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Development and validation of an ultra performance liquid chromatography–tandem mass spectrometry method for the quantification of daptomycin in human plasma

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ABSTRACT

A rapid, simple and accurate analytical method based on ultra performance liquid chromatography (UPLC) combined with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) on a hybrid q TOF instrument has been developed and fully validated for the quantification of daptomycin (DPT) in human plasma. The samples were analyzed after simple pretreatment involving protein precipitation, while chromatographic separation of DPT and the internal standard (reserpine) was achieved on an Acquity BEH C18 column (100 mm × 2.1 mm, 1.7 μm) using gradient elution with 0.1% aqueous formic acid (FA) and acetonitrile with 0.1% FA (with DPT eluting at 2.60 min). The method presented good fit ($r > 0.999$) over the quantification range of 0.01–10 μg mL^{-1} with the lower limit of quantitation (LLOQ) being 0.01 μg mL^{-1} of human plasma for DPT. The intra- and inter-day precision, measured as % relative standard deviation, was less than 11% for DPT. The validation results showed that the developed method demonstrated adequate selectivity, sensitivity, precision and accuracy and therefore was successfully applied to the analysis of clinical samples following intravenous (iv) administration of 5.4 mg kg^{-1} DPT to patients suffering from post-traumatic osteomyelitis induced by methicillin-resistant *Staphylococcus aureus* (MRSA). The developed methodology is the first report of an accurate mass tandem MS method for the analysis of this potent antibiotic in human plasma and can be used to further study pharmacokinetic, bioequivalence and even metabolic aspects related to this drug.

1. Introduction

Daptomycin (DPT) (Fig. 1a) is a potent cyclic lipopeptide antibiotic for the treatment of complicated skin and soft tissue infections caused by Gram-positive organisms, including both susceptible and methicillin resistant strains of *Staphylococcus aureus* (MRSA) [1]. In addition, it is used for the treatment of various other infections, including serious and life-threatening Gram-positive infections, glycopeptide-intermediate *S. aureus* (GISA) [2], vancomycin-resistant enterococcal (VRE) infections [3] and right-sided endocarditis with associated bacteraemia [1]. The development of new antibiotics like DPT are increasingly in demand, particularly in light of recent queries regarding the resistance against Gram-positive bacteria [4,5], thus necessitating the development of effective analytical methodologies for the determination and further studies (*e.g.* pharmacokinetic) thereof.

Despite the fact that DPT was discovered in the late 1980s it was introduced into clinical practice in 2003 [6,7] and since then little information is available in the literature for the analysis of this antibiotic in biological fluids. Mainly HPLC-UV based analytical methodologies [8–10] have been reported for the determination of DPT with further applications in pharmacokinetic studies [11–13]. Recently UPLC-UV instrumentation has been employed for the determination of DPT in plasma [14,15], while the use of LC–MS for the determination of DPT in plasma, urine and peritoneal fluid has been reported [16]. It should be noted that only recently a tandem MS based analytical methodology for the determination of DPT in biological fluids has appeared in the literature [17]. However, there is no analytical method based on high-resolution tandem MS combined with UPLC, which has the advantage of higher detection

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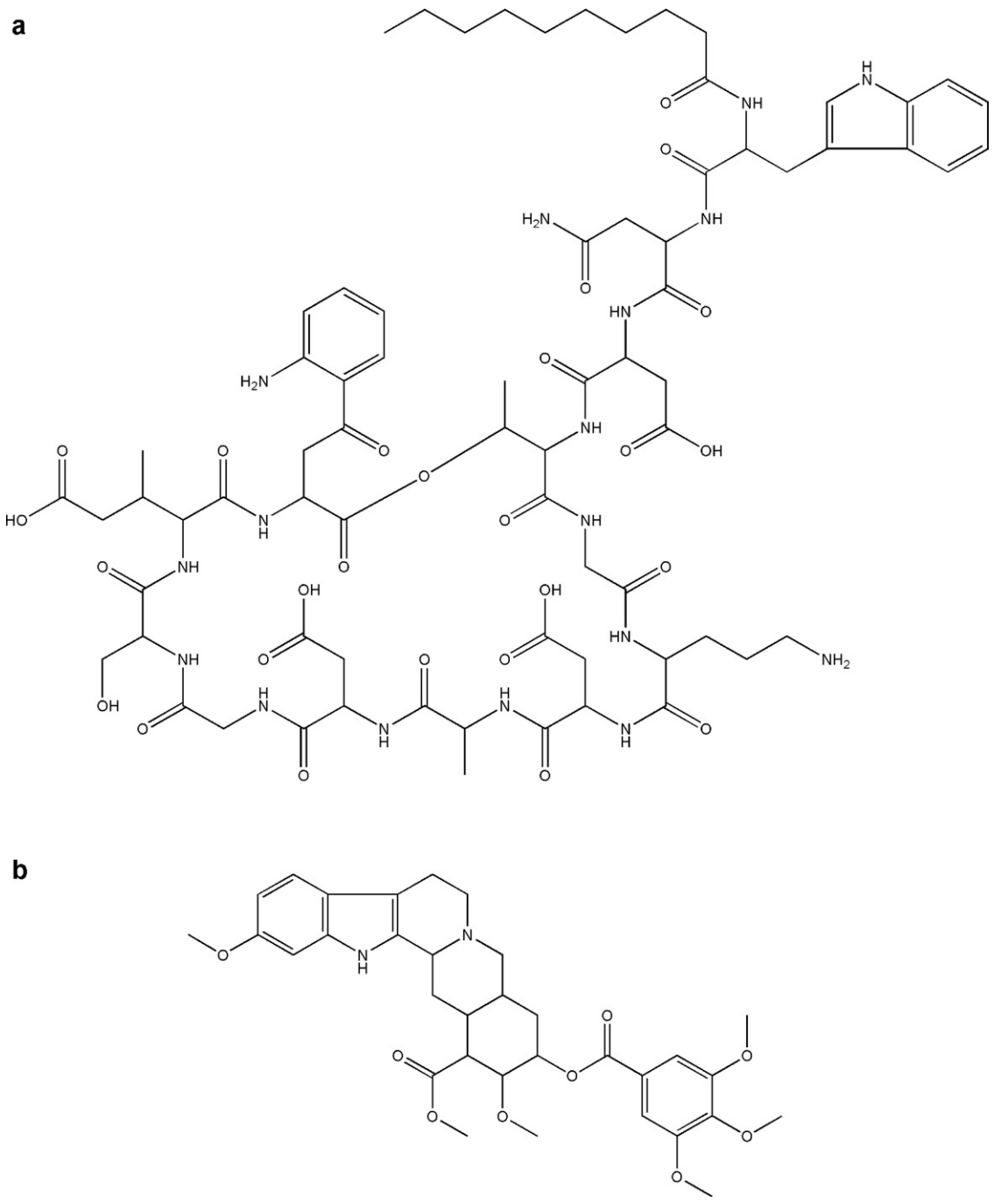

