasmid is a novel in-house developed anti-cancer vaccine which encodes a melanoma associated epitope (Mart-1) and an immuno stimulatory sequence (tetanus toxin fragment-c). The pharmaceutical development of pDERMATT necessitated the availability of an assay for the quantification and purity determination of pDERMATT active pharmaceutical ingredient (API), the produced bulk drug and its pharmaceutical dosage form. An anion-exchange liquid chromatographic method (AEX-LC) with ultraviolet (UV) detection was developed, which is based on separation on a non porous anion-exchange (NPR AEX) column with a mobile phase gradient of 0.45–0.53 M NaCl in 20 mM Tris–HCl 10% isopropanol (IPA) pH 9 and UV detection at 260 and 280 nm. The method was found to be precise, accurate and linear over a concentration range of 5–150 μg/ml. The supercoiled topoisoform of pDERMATT was well separated from the linear and open-circular form, the main degradation products formed during stress testing, confirming its stability-indicating capability. The use of photo diode array (PDA) detection enabled us to confirm all visible peaks to contain DNA. Additionally capillary gel electrophoresis (CGE) showed the same peak profile as the developed HPLC method. The developed LC-UV method will be used for the pharmaceutical quality control of pDERMATT API, bulk drug and its pharmaceutical dosage form.

1. Introduction

pDERMATT (plasmid DNA Encoding Recombinant MART-1 and Tetanus toxin fragment-c) is a novel in-house developed anti-cancer vaccine (Fig. 1). MART-1 is a melanoma associated antigen (melanocyte lineage-specific) which is expressed in a large fraction of melanomas [1]. The plasmid will be administered using a newly developed tattoo strategy [2]. This short-interval intradermal DNA vaccination leads to the rapid and sustained development of both T-and B-cell responses. After administration of the plasmid, the encoding protein is expressed in the skin and can be taken up by Langerhans cells, a subset of Dendritic Cells (DCs) in the epidermal layer of the skin. Upon maturation, and activation, Langerhans cells migrate to the lymph nodes and present antigens displayed by MHC class molecules to naive T-cells. This action is essential in initiating an acquired immune response [3].

The pharmaceutical development of a stable, sterile, intradermal dosage form for the scheduled phase I clinical trials in patients with melanoma, necessitated the availability of an assay [4] for the quantification and purity determination of pDERMATT active pharmaceutical ingredient (API) and its pharmaceutical dosage form. The use of pDNA chromatography has been reviewed by Diogo et al. [5]. Of the techniques reviewed anion-exchange (AEX), hydrophobic interaction chromatography (HIC) and reversed phase ion-pair chromatography can be used for quantitation. Hydrophobic interaction chromatography has proven a successful separation method [6–8], however AEX high performance liquid chromatography (HPLC) is still most widely used [5,9]. Several groups have successfully separated the plasmid DNA forms using AEX-HPLC [10–14]. However none of these groups have used isopropanol (IPA) in the mobile phase. The addition of IPA to the mobile phase prevents solvophobic interactions between the DNA and the stationary phase, resulting in better separation [9]. Validation of the analytical method was performed according to international guidelines [15]. Agarose gel electrophoresis (AGE) and on-line photo diode array (PDA) detection were used to attain more information on the observed impurities and degradation products of pDERMATT.

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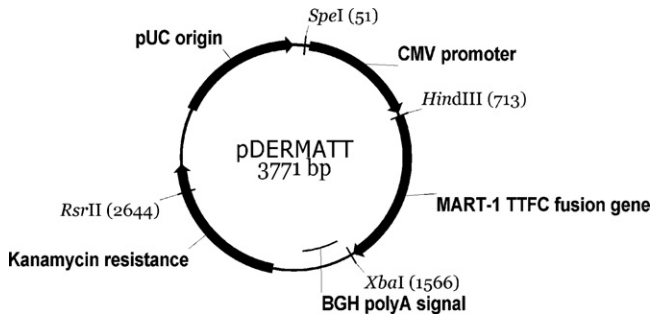

Fig. 1. Plasmid DNA map of pDERMATT (plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c) including selected restriction sites.

2. Materials and methods

2.1. Chemicals

The inset of the pVAX-based plasmid pDERMATT (Fig. 1), a plasmid of 3.8 kb with a kanamycin resistant marker, was designed at the Netherlands Cancer Institute (NKI-AvL, Amsterdam, The Netherlands) and subsequently manufactured by GeneArt (Regensburg, Germany) to obtain a small quantity of plasmid produced under Good Manufacturing Practice (GMP)/Good Laboratory Practice (GLP). pDERMATT is replicated in-house (AmBTU, Amsterdam, The Netherlands) using *E. coli* fermentation (bulk drug) [16]. pDERMATT final product was manufactured in-house (Department of Pharmacy & Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands) by freeze-drying. Tris, NaCl and water for chromatography were obtained from Merck (Darmstadt, Germany), isopropanol and 25% (w/v) HCl from Biosolve B.V. (Amsterdam, The Netherlands). All chemicals obtained were of analytical grade and used without further purification.

