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HPLC-DAD validated method for DM4 and its metabolite S-Me-DM4 quantification in biological matrix for clinical and pharmaceutical applications

Giulio Lovato [a,c,1], Luigi Ciriolo [b,1], Miryam Perrucci [b,1], Luca Federici [a,c], Rodolfo Ippoliti [d], Stefano Iacobelli [e], Emily Capone [a,c], Marcello Locatelli [b,*], Gianluca Sala [a,c]

[a] Department of Innovative Technologies in Medicine and Dentistry, Center for Advanced Studies and Technology (CAST), University of Chieti-Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy
[b] Department of Pharmacy, University of Chieti-Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy
[c] Center for Advanced Studies and Technology (CAST), University of Chieti-Pescara "G. d'Annunzio", Via dei Vestini 31, Chieti 66100, Italy
[d] Department of Life, Health and Environmental Sciences, University of L'Aquila, 67100 Coppito, Italy
[e] MediaPharma s.r.l., Via Colle dell'Ara, Chieti 66100, Italy

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ABSTRACT

The present study focuses on the development and validation of an HPLC-DAD methodology for the detection of a potent chemotherapeutic agent, Maytansinoid Ravtansine (DM4), and its metabolite, S-methyl-DM4 (S-Me-DM4), in plasma samples. Methodologically, after a simple protein precipitation with acetonitrile and after drying 1 mL of supernatant, the sample (suspended with N,N-Dimethylacetamide, DMA) was directly analyzed by HPLC under isocratic elution using a mobile phase comprising milliQ water and methanol (25:75, *v:v*), both acidified with 0.1 % *v:v* formic acid. Employing a flow rate of 1.0 mL/min and a reversed-phase GraceSmart RP18 column thermostated at 40 °C, we achieved complete resolution and separation of DM4 and S-Me-DM4 within 13 min. The optimized injection volume of 20 μL and the wavelength set at 254 nm were utilized for quantitative analyses. Rigorous validation has not only ensured its reliability and reproducibility but has also addressed potential limitations associated with methodological inconsistency. The limit of detection and quantification of the method were 0.025 and 0.06 μg/mL for both the analytes, respectively. The calibration curve showed a good linearity in the range 0.06–20 μg/mL. For both analytes, the intraday precision and trueness were 2.3–8.2 % and −1.1 to 3.1 %, respectively, while the interday values were 0.7–10.1 % and −10.4 to 7.5 %, respectively. The developed methodology enables the concurrent determination and quantification of free DM4 and its metabolite, free S-Me-DM4, making it a valuable tool for assessing the pharmacokinetics and pharmacodynamics of DM4-based therapies. In addition, the procedure was successfully applied to analyse the presence of free DM4 or its metabolite, free S-Me-DM4, in human plasma samples spiked with the 1959-sss/DM4 antibody-drug conjugate (ADC). The utilization of the herein validated methodology allowed to confirm the presence of these analytes, thereby providing insights into their potential release from the ADC structure.

1. Introduction

Maytansinoids, a class of compounds derived from the natural product maytansine, have garnered significant attention in the field of drug development owing to their potent antimitotic activity and promising applications as payloads in antibody-drug conjugates (ADCs) [1]. Among these maytansinoids, N2′-Deacetyl-N2′-(4-mercapto-4-methyl-1-oxopentyl)-maytansine or Ravtansine or simply DM4 has emerged as a particularly compelling candidate, exhibiting improved biological activity and enhanced stability when conjugated to antibodies [1].

Structurally, maytansinoid derivatives, DM1, DM3, and DM4, differ from each other based on the number of methyl groups present on the carbon adjacent to sulfur—zero, one, or two, respectively. The

* Corresponding author.
E-mail address: marcello.locatelli@unich.it (M. Locatelli).
[1] These authors contributed equally.

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introduction of methyl groups contributes to the heightened potency of the free drug, likely due to increased hydrophobicity and improved membrane permeability [2].

DM4 is a thiol-containing compound that undergoes methylation within cells through the activity of methyltransferases, resulting in the formation of S-Me-DM4 [2]. This metabolite demonstrates notable cytotoxicity. Importantly, both DM4 and S-Me-DM4 possess the ability to pass through cell membranes, leading to an enhanced phenomenon known as the "bystander effect" where the drug can diffuse out of a targeted cell into adjacent cells [3]. S-Me-DM4, exert its cytotoxic effect by inhibiting microtubule formation and disrupting the assembly of the mitotic spindle. The presence of a further methyl group results in increasing potency of free drug as a higher hydrophobicity induces major membrane permeability [2,4]. However, due their extremely high cell killing potency (with IC_{50} in the picomolar range), such compounds cannot be used as free drugs.