Fig. 1. Chemical structures of DPT [Decanoyl-WNDTGODADGS-Me-E-anthraniloyl-A] (a) and IS (reserpine) (b).

sensitivity and the ability to exclude false positives from nominal mass endogenous components in biological matrices.

The objective of this study was to develop and validate (in terms of calibration model, precision, accuracy, selectivity, sensitivity, lower limit of quantitation, recovery, matrix effect, stability, sample dilution) a UPLC–ESI MS/MS analytical method for the quantification of DPT in human plasma. Tandem mass spectrometry with accurate mass measurements was used to improve sensitivity and specificity. The validation results demonstrated that the proposed methodology is suitable for monitoring DPT in human plasma. The developed methodology could meet the criteria for high-throughput analysis requirements in the context of simple and efficient extraction with small plasma volume, reduced analysis time and high sensitivity.

2. Experimental

2.1. Materials and reagents

Acetonitrile and methanol (gradient HPLC grade) were purchased from Merck (Darmstad, Germany). Ultra pure water was produced in the laboratory by a Direct-Q system (Millipore, France) and was used throughout the study. DPT (Decanoyl-WNDTGODADGS-Me-E-anthraniloyl-A; $M_{r,monoisotopic}$ 1619.7086) was generously donated by Novartis Hellas. Reserpine (RSP) ($M_{r,monoisotopic}$ 608.2733), used as internal standard (IS) (Fig. 1b) and formic acid (FA) were purchased from Sigma Aldrich (Steinheim, Germany). All substances and solvents have been used without any further purification.

2.2. Liquid chromatography

All analyses were performed on an Acquity UPLC™ system (Waters Corp., Milford, MA, USA) comprising a Binary Solvent Manager and an autosampler (Sample Manager) capable of maintaining the sample temperature from −5 to 40 °C. Chromatographic separation was achieved on a C18 BEH column (Waters Acquity, 100 mm × 2.1 mm, 1.7 μm), preceded by a precolumn (Waters VanGuard 5 mm × 2.1 mm, 1.7 μm) of the same packing material and an on-line filter. Mobile phases consisting of 0.1% aqueous FA (Solvent A) and acetonitrile with 0.1% FA (Solvent B) were selected for the chromatographic separation. The total analysis time, including column equilibration was 3.5 min per injection and the flow rate was 0.3 mL min^{-1}. The gradient elution program used was as follows: from 5% B to 40% in 0.5 min; 1.0 min at 40% B; from 40% to 100% B in 0.5 min; 1.0 min at 100% B; from 100% to 5% B in 0.1 min; equilibration at 5% B for 0.4 min. The column temperature was maintained at 40 °C throughout all experiments, whereas the sample temperature was maintained at 10 °C avoiding light exposure in order to prevent any possible degradation. Samples were injected using a 10 μL loop using the full loop injection mode.