2.2. Agarose gel electrophoresis including restriction analysis

Agarose gels were run in a Horizon 20·25 horizontal gel electrophoresis unit coupled to a Whatmann Biometra power supply (Westburg B.V., Leusden, The Netherlands).

In process controls (IPCs) and final product were analysed by electrophoresis using 25 cm, 1% agarose (ABGene, Epsom, Surrey, UK) self cast gels. Running buffer was a 40 mM Tris–acetate, 1 mM EDTA, pH 8.3 solution and electrophoresis was carried out at 30 V for 21 h. After electrophoresis, gels were stained for 1.5 h with a 1× Sybr green I solution (Sigma–Aldrich Chemie, Zwijndrecht, The Netherlands) and then visualized and photographed under UV light (GeneGenius, Westburg B.V., Leusden, The Netherlands).

Restriction enzyme analysis was performed using the following enzyme combinations HindIII and XbaI, SpeI and XbaI, RsrII and SpeI, resulting in bandsizes of 853 and 2918, 1515 and 2256, 1179 and 2592 base pairs respectively. Digestions were performed according to instructions of the manufacturer.

For agarose gel electrophoresis the samples were diluted, if necessary, to approximately 20 ng pDERMATT/μl; for determination of residual *E. coli* host RNA/DNA in the bulk and lyophilised product the sample was diluted to 50 ng pDERMATT/μl. Of the resulting solutions 10 μl was mixed with 2 μl of 6× loading dye and this was subsequently loaded onto the gel.

2.3. Sequencing

Primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [17]. A total of 26 primers were designed to cover the whole sequence. First, polymerase chain reaction (PCR) products of the forward and reverse couples were generated. Results of the PCR reaction were analysed on a 2% agarose gel. For sequencing the PCR products were purified with ExoSAP-IT (GE-Healthcare, Diegem, Belgium). After purification, DNA cycle sequencing was carried out as described by the manufacturer (Applied Biosystems, Foster City, CA, USA) in 20 μl reactions on a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA). The forward and reverse primers were identical to those used in the PCR amplifications. Both DNA strands were sequenced. Sequences were analysed on an Applied Biosystems 3100-Avant DNA sequencer. For sequence alignment Seqscape v2.1 (Applied Biosystems, Foster City, CA, USA) was used.

2.4. Sample preparation

2.4.1. pDERMATT standard reference solution

pDERMATT standard reference solution was prepared by diluting the plasmid DNA solution obtained from the first pDERMATT production [16]. First the concentration was determined using the double strand DNA (dsDNA) program of a Biophotometer (Eppendorf, Hamburg, Germany). Subsequently the solution was diluted with mobile phase buffer A to a final concentration of 100 μg/ml pDERMATT. The exact concentration was again determined using the Biophotometer. The standard reference solution was stored, aliquoted in micro test tubes (Eppendorf, Hamburg, Germany), at −80 °C until analysis.

2.4.2. Open circle plasmid species generation

Open-circular (OC) topoisomers were prepared by incubating supercoiled (SC) plasmid DNA with the nicking enzyme (cuts only one strand of double stranded DNA) N.BstNBI (New England Biolabs, Hertfordshire, England, UK) at a concentration of 1 U/μg. The reaction was carried out in 1× NEBuffer 3 (supplied with the enzyme) at 55 °C for 1 h. The enzyme was inactivated by incubation at 80 °C for 20 min. The pDERMATT concentration for digestion was 20 ng/μl, according to the protocol of the manufacturer. Successful digestion was confirmed by AGE, with only one band present at approximately 6740 bp ±10% (compared to the supercoiled DNA ladder). For HPLC analysis 1 ml of the sample was precipitated with isopropylalcohol (IPA) and resuspended in 20 μl giving a concentration of 1 mg/ml. OC plasmid was stored at −20 °C until analysis.

2.4.3. Linear plasmid species generation

Linear (Lin) plasmid preparations were generated by incubating SC plasmid DNA with HindIII, a single-cutting restriction enzyme (New England Biolabs, Hertfordshire, England, UK) in the buffer recommended by the manufacturer at a concentration of 1 U/μg. The reactions were incubated at 37 °C for 1 h. The enzyme was inactivated by incubation at 65 °C for 15 min. The plasmid DNA concentration for digestion was 40 ng/μl, according to the protocol of the manufacturer. Successful linearization of the plasmid was confirmed by AGE, with only one band present at 3771 bp ±10% (compared to the Lambda DNA BstEII digest ladder). For HPLC analysis 1 ml of the sample was precipitated with IPA and resuspended in 40 μl giving a concentration of 1 mg/ml. Lin plasmid was stored at −20 °C until analysis.