Indeed, to mitigate cytotoxic effects and enhance selectivity, Antibody-Drug Conjugates (ADCs) were developed, providing a targeted approach to specifically deliver the drug to the tumour site [5]. The first ADC incorporating a maytansinoid payload was approved and introduced to the market in 2013, marking a significant milestone in the advancement of targeted cancer therapies [6]. ADCs consist of three components: a monoclonal antibody (mAb), a cytotoxic drug, and a linker that connects both components. The antibodies recognize and bind to specific target antigens present on the surface of tumour cells, enabling the selective delivery of the potent drug [6–11].

Simultaneously, it is crucial for the ADC to remain stable during circulation but release its payload upon reaching the intended target site. The combination of antibody's selectivity and drug's potency contributes to the therapeutic efficacy of ADCs in cancer treatment [5–8]. The evaluation of ADC efficacy requires the assessment of various parameters, including *in vivo* activity on cell and animal lines, Drug-Antibody Ratio (DAR), and bond stability. These analyses are crucial to prevent drug losses before reaching the tumour site and to minimize adverse toxicity reactions [7,10,11]. In Fig. 1, a schematic representation of linkers used in internalizing and non-internalizing ADCs was reported, as also highlighted by Ashman et al. [12].

Thanks to these characteristics there are some ADC available on the market: Gemtuzumab ozogamicin (Mylotarg®), the first ADC available from 2000, Brentuximab Vedotin (Adcetris®), trastuzumab emtansine (Kadcyla™), and Inotuzumab Ozogamicin (Besponsa®), Mirvetuximab Soravtansine (Elahere).

While liquid chromatography coupled to mass spectrometry (LC-MS) is commonly employed for such assessments, it often requires complex