2.3. Mass spectrometry

Mass spectrometric analysis was carried out on a Waters Micromass (Milford, USA) hybrid quadrupole Time of Flight mass spectrometer (q ToF Premier) equipped with an electrospray ionization (ESI) interface and operated in positive ion mode. The electrospray voltage was 3.5 kV and the sample cone voltage was 35 V. The extraction cone voltage was 2 V and the MCP plates were operated at 1850 V. The source temperature was 120 °C and the desolvation temperature was set at 350 °C. Nitrogen was used as the desolvation and cone gas and was set at 650 and 50 L h^{-1}, respectively. The analyzer was operated in the V optics mode at a resolution (FWHM) of 9500. The collision gas was argon. For tandem MS acquisitions, the collision energy was optimized at 25 eV for DPT and 35 eV for RSP, respectively. Full scan spectra of the analyte and the IS were acquired both in continuum and centroid mode and were collected from 90 to 1800 amu, whereas the MS/MS spectra were collected in centroid mode for a mass range of 90–1000 amu. The scan time was 0.4 s with an interscan delay of 0.02 s. The lock mass compound was leucine enkephalin (m/z 556.2771) and was infused at 2 μL min^{-1} at a concentration of 500 pg μL^{-1}. For the lock mass spectrum the scan time was set to 0.2 s with a frequency of 10 s, averaging 2 scans. A solution of sodium formate was used for mass calibration. A Harvard Apparatus Pump II syringe pump (Holliston, USA) was used for the infusion of the analytes and the calibration compound, whereas the embedded instrument syringe pump was used for the infusion of the lock mass compound. The MassLynx V 4.1 software (Waters) has been used for instrument control, data acquisition and handling.

2.4. Preparation of standard solutions and quality control samples

Stock standard solutions of DPT and the IS were prepared in methanol at a concentration of 1 mg mL^{-1} and stored at −35 °C. Working standard solutions of the analyte at concentrations of 100, 10 and 1 μg mL^{-1} and the IS at a concentration of 1 μg mL^{-1}, were prepared by dilution of the aforementioned stock solutions in water:methanol (50:50, v/v) and were kept refrigerated at −35 °C in dark. The solutions of the analyte and the IS were stable for at least twenty days, under the described conditions.

The plasma calibration standards were prepared at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg mL^{-1} for DPT and 0.5 μg mL^{-1} for IS by spiking appropriate aliquots of working solutions to 100 μL of blank pooled drug-free human plasma. Low, medium and high concentration quality control (QC) samples at concentrations of 0.03, 0.75 and 7.5 μg mL^{-1} for DPT together with 0.5 μg mL^{-1} for IS were prepared by spiking blank plasma samples. The spiked samples were then treated as described in the Section 2.5.

2.5. Plasma sample collection and preparation

Plasma samples were collected from Evangelismos Hospital (Athens, Greece) from patients suffering from MRSA resistant induced post-traumatic osteomyelitis and were stored at −35 °C until analysis. Collection of plasma samples was performed with the consent of patients and under the approval of the Evangelismos scientific and ethical committee (hospital's ethical committee approval number 47/30-01-08). Plasma samples were analyzed within three days from sample collection according to the following procedure.

Frozen plasma samples were thawed at room temperature and subjected to protein precipitation as follows. Plasma aliquots of 100 μL (or a calibration standard or a QC sample) and 50 μL of 1 μg mL^{-1} IS working solution were added to a 1.5 mL eppendorf tube and the mixture was vortexed for 2 min. Then, 600 μL of a methanol:acetonitrile (1:2, v/v) mixture was added and the mixture was vortexed for 2 min followed by centrifugation at 10,000 rpm for 2 min at 15 °C. The supernatant was transferred into a clean eppendorf tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of the initial mobile phase composition (*i.e.* 95% Solvent A–5% Solvent B), vortexed for 1 min, centrifuged again under the same conditions, transferred into plastic autosampler vials with preslit septa (Waters, USA), and 10 μL was injected into the UPLC–ESI MS/MS system.

2.6. Assay validation

The assay validation was performed to meet the acceptance criteria according to the FDA guidelines for validation of bioanalytical methods [18–20]. The assay was validated in terms of specificity, selectivity, calibration model, precision and accuracy (intra- and inter-day), lower limit of quantitation (LLOQ), recovery from spiked human plasma samples, matrix effect, stability (short and long term, autosampler and freeze–thaw) and sample dilution.

2.6.1. Selectivity

Selectivity was studied by comparing chromatograms of six different batches of blank drug-free human plasma from six subjects with those of corresponding calibration standard plasma samples spiked with DPT and IS.

2.6.2. Calibration model and LLOQ

The construction of the calibration curve was based on the analysis of the calibration standards ($n = 5$) at nine concentration levels ranging from 0.01 to 10 μg mL^{-1} and plotting the peak area ratios of DPT to IS against the nominal calibration standard concentration. Non-linear regression analysis employing a quadratic model with $1/y^2$ weighting factor was used for curve fitting. The 0.0 value was neither included as a point nor the calibration curve was forced to pass through it.

The lower limit of quantitation (LLOQ) of the assay was the lowest analyte concentration on the calibration curve that could be determined with a precision below 20% and accuracy between 80 and 120%.

The response of a blank plasma sample was at least five times lower compared to that of a spiked sample at the LLOQ at the t_R of DPT and IS.

2.6.3. Intra- and inter-day precision and accuracy

The intra- and inter-day precision expressed as relative standard deviation (%) (RSD%) and the accuracy expressed as relative error (%) (RE%) were evaluated by repeated analysis of five replicates ($n = 5$) of QC samples at five levels (LLOQ, low QC, medium QC, high QC, upper limit of quantitation (ULOQ)). The inter-day precision and accuracy were assessed by analyzing five sets of all QC samples on three consecutive days. The concentration of each sample was calculated using a calibration curve of calibration standards prepared and analyzed on the same day.