2.4.4. pDERMATT calibration and quality control samples

pDERMATT stock solution was prepared by dilution with mobile phase buffer A (20 mM Tris, 10% isopropyl alcohol (IPA), pH 9.0) of the plasmid DNA solution obtained with the first pDERMATT production [16] to 200 μg/ml. Calibration and quality control samples were prepared from two separately diluted stock solutions. To obtain sample solutions containing 5, 10, 20, 50, 70, 100, 130 and 150 μg/ml pDERMATT, subsequently 25, 50, 100, 250, 350, 500, 650 and 750 μl of stock solution was transferred to a micro test

tube. Mobile phase buffer A was added to a final volume of 1000 μl. Samples were stored at −80 °C until analysis.

2.4.5. pDERMATT API

pDERMATT drug substance sample was prepared by diluting the pDERMATT solution prepared by GeneArt to a final concentration of 100 μg/ml with mobile phase buffer A. Samples were diluted prior to analysis.

2.4.6. pDERMATT bulk drug

pDERMATT bulk drug sample was prepared by determining the concentration of the produced batches [16] with a Biophotometer (Eppendorf, Hamburg, Germany) and subsequent dilution with mobile phase buffer A to a final concentration of 100 μg/ml. Samples were diluted prior to analysis.

2.4.7. Pharmaceutical dosage form

pDERMATT 2 mg/vial lyophilised powder for intradermal use was dissolved in 20 ml of mobile phase buffer A. An aliquot of the solution was transferred to a micro test tube. Samples were stored at −80 °C until analysis.

2.5. Liquid chromatography

The HPLC system consisted of an 1100 Series binary HPLC pump, Model G1312A (Agilent technologies, Amstelveen, The Netherlands), a model SpectraSERIES AS3000 automatic sample injection device, equipped with a 20 μl sample loop (Thermo Separation Products, Breda, The Netherlands), and a photodiode array detector Model Waters™ 996 (Waters Chromatography B.V., Etten-Leur, The Netherlands). Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA). Separation was achieved using a TSKgel DNA-NPR analytical column (75 mm × 4.6 mm I.D. particle size 2.5 μm) protected by a TSK DNA-NPR guard column (5 mm × 10 mm, Anachem House, Luton, England). The mobile phase consisted of two buffers, buffer A, Tris (20 mM)–IPA (90:10, v/v) (pH* 9.0) and buffer B, Tris (20 mM)–NaCl (1 M)–IPA (90:10, v/v) (pH* 9.0). Injection of 10 μl of sample was followed by a linear gradient of 0.45–0.53 M NaCl for 32 min. The flow rate was 0.5 ml/min and total run time 35 min. Absorbance was monitored at 260 and 280 nm. Furthermore a UV spectrum was recorded from 340–190 nm using a diode-array detector.

2.6. Validation procedure

The LC method was validated with respect to the following parameters: linearity, accuracy, precision, stability-indicating capability, and stability of standard reference solutions [15].

2.6.1. Linearity

Calibration curves at six concentration levels (5, 10, 20, 50, 100 and 150 μg/ml pDERMATT in mobile phase buffer A) were analysed in duplicate in three separate runs. Least-squares analysis of concentration, versus the area of the pDERMATT peak was applied. The linearity of the calibration curves was evaluated by means of back-calculated values of the calibration standards, the deviation of response factors at different concentration levels and the observed correlation coefficients of the calibration curves [18].

2.6.2. Accuracy and precision

Accuracy, within-run and between-run precisions of the method were determined by assaying quality control samples at three concentration levels (70, 100 and 130 μg/ml pDERMATT in mobile phase buffer A) 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 the analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable. From the ANOVA analysis the day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained. Within-run and between-run precisions were defined using equation 1 and 2 respectively, where n is the number of replicates.

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

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

2.6.3. Selectivity, specificity and stability-indicating capability

Selectivity was tested in the presence of the excipient sucrose present in the pharmaceutical dosage form.

The specificity of the LC method was tested by spiking pDERMATT stock solutions in the concentration of 1 mg/ml SC pDERMATT in water with Lin and OC pDERMATT in different concentrations. OC and Lin pDERMATT could be generated by restriction analysis, as described above. Results were compared with unspiked samples [15]. Lin and OC concentrations of 5, 10, 20, 40, 80 and 160 μg/ml were used for spiking the 1 mg/ml stock solution. All samples were diluted with mobile phase buffer A to a theoretical concentration of 100 μg/ml SC pDERMATT before analysis, resulting in Lin and OC concentrations of 0.5, 1, 2, 4, 8, 16 μg/ml.