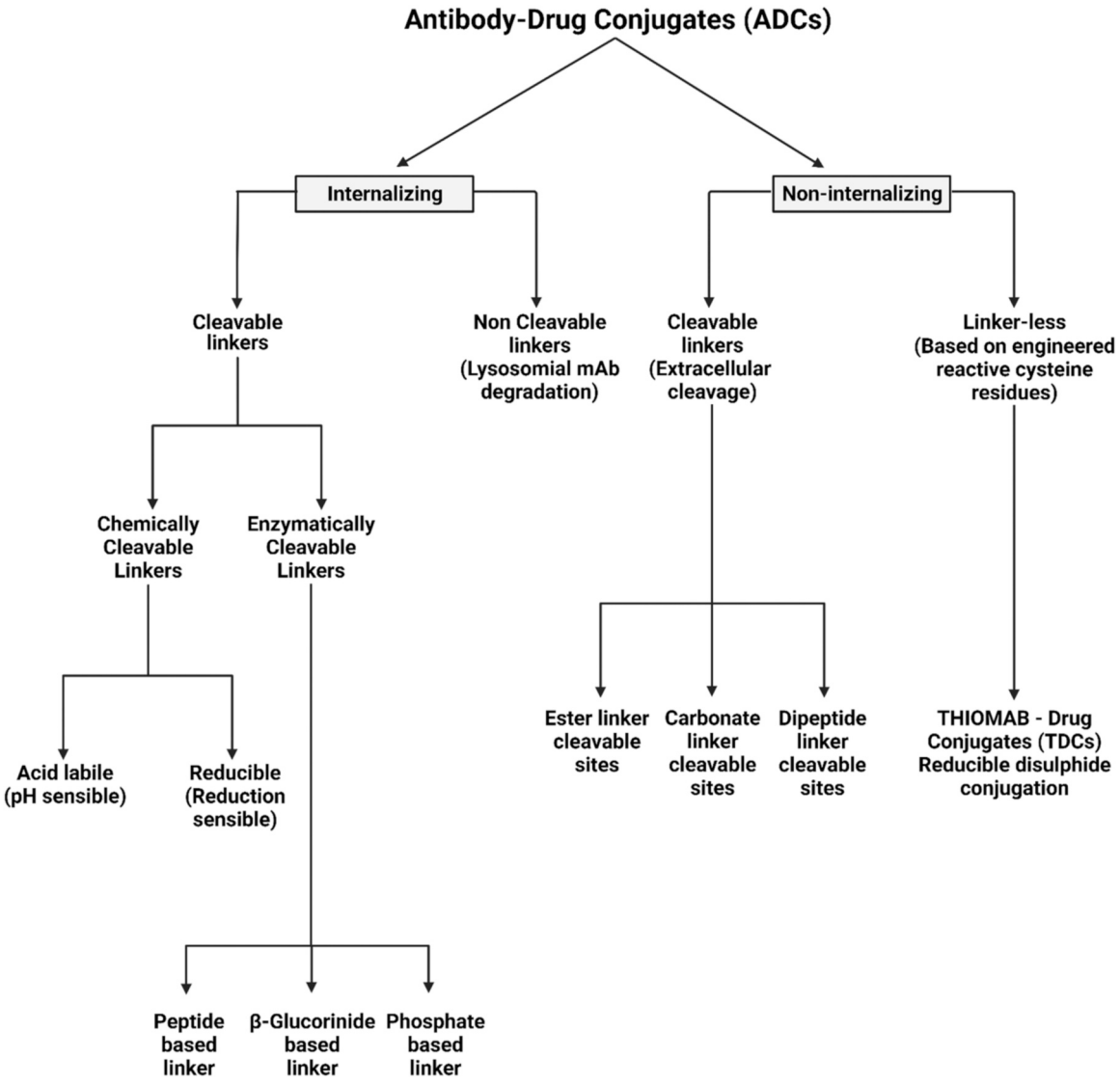

Fig. 1. Schematic representation of linkers.

instrumentation, substantial maintenance costs, and specialized expertise. Considering these factors, the primary objective of this study is to develop and validate a high-performance liquid chromatography (HPLC)-based method for the simultaneous quantification of free DM4 and its primary metabolite, S-Me-DM4, in human plasma samples. The method was further applied to a DM4 containing ADC, 1959-sss/DM4, which is a linkerless non-internalizing ADC previously described [5,9,13–16]. Non-internalizing ADCs are an emerging class of compounds designed to release the payload directly in the extracellular tumour microenvironment (TME), in contrast to classical internalizing ADCs in which the cytotoxic moiety is delivered into the cancer target cells [17]. A particularly innovative aspect of the 1959sss/DM4 ADC is its linker-less design, where the payload (SH-DM4) is directly bonded to the residual cysteines in the engineered 1959sss antibody [5].

The underlying hypothesis behind this conjugation strategy is that the reduction conditions in the tumour's extracellular environment will trigger the release of the payload, DM4. Due to its ability to diffuse into cancer and cancer-associated stroma cells, DM4 exhibits potent anti-tumor activity within the tumour tissues, as supported by various preclinical models of different human cancers [5,9,13–16].

The analytical method should be employed in future non-clinical and clinical studies to evaluate the stability of both the free drug and its metabolite, as well as the ADC, in biological matrices. By establishing a robust HPLC methodology, the aim of the present study is to provide an accessible and cost-effective analytical approach that can support comprehensive evaluations of free DM4 and S-Me-DM4 in several contexts. This research may will contribute to the advancement of DM4-based therapies and ADC development, ultimately improving the efficacy and safety of targeted cancer treatments.

2. Materials and methods

2.1. Chemicals and reagents

For the HPLC analysis, Ravtansine (DM4) and its main metabolite S-methyl-DM4 (S-Me-DM4) were obtained from MedChemExpress (New Jersey, USA). N,N-Dimethylacetamide (DMA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) and Acetonitrile (AcN) (HPLC grade) were bought from VWR Chemicals (Pennsylvania, USA) and Formic Acid (HPLC grade) was obtained from Carlo Erba Reagents (Milan, Italy). The water was purified using Milli-Q Lab Water by Merck (Darmstadt, Germany).

2.2. Preparation of standard solutions

The stock solution of DM4 and S-Me-DM4, each at a concentration of 1 mg/mL, was prepared by accurately weighing 1 mg of each compound and dissolving it in 1 mL of DMA. To aid in solubilization, the stock solution was subjected to sonication in an ultrasonic bath for 2 min at 20 °C, following the guidelines provided by MedChemExpress. The working solutions concentration range was evaluated considering a 10-folds matrix dilution (10 % matrix modification, as allowed for bioanalytical method validation). In consideration of this, the working solutions have a concentration range from 0.25 to 200 μg/mL and were prepared by diluting the stock solutions with DMA. Both the stock and working solutions were stored at −20 °C and showed stability for a period of one month.