2.6.4. Recovery

The % relative recovery (R%) of DPT from human plasma was evaluated by comparing five replicates of QC samples at the three QC concentration levels (0.03, 0.75 and 7.5 $\mu g\,mL^{-1}$) for DPT and at 0.5 $\mu g\,mL^{-1}$ for the IS with those of post-extraction spiked plasma samples.

2.6.5. Matrix effect

In order to evaluate the matrix effect (ME) on the ionization of DPT, *i.e.* the potential ion suppression or enhancement due to human plasma components, five sets of blank human plasma were processed according to the sample pretreatment procedure and then reconstituted with suitable volumes of DPT working solutions at three concentration levels (0.03, 0.75 and 7.5 $\mu g\,mL^{-1}$) and the IS (0.5 $\mu g\,mL^{-1}$). The corresponding peak areas of these samples (A), were compared with those of equivalent concentrations of DPT and IS working standard solutions in mobile phase (B). The ratio ($A/B \times 100$)% was used to evaluate the ME on the ionization efficiency of DPT. Similarly, the ME of the internal standard was also assessed.

2.6.6. Stability

Analyte stability in human plasma was studied by repeated analysis (five replicates) of QC samples at three concentration levels (low, medium and high) after various storage and handling conditions. *Short-term stability* was assessed at room temperature for 6 h, whereas *long-term stability* was evaluated at −70 °C for 20 days. *Autosampler* (in process sample) *stability* was assessed by analyzing pretreated QC samples stored in the autosampler at 10 °C for 20 h, as well as *freeze–thaw stability* was evaluated after three freeze–thaw cycles, which consisted of storage at −35 °C for a minimum of 12 h, followed by thawing at room temperature.

2.6.7. Sample dilution

In order to demonstrate the ability to dilute and analyze samples containing DPT concentrations above the ULOQ, a set of spiked plasma samples were prepared with DPT concentrations of 15, 37.5, 75 $\mu g\,mL^{-1}$ and were stored at −35 °C before analysis. The spiked samples were thawed at room temperature, diluted appropriately with blank drug-free human plasma, processed according to the sample pretreatment procedure and analyzed by the developed methodology.

2.7. Application to the analysis of clinical samples

The validated UPLC–ESI MS/MS method that has been developed was applied to quantify the plasma concentrations of DPT after a single intravenous dose of 5.4 $mg\,kg^{-1}$. Blood samples (about 1 mL) were withdrawn from the patient before and 30 min after dosing and were collected in tubes with EDTA as the anticoagulant. Plasma samples were obtained after immediate centrifugation of blood at 4 °C and were stored at −35 °C until analysis.

3. Results and discussion

3.1. Analytical method development/optimization of UPLC–ESI MS/MS conditions

In order to develop the ESI MS/MS method for the determination of DPT, the full scan precursor (Fig. 2a) and product ion spectra (Fig. 2b) of the analyte (Fig. 2i) and the IS (Fig. 2ii) were acquired both in positive ion mode by infusing each compound separately at a concentration of 10 $\mu g\,mL^{-1}$ in MeOH:H_2O (50:50)–0.1% FA at a flow rate of 5 $\mu L\,min^{-1}$. The infusion of the compounds was performed with the lockmass spray enabled. DPT presented greater ionization efficiency in positive rather than in negative ESI mode, giving rise to singly $[M+H]^+$ and doubly charged $[M+2H]^{2+}$ ion peaks at m/z 1620.7178 and m/z 810.8638, respectively (Fig. 2a-i). For the quantification, the doubly charged ion of DPT was selected as precursor ion for the tandem mass measurements, mainly because of its predominant abundance in the spectrum (intensity of $[M+2H]^{2+}$ was ∼34-fold higher than that of $[M+H]^+$), thus enhancing the sensitivity of the method. In the positive ESI mode, the IS yielded a predominantly protonated molecular ion $[M+H]^+$ at m/z 609.2822 (Fig. 2a-ii), which was used as the precursor ion for the tandem mass measurements. The collision energy voltage for the effective fragmentation of the selected precursor ions of DPT and IS was optimized over the range of 5–35 V with a 2.5 V step, and it was shown that 25 V provided optimum fragmentation of DPT, whereas the corresponding value for RSP was found to be 35 V. The product ion mass spectra of the DPT doubly charged ion showed predominant ions at m/z 640.9492, 341.2234 and 187.0878 (Fig. 2b-i), corresponding to the Decanoyl-WNDT, Decanoyl-W and W peptide fragments of DPT, respectively. In addition, subsequent loss of CO from the last two fragment ions resulted to the ion signals at m/z 313.2238, and 159.0934 (Fig. 2b-i). For the sensitive and specific quantification of DPT in positive ESI MS/MS mode the transitions 810.8638 → 640.9492, 341.2234, 313.2238, 187.0878, 159.0934 (sum of all the product ions) were monitored for DPT, whereas those of 609.2822 → 448.2004, 397.2146, 365.1896, 236.1299, 195.0677, 174.0951 for the IS (Fig. 2b-ii). The ESI interface parameters and the ESI probe position were optimized for maximum abundance of the analyte's ions by infusing a 10 $\mu g\,mL^{-1}$ solution of the analyte in 40% (A)–60% (B) (i.e., the composition of the mobile phase at the t_R of DPT) at a flow rate of 5 $\mu L\,min^{-1}$.