The stability-indicating capability of the LC method was tested by subjecting pDERMATT stock solutions in the concentration of 1 mg/ml pDERMATT in water to several stress conditions for 1 h (oxidation) or 24 h (acid, alkaline, and heat) [15]. Heat: 1 ml of pDERMATT stock solution was exposed to 55 °C. Oxidation: to 1 ml of pDERMATT stock solution, 1 ml of a 30% hydrogen peroxide solution was added. Acidic: to 1 ml of pDERMATT stock solution 0.5 ml 0.1 M hydrochloric acid (HCl) was added. Samples were neutralised using 0.1 M sodium hydroxide (NaOH). Alkaline: similar to the method described under "acidic", using 0.1 M NaOH as alkaliser and 0.1 M HCl to neutralise the sample solution. All samples were diluted with mobile phase buffer A to a theoretical concentration of 100 μg/ml pDERMATT before analysis.

2.6.4. Stability of standard reference solutions

Standard reference solutions were stored at ambient temperature (20–25 °C) and ambient light conditions in both micro test tubes (polypropylene, Eppendorf) and glass autosampler vials, pDERMATT area and purity were monitored in time. The pDERMATT content and purity of pDERMATT standard reference solutions stored at −20 °C (in the dark) were determined after 8.5 months and compared with freshly prepared standard reference solutions.

2.7. CD measurements

Spectra of P(DMAEMA)–DNA 3:1 (w/w) polyplexes in 20 mM HEPES buffer (pH 7.4) were recorded with a 1 cm path length cuvette using a spectropolarimeter J-600 (Jasko, Tokyo, Japan) at ambient temperature and subtracted from the spectrum of buffer alone. The integration time was set to 1 s, the slit was set to 2 nm, and each measurement was the average of three repeated scans in steps of 1 nm. The P(DMAEMA)–DNA complexes were prepared by adding 1.50 ml of 80 μg/ml P(DMAEMA) solution to 1.50 ml of 26.6 μg/ml DNA (=6×10^{-3} M DNA) solution and incubating for 30 min at ambient temperature prior to measurements. The measured CD signals were converted to molar absorbance differences $\Delta\varepsilon$ ($M^{-1}\,cm^{-1}$), based on the molar concentration of DNA bases.

2.8. Capillary gel electrophoresis

Capillary gel electrophoresis was performed with samples of pDERMATT as published earlier [19].

3. Results and discussion

3.1. Characterisation of pDERMATT drug substance

pDERMATT drug substance was characterised by agarose gel electrophoresis and sequencing. The sequence showed a few point mutations as compared to the reference sequence supplied by the manufacturer. However all mutations were in non coding regions and therefore considered non critical. Gel electrophoresis revealed expected band sizes of SC intact plasmid and plasmid subjected to restriction analysis (see Table 1).

3.2. LC-UV method development

The separation principle of AEX-HPLC is based on the interaction between the negatively charged phosphate groups on the pDNA backbone and the positively charged groups on the stationary phase [11]. Although the overall charge and molecular weight of the different topologies might be similar, the different isoforms will possess different conformations and thus different local charge densities [10,11,20]. Since the overall interaction between pDNA and the stationary phase is thought to be a local attraction generated by opposite charges in close proximity [9], the isoforms will have different retention times in an increasing salt gradient [11]. We selected two columns and different mobile phases from literature for the pDERMATT analysis [11–13]. First a Zorbax SAX column (Agilent, Amstelveen, The Netherlands) 4.6 mm × 250 mm I.D., 70 Å pore, 5-μm particle size was tested. The pH range of this column is 2–7 and since the sugar-phosphate backbone of DNA is hydrolysed in an acid environment, the mobile phase consisted of a phosphate buffer of pH 7. Buffer A was composed of monobasic sodium phosphate (20 mM)—dibasic sodium phosphate (30 mM). Buffer B was composed of buffer A with 2 M NaCl [12]. However with different gradients tested no DNA peak was visible. Therefore based on the results of Molloy et al. [11], and Yamakawa et al. [13] a TSKgel DNA-NPR column was tested. With this system a 25 mM borate-based buffer (pH 9) and a linear gradient of 0.55–0.75 M NaCl for 5 min was tested. Flow rate was set at 1 ml/min and the column was heated to 40 °C [11]. Injection of 400 ng onto the column resulted in a single peak, which eluted relatively fast at 2.7 min. Smith et al. have shown that with increasing gradient steepness the SC peak eluted closer to the OC peak [14]. Possibly the used gradient in our experiment was too steep, resulting in co-elution of OC and SC pDNA in one peak. Therefore a more shallow gradient was designed, which resulted in the appearance of a little peak (OC DNA) not baseline separated from the main peak (SC DNA). Two different mobile phase compositions were tested, the mobile phase used by Horn et al. [12] (described above) and a 20 mM Tris-based buffer (pH 9). Linear and OC standards were used for determining the retention times of the different topoisomers. A mixture of all three available topoisomers was also injected into the system. With the phosphate-based mobile phase the linear and OC forms could not be separated from each other. However the Tris-based mobile phase gave a better resolution, but it was still not optimal. Solvophobic interactions between the DNA and the stationary phase could be the cause of the inadequate separation. To prevent solvophobic interactions 10% isopropanol can be added to the mobile phase buffers [9]. The addition of 10% isopropanol to the mobile phase buffers resulted in such a pressure increase that the flow had to be reduced to 0.5 ml/min. By reducing the column temperature from 40 °C to ambient, separation of the three different topoisomers was accomplished (Fig. 2). On weak-base anion-exchangers, like DEAE, retention times increase at higher column temperature resulting in co-elution of similar size fragments. The increase in retention time is probably due to the partial desolvation of the DNA and better accessibility of the stationary phase at higher temperature. For separation of double stranded DNA (dsDNA) on weak-base anion-exchanger temperatures between ambient and 35 °C are optimal [9]. The gradient was set from 0.45–0.53 M NaCl in 32 min, with a total run time of 35 min. These settings resulted in acceptable tailing factors (<1.5), theoretical plates (>2,000), and SC peak width at baseline (<3.0 min). The system was tested for linearity by using a calibration curve with a range of 5–150 μg/ml. This concentration range resulted in linear concentration response curves. The lower

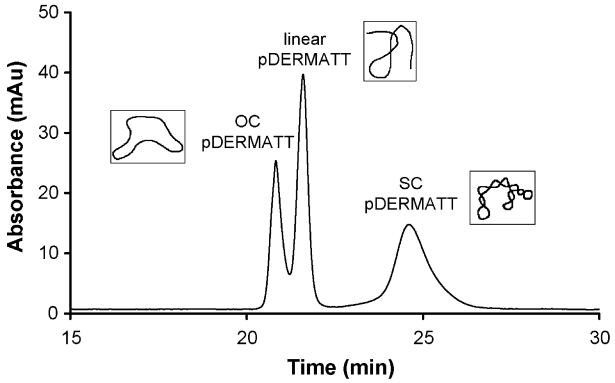

Fig. 2. Representative chromatogram of linear, open circular and SC pDERMATT (each 36 μg/ml, 10 μl injection). The retention time of the SC form is approximately 25 min (k' 14.6). Detection: 260 nm. Inserts provided by PlasmidFactory (www.plasmidfactory.com).

Table 1
Characterisation of pDERMATT drug substance.

Analytical method	Results			
Sequencing	Conforms to reference sequence with exception of the following point mutations: A2C, C3T, T4G, C241T, C1931T, one bp deletion at 2865, C3266T and G3742C. All mutations are in non coding regions of the plasmid with the exception of 3266 and 3742, which are in the pUC origin of replication. The plasmid has duplicated in the bacteria, therefore these mutations have no consequences.			
AGE analysis	Measured size: 3657 bp (3% deviation of expected size 3771 bp)			
	Restriction enzymes	Expected (bp)	Measured (bp)	Deviation
	HindIII/XbaI	853	817	4.2%
		2919	2891	1.0%
	SpeI/XbaI	1515	1490	1.7%
		2257	2225	1.4%
	RsrII/SpeI	1179	1160	1.6%
		2593	2582	0.4%