The chromatographic conditions were optimized for the rapid and efficient separation of DPT and IS from plasma components. For this reason, a C_{18} stationary phase and acetonitrile as the organic modifier of the mobile phase were chosen, over C_8 and methanol respectively, as this combination gave the best results in terms of peak shape (width, symmetry, sharpness). Further optimization was performed by adding 0.1% FA in the mobile phase and increasing the column temperature, which improved even more the peak shape of the analyte, accelerated analysis time and enhanced ionization efficiency. Finally, under the optimized chromatographic conditions DPT eluted at 2.60 min and RSP (IS) at 2.25 min, respectively, as shown in Fig. 3b.

3.2. Selection of internal standard

RSP was selected as the IS of the assay as it is well separated from the analyte, it shows stable ionization efficiency and adequate and reproducible extraction recovery from plasma employing the sample pretreatment protocol described above. In addition, it is not an endogenously occurring substance, it is stable under the described conditions, it is not harmful and it is inexpensive.

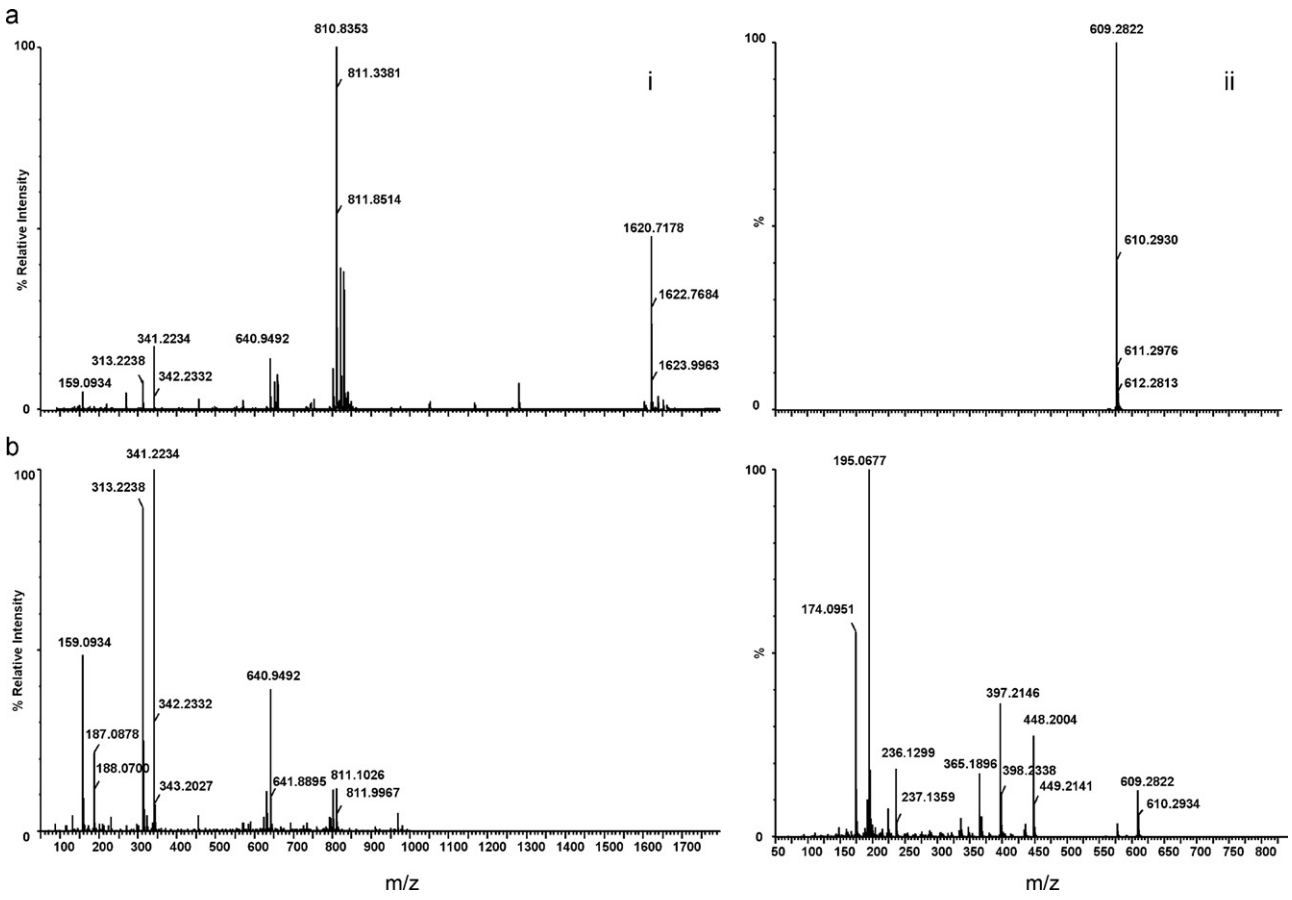

Fig. 2. Precursor (a) and product (b) ion ESI mass spectra of protonated DPT (i) and reserpine (ii).

3.3. Sample preparation

The protein precipitation of plasma samples with organic solvents was selected over liquid–liquid and solid phase extraction for fast, simple and inexpensive sample clean-up. It was found that the addition of a 2:1 (v/v) AcN:MeOH mixture provided almost quantitative recovery of DPT and no interferences from plasma matrix. Furthermore, due to its simplicity and speed, this sample pretreatment protocol can be considered compatible with high throughput analysis.

3.4. Method validation

3.4.1. Selectivity

The calibration standard plasma samples analyzed by the developed analytical methodology showed that the retention time (t_R) of DPT and IS was 2.60 min and 2.25 min, respectively. All batches of blank drug-free human plasma analyzed under the same conditions were found to be free of interference peaks from endogenous plasma substances at the t_R of the analyte and the IS, demonstrating the selectivity of the assay. Representative chromatograms of blank human plasma and spiked blank plasma with DPT at the LLOQ are shown in Fig. 3a and b, respectively.

3.4.2. Calibration model and LLOQ

Calibration curves were constructed by plotting the peak area ratios of DPT to IS of plasma calibration standards versus nominal concentrations of the analyte. The calibration model was selected based on the analysis of the data by linear and non-linear regression as well as with and without weighting. Calibration curves of five different lots of plasma calibration standards were constructed over the concentration range 0.01–10 $\mu g\,mL^{-1}$. The best fit and least square residuals for the calibration curves were achieved when a quadratic model was incorporated with $1/y^2$ weighting factor. The correlation coefficient (r) of the calibration curve was 0.9994, indicating good correlation (Fig. 4), whereas the equation of the fitted model is shown in Table 1. The back calculated values obtained are within the proposed 15% margin of the nominal concentrations [18–20], indicating the adequacy of the proposed non-linear model.

The LLOQ of DPT with the proposed method was 0.01 $\mu g\,mL^{-1}$ (Table 1) with signal to noise ratio (S/N) > 5 [18–20].

3.4.3. Intra- and inter-day precision and accuracy

The method was evaluated in terms of intra-day precision and accuracy by assaying ($n = 5$) QC samples at LLOQ (0.01 $\mu g\,mL^{-1}$), low QC (0.03 $\mu g\,mL^{-1}$), medium QC (0.75 $\mu g\,mL^{-1}$), high QC (7.5 $\mu g\,mL^{-1}$) and ULOQ (10 $\mu g\,mL^{-1}$). As shown in Table 2 the

Table 1
Calibration equation parameters for the determination of DPT in human plasma by the developed UPLC–ESI MS/MS methodology.

Calibration equation[a]	$y = -0.39 \times 10^{-2}x^2 + 0.16x + 0.22 \times 10^{-2}$
Slope X^2 (±SD[b])	$-0.39 \times 10^{-2} \pm 0.76 \times 10^{-3}$
Slope X (±SD[b])	$0.16 \pm 0.41 \times 10^{-2}$
Intercept (±SD[b])	$0.22 \times 10^{-2} \pm 0.19 \times 10^{-4}$
SE[c]	0.05042
Correlation coefficient (r)	0.9994
Range ($\mu g\,mL^{-1}$ of plasma)	0.01–10
LLOQ ($\mu g\,mL^{-1}$ of plasma)	0.01

[a] The quantification of DPT and the construction of the calibration curve were performed as an area ratio versus IS.
[b] SD, standard deviation.
[c] SE, standard error of the estimate.