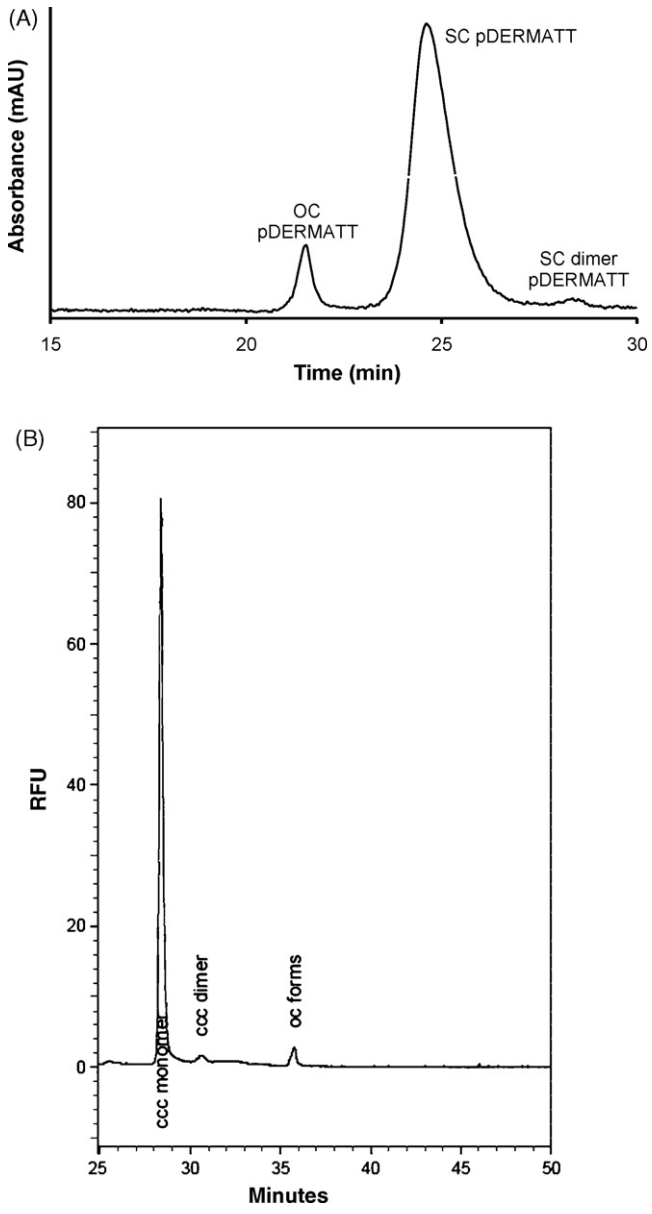

Fig. 3. Representative chromatograms of pDERMATT drug substance. HPLC, 100 μg/ml, 10 μl injection and 260 nm detection (A) and CGE, 100 ng (B). Capillary gel electrophoresis (CGE) was carried out at PlasmidFactory, Bielefeld, Germany.

limit of quantification was 2 μg/ml and the upper limit 250 μg/ml. A representative chromatogram of pDERMATT 100 μg/ml is given in Fig. 3A, Fig. 3B shows the CGE chromatogram. There is a difference in retention time of the topoisoforms between both methods. This difference could be due to different separation mechanisms. In CGE separation is based on charge and size. Also the use of intercalating dyes can change migration time [21]. Possibly the intercalating dye can access the OC form better than the SC form or the SC dimer, therefore the OC molecule is bigger than would be expected and migrates more slowly.

3.3. Validation

3.3.1. Linearity, accuracy and precision

Linearity, accuracy, within-run and between-run precision of the LC-UV method over the concentration range of 5–150 μg/ml were examined. This range corresponds to 5–150% of the intended test concentration of 100 μg/ml for the pharmaceutical quality control

Table 2
Results of pDERMATT quality control samples ($n = 3$ per concentration level).

Concentration (nominal μg/ml)	Within-run precision (%)	Between-run precision (%)	Accuracy (%)
64.79	0.8	1.6	102.5
92.45	0.6	0.2	102.4
119.96	1.0	1.2	102.8

of pDERMATT API, bulk drug and the drug in its pharmaceutical dosage form. The assay was linear with a relative standard deviation of 3.4% in the response factors (area divided by concentration) obtained in the tested concentration range and correlation coefficients >0.9997 found for all three calibration curves. The average back-calculated concentration for the different calibration concentration levels in the six analytical runs varied between 97.0% and 101.5% of the theoretical concentration for the tested concentration range. The maximal deviation from the nominal concentration was 7.9%, which was found in the lowest calibration level. Within- and between-run precisions were ≤1.6% with accuracies between 102.4% and 102.8% (Table 2).

3.3.2. Stability of the standard reference solution

The pDERMATT SC area of the standard reference solutions and purity were constant during at least 9 h when stored in a glass autosampler vial at ambient light and temperature (20–25 °C). When the standard reference solution was stored in a micro test tube the pDERMATT SC area and purity were constant during at least 22 h. After 51 h of storage in a micro test tube the purity of the standard reference solution is still constant, however the SC concentration has increased. The chromatograms show that the peak at approximately 29 min resolves and the area of the SC peak increases slightly, suggesting that the dimer is converted into two monomer SC plasmids (Fig. 4). During plasmid DNA replication in bacteria formed dimers can be resolved to monomers [22], however if monomers can be formed spontaneous from dimers is not known. Since this is the only intact and undamaged plasmid form [23], the SC form is the desired form and considered as most appropriate for therapeutic applications [24,25].

The pDERMATT content of standard reference solutions ($n = 2$) stored at −20 °C (dark) was stable for at least 8.5 months (98.3 ± 0.6% of the initial content) with no significant increase in degradation products.

3.3.3. stability-indicating capability

pDERMATT is pharmaceutically formulated as a lyophilised dosage form containing the excipient sucrose. Sucrose was added to

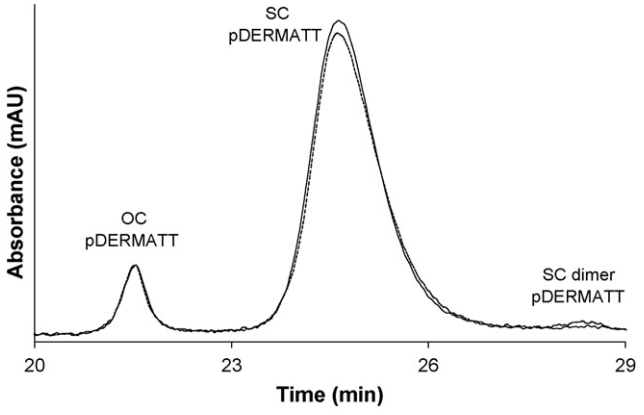

Fig. 4. Representative chromatograms of pDERMATT (100 μg/ml, 10 μl injection). Freshly prepared reference standard (dotted line), reference standard after 51 h storage in PP micro test tube (continuous line) In time the dimer converts to the SC form.

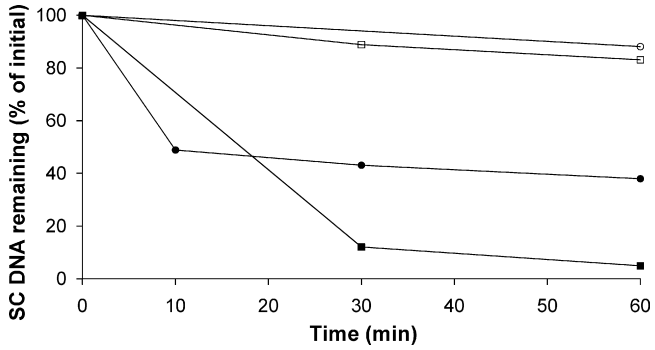

Fig. 5. Degradation of pDERMATT under different stress conditions ((○) heat: 55 °C); (□) alkaline: 0.1 M NaOH; (●) oxidation: 30% hydrogen peroxide; (■) acid: 0.1 M HCl).

pDERMATT quality control samples in a concentration corresponding to the pDERMATT excipient ratio present in the formulation. Sucrose was not recovered in the chromatogram and did not affect the response of pDERMATT.

The selectivity of the LC-UV method was examined by analysing the spiked samples and comparing the measured concentration of the SC peak with the measured concentration obtained in unspiked samples. In all Lin and OC levels tested, the measured concentration of the SC peak was not affected. Furthermore the areas measured for the Lin and OC peak showed a good linearity, considering that we are measuring below the lower limit of quantification (5 μg/ml pDERMATT), with regression coefficients of >0.995 (data not shown). It was not possible to design a gradient that could baseline separate the Lin and OC topoisomer (Fig. 2), however based on the results mentioned above, the designed method was specific for the different topoisomers as well.

Fig. 5 shows the percentage SC pDERMATT remaining with respect to the initial concentration at several time points under the different stress conditions. Fast degradation was observed for pDERMATT when exposed to oxidising or acidic conditions, with the highest degradation rate under acidic conditions. After 3 h exposure to acidic conditions no SC pDERMATT remained. pDERMATT was more stable after exposure to alkaline conditions and heat (55 °C) at neutral pH. Even after storage for 24 h at these conditions more than 80% was in the SC form. Upon degradation mainly OC DNA is formed. In addition denatured DNA is visible in the samples stored under alkaline conditions [26].

3.3.4. System suitability parameters

The observed linearity of the analytical method supports the use of a single standard reference concentration for the standardised quantitative analysis of pDERMATT. The system suitability will consist of one blank injection (mobile phase), six injections of the pDERMATT standard reference solution and one injection of a separately diluted standard reference solution. Criteria for retention factor, theoretical plates, tailing factor, % rsd in area (six injections), and ratio between the area of the separately diluted standard reference solutions were defined as: $13.8 \leq k' \leq 15.4$, $N > 2500$, $1.1 \leq T \leq 1.5$, % rsd < 3.0% and ratio 0.97–1.03 respectively. The SC pDERMATT content of sample solutions will be calculated using the bracket mode, in which the areas of two preceding and two following standard reference solutions are used.

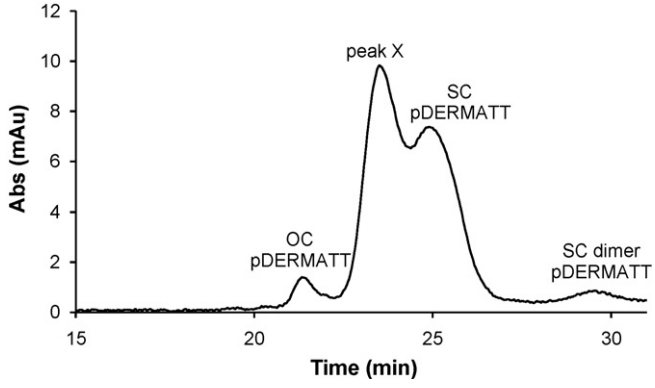

Fig. 6. Anion-exchange HPLC profile of pDERMATT batch with double peak.