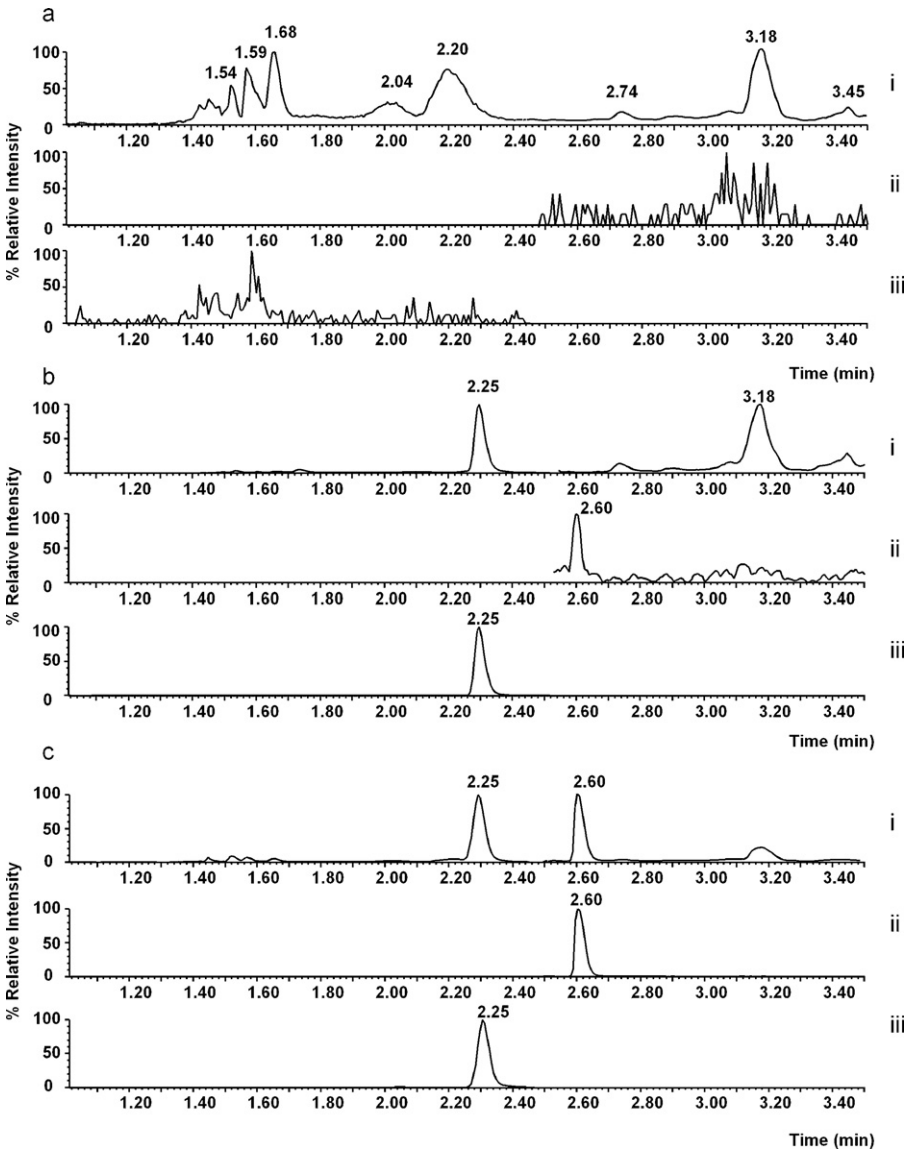

Fig. 3. Representative UPLC–ESI MS/MS chromatograms of: (a) blank plasma sample; (b) LLOQ human plasma sample spiked with DPT (0.01 μg mL^{-1}) and IS (0.5 μg mL^{-1}), and (c) human plasma sample collected 0.5 h after intravenous administration of DPT (5.4 mg kg^{-1}) to a patient. The t_R for DPT and IS are 2.60 and 2.25 min, respectively. The traces of the chromatograms correspond to: (i) the total ion chromatogram (TIC); (ii) the extracted ion chromatogram (XIC) of DPT (810.8638 → 640.9492, 341.2234, 313.2238, 187.0878, 159.0934), and (iii) the XIC of IS (609.2822 → 448.2004, 397.2146, 365.1896, 236.1299, 195.0677, 174.0951).

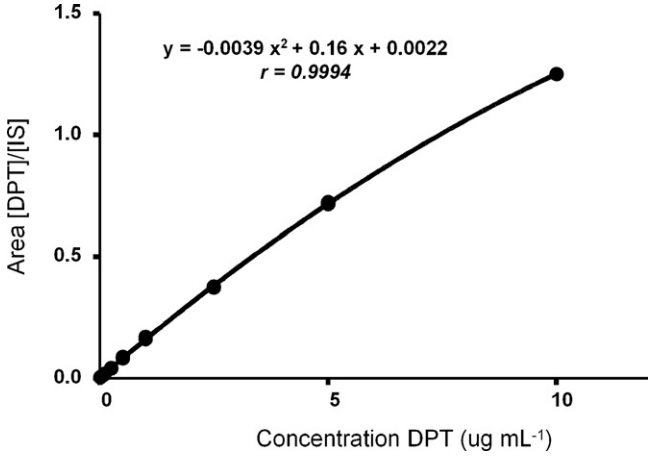

Fig. 4. Calibration curve of DPT in human plasma for the concentration range of 0.01–10 μg mL^{-1}.

intra-day precision for DPT was less than 9.91% and the accuracy was better than 9.01%. The inter-day precision was assessed by analyzing five sets of all QC samples on three consecutive days and was less than 10.82% whereas the corresponding accuracy was better than 5.67% (Table 2).

The RSD% of the peak t_R was found to be less than 0.2% within intra-day runs and less than 0.3% within 10 consecutive days of analysis for both DPT and IS.

3.4.4. Recovery

The extraction recovery data (R%, ±RSD%) of DPT from human plasma at three concentration levels (low, medium and high QC) are shown in Table 3 together with the recovery of the IS at the concentration of 0.5 μg mL^{-1} (n = 5). The data show that the recovery is almost quantitative for both DPT and IS.

3.4.5. Matrix effect

The matrix effect assessment of the proposed UPLC–ESI MS/MS method was performed on the basis that salts and endogenous

Table 2
Precision and accuracy data (intra- and inter-day) for DPT determination in human plasma ($n = 5$).

Level	Spiked concentration ($\mu g\,mL^{-1}$)	Intra-day ($n = 5$)			Inter-day ($n = 15$)		
		Mean concentration found ($\mu g\,mL^{-1}$)	RE (%)	RSD (%)	Mean concentration found ($\mu g\,mL^{-1}$)	RE (%)	RSD (%)
LLOQ	0.01	0.011	9.01	5.26	0.011	5.67	10.40
LQC	0.03	0.031	4.74	8.90	0.031	3.51	7.71
MQC	0.75	0.729	−2.87	6.66	0.717	−4.40	9.32
HQC	7.5	7.957	6.10	3.57	7.425	−1.00	10.82
ULOQ	10	9.563	−4.37	9.91	10.385	3.85	10.62

Table 3
Recovery % (R%) and matrix effect % (ME%) of DPT and IS in human plasma ($n = 5$).