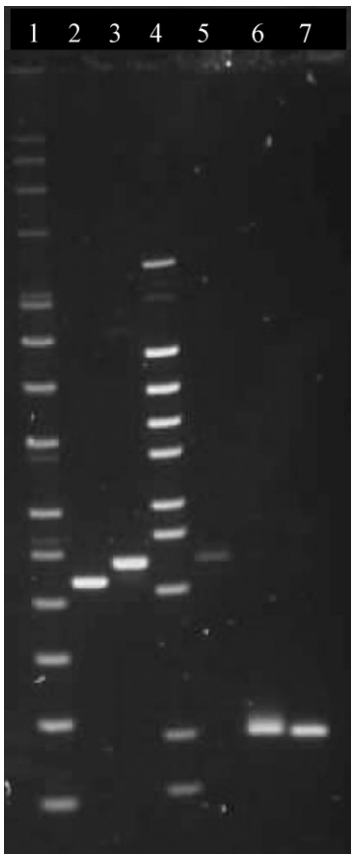

Fig. 7. HPLC-fractions of plasmid DNA from the batch that showed a double peak. Lane 1, SC DNA ladder; lane 2, linear pDERMATT standard; lane 3, OC pDERMATT standard; lane 4, Lambda DNA BstEII digest; lane 5, OC fraction; lane 6, peak X; lane 7, SC peak.

3.4. Analysis of pDERMATT batches

The analysis of the in process controls taken during pDERMATT production showed that impurities of the production process are

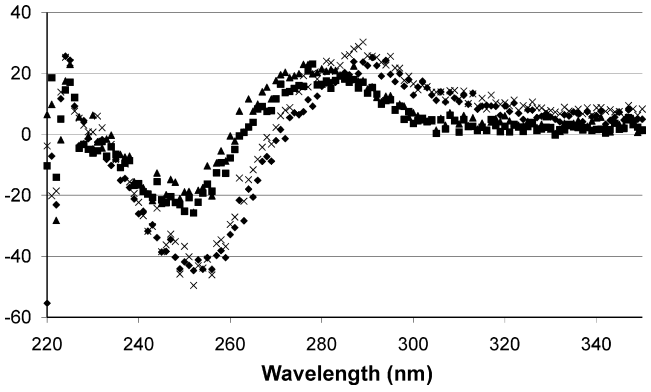

Fig. 8. CD spectra of different topologies in the presence of P(DMAEMA) at a P(DMAEMA): DNA 3:1 (w/w) ratio ((▲) SC pDERMATT treated with HindIII; (■) OC pDERMATT; (♦) SC pDERMATT; (✕) DNA of lot with double peak).

not retained on the column [16]. The IPCs taken after lysis, but before purification showed a large flow through peak compared to the purified samples. Supercoiled DNA was visible in all IPCs except for the IPCs taken during loading and the wash step of the purification. Since the last sample only contains RNA [16], this shows that RNA does not interfere in the developed method of analysis.

For the bulk drug a specification of ≥90% SC pDERMATT is adopted [16]. All bulk batches had a SC content of >90% with exception of one batch. This batch showed a double peak at the retention time of the SC peak (Fig. 6). Possibly peak X consists of SC plasmid DNA with a lower amount of supercoils, as has been described earlier [27]. To prove this statement additional analysis was performed. AGE analysis showed a slight difference in migration between both peaks (Fig. 7). The size of peak X is 3965 bp and of the SC band 3915 bp compared to the SC DNA ladder. When DNA contains a smaller amount of supercoils it will migrate more slowly as can be seen with OC DNA, which contains no supercoils. CD analysis of the pDERMATT lot showed a similar pattern as seen with SC pDERMATT (Fig. 8). If peak X would be an OC or linear species, the spectrum would deviate from the SC pDERMATT spectrum and be positioned between the spectra of the standards [27].

4. Conclusion

In conclusion, a stability-indicating analytical method comprising of separation with AEX-LC and subsequent UV detection was developed for the novel investigational anti-cancer plasmid pDERMATT. The method was found linear in the range of 5–150 μg/ml, precise and accurate. Specificity tests with known amounts of OC or Lin pDERMATT showed degradation products well separated from the parent compound, confirming stability-indicating capability. The developed stability-indicating LC-UV method is successfully used for the pharmaceutical quality control of pDERMATT API, bulk drug and its pharmaceutical dosage form.

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