Statistical Variable	Nominal DPT concentration ($\mu g\,mL^{-1}$)			Nominal IS concentration ($\mu g\,mL^{-1}$)
	0.03	0.75	7.5	0.5
Mean R (%) ± SD	96.1 ± 8.3	98.6 ± 7.5	93.5 ± 5.3	92.7 ± 5.0
Mean ME (%) ± SD	96.8 ± 3.1	95.6 ± 2.6	93.2 ± 4.0	94.0 ± 2.7

Table 4
Stability study of DPT in human plasma ($n = 5$).

Storage conditions	Nominal concentration ($\mu g\,mL^{-1}$)	Mean value ± SD[a]	RSD (%)	% Nominal[b]	RE (%)
Freeze–thaw (from −35 to 25 °C, 3 cycles)	0.03	0.03 ± 0.003	9.4	109	−9.6
	0.75	0.86 ± 0.06	7.3	114	−14.5
	7.5	8.6 ± 0.3	2.9	115	−15.3
Short-term (6 h, 25 °C)	0.03	0.03 ± 0.002	9.3	103	−3.2
	0.75	0.75 ± 0.06	7.7	100	−0.01
	7.5	7.1 ± 0.4	5.9	94.9	5.1
Autosampler (20 h, 10 °C)	0.03	0.03 ± 0.002	7.2	98.2	1.8
	0.75	0.73 ± 0.04	5.8	97.6	2.4
	7.5	7.6 ± 0.4	5.5	101	−1.9
Long-term (20 days, −70 °C)	0.03	0.03 ± 0.002	5.7	110	−10.2
	0.75	0.85 ± 0.06	7.2	113	−13.3
	7.5	8.3 ± 0.6	6.8	111	−11.1

[a] SD, standard deviation.
[b] Accuracy expressed as 100 × (mean calculated concentration)/(nominal concentration).

material present in the supernatant from protein precipitated plasma samples can cause ionization suppression with higher variation [21–23] compared to that of liquid–liquid or solid phase extracts. As shown from the results included in Table 3 the percentage matrix factors (ME%) calculated indicate low ion suppression and are in agreement with the international guidelines [18–20].

3.4.6. Stability

The stability of DPT was studied under various conditions as described in Section 2 for the low, medium and high QC samples in quintuple and the results are shown in Table 4. The RSD% values of *short- and long-term stability* were found to be less than 9.3% and 10.9%, respectively. The *autosampler stability* RSD% values obtained were found to be less than 7.2%, whereas the deviation found for the QC samples undergone *freeze–thaw stability* was less than 15.3% of the nominal concentration.

3.4.7. Sample dilution study

The results of the sample dilution study for sample concentrations of 15, 37.5 and 75 $\mu g\,mL^{-1}$ are expressed as recovery and are shown in Table 5. These data indicate that diluting high concentration samples above the ULOQ has little effect on the result.

3.5. Application to the analysis of clinical samples

The proposed analytical methodology was applied to the analysis of clinical samples obtained after iv administration of DPT. In particular, a 27-year-old patient suffering from post-traumatic osteomyelitis induced by MRSA was receiving 5.4 mg kg^{-1} of DPT (Cubicin®, Cubist Pharmaceutical) daily for a three-month treatment. Blood samples were collected from the patient at two different days before and 30 min after the iv administration of DPT in a tube containing EDTA as anticoagulant. The blood samples were

Table 5
Human plasma sample dilution study spiked with DPT ($n = 5$).

Dilution factor	Twofold[a]	Fivefold[b]	Tenfold[c]
Assayed concentration ($\mu g\,mL^{-1}$)	7.23 ± 0.55	7.24 ± 0.22	7.43 ± 0.19
Calculated concentration ($\mu g\,mL^{-1}$)	14.46 ± 1.10	36.22 ± 1.10	74.34 ± 1.90
Accuracy (RE%)	−3.6	−3.4	−0.9
RSD%	7.6	3.1	2.7

[a] Nominal concentration: 15 $\mu g\,mL^{-1}$.
[b] Nominal concentration: 37.5 $\mu g\,mL^{-1}$.
[c] Nominal concentration: 75 $\mu g\,mL^{-1}$.

shaken gently and centrifuged at 4000 rpm for 10 min at 25 °C, immediately after drawing. Plasma samples were refrigerated at −35 °C until analysis. Three aliquots (100 μL) of each plasma sample were analyzed by the developed UPLC–ESI MS/MS methodology after IS addition and sample pretreatment in order to quantify DPT. Plasma samples found with concentration levels above the calibration range were diluted appropriately and then re-analyzed. The concentration of DPT before iv administration was 2.62 ± 0.14 and 1.3 ± 0.07 μg mL^{-1} of human plasma, whereas the concentration of the antibiotic 30 min after DPT iv administration was found to be 14.4 ± 0.63 and 18.4 ± 0.71 μg mL^{-1} of human plasma, respectively, for the two sets of plasma samples. Representative chromatograms of human plasma sample collected 0.5 h after iv administration of DPT (5.4 mg kg^{-1}) to the patient are shown in Fig. 3c.

4. Conclusions

The described UPLC–ESI MS/MS validated analytical methodology enables the rapid and selective assay of DPT in human plasma. The proposed method presents high sensitivity, accuracy, precision, recovery and stability combined with high accuracy mass measurement, thus being suitable for monitoring of pharmacokinetic, bioavailability and bioequivalence studies. The simplicity of the sample pretreatment and the rapid analysis time could render this method as an ideal high-throughput method for the efficient analysis of a large number of clinical samples.

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