The range was evaluated in order to obtain the minimum concentration detectable (LOD) and quantifiable (LOQ) with the present method and with the concentrations used in the present study, while the maximum concentration (ULOQ, upper limit of quantification) was chosen in order to validate a linear range as wide as possible in order to limit the possibility of having "over range" samples, for which a reanalysis is required after the dilution process.

2.3. Plasma samples preparation

Pooled plasma samples were obtained from the whole blood of healthy voluntary employees affiliated with the Center for Advanced Studies and Technology (CAST), University of Chieti-Pescara "G. d'Annunzio," using citrate as an additive. Prior to participation in this study, all individuals were contacted and provided explicit and informed consent for the analysis and utilization of their plasma samples.

Plasma samples holding the analytes (obtained from whole blood with citrate as an additive) were prepared at concentrations of 0.025, 0.06, 0.08, 0.10, 0.20, 0.50, 1.00, 4.00, 5.00, and 20.00 μg/mL. Quality control (QC) samples were also prepared at concentrations of 0.25, 2, and 10 μg/mL. To prepare each sample, an aliquot of 270 μL of blank human plasma was taken, and 30 μL of the corresponding concentration of the working solution holding a mixture of DM4 and S-Me-DM4 in DMA was added. The chosen volumes ensured that the matrix modification remained below 15 %, as required for bioanalytical method validation. After spiking, the samples were gently shaken using a vortex mixer (VELP Scientifica, Monza, Italy). Subsequently, three volumes (900 μL) of AcN were added to achieve protein precipitation. All samples were vortexed and centrifuged at 12,000 *x g* for 15 min. From each sample, a standardized volume of supernatant (1 mL) was collected and dried under vacuum using the SC110A SPEEDVAC® concentrator (SpeedVac). The dried samples were reconstituted with 300 μL of DMA, followed by vortexing and centrifugation at 12,000 *x g* for 3 min to remove non-soluble particulates. The resulting supernatant was directly injected into the HPLC instrumentation.

2.4. HPLC-DAD method validation

The analysis of DM4 and S-Me-DM4, both in DMA and human plasma citrate, as well as the subsequent monitoring of analyte stability in human plasma citrate, were conducted using an Agilent 1100 liquid chromatography system (Agilent Technologies, Waldbronn, Germany). The system consisted of a solvent pump, *on-line* degasser, thermostated autosampler and column compartment, and diode array detector (DAD). Data acquisition and processing were performed using Chemstation Software (Agilent).

During method development, various chromatographic parameters and mobile phases were evaluated. A C18 reversed-phase packing column (GraceSmart RP18, 4.6 × 150 mm, 5 μm; Grace, Deerfield, IL, USA) was employed for the separation. Different temperatures (40 °C and 50 °C) and flow rates (0.8 mL/min, 1.0 mL/min and 1.2 mL/min) were tested. The quantitative analyses were conducted at 254 nm, which was determined to be the optimal wavelength for maximum sensitivity of DM4 and S-Me-DM4. At this wavelength the maximum absorbance value is observed in the mobile phase used for the chromatographic elution, obtaining the maximum signal-to-noise ratio (S/N) and consequently maximizing the methodological sensitivity. The choice of the chromatographic column was based on the type of analytes of interest and based on LogP and pKa values. The column used has a reduced film thickness (3 μm) with a pore size of 120 Å (USP Type L1) particularly suitable for general purpose and routine applications. It also has a large surface area (220 m^2/g) with a carbon load equal to 10 %, as well as being endcapped (reducing peak symmetry problems due to interactions with free silanols).

For the analysis in both DMA and human plasma, the best results were achieved using the GraceSmart RP18 column (4.6 mm × 150 mm × 5 μm) thermostated at 40 °C (± 1 °C). The mobile phase consisted of a mixture of milliQ water and methanol (25:75, *v:v*), both acidified with 0.10 % *v:v* formic acid. Isocratic elution at a flow rate of 1.00 mL/min was employed, and the injection volume was set at 20 μL. Fig. 2 illustrates the chromatograms obtained at 254 nm for a single analyte in the solvent (DMA) and for the analyte mixture. The method was validated according to International Guidelines, including assessments of linearity, selectivity, precision and trueness (both intraday and interday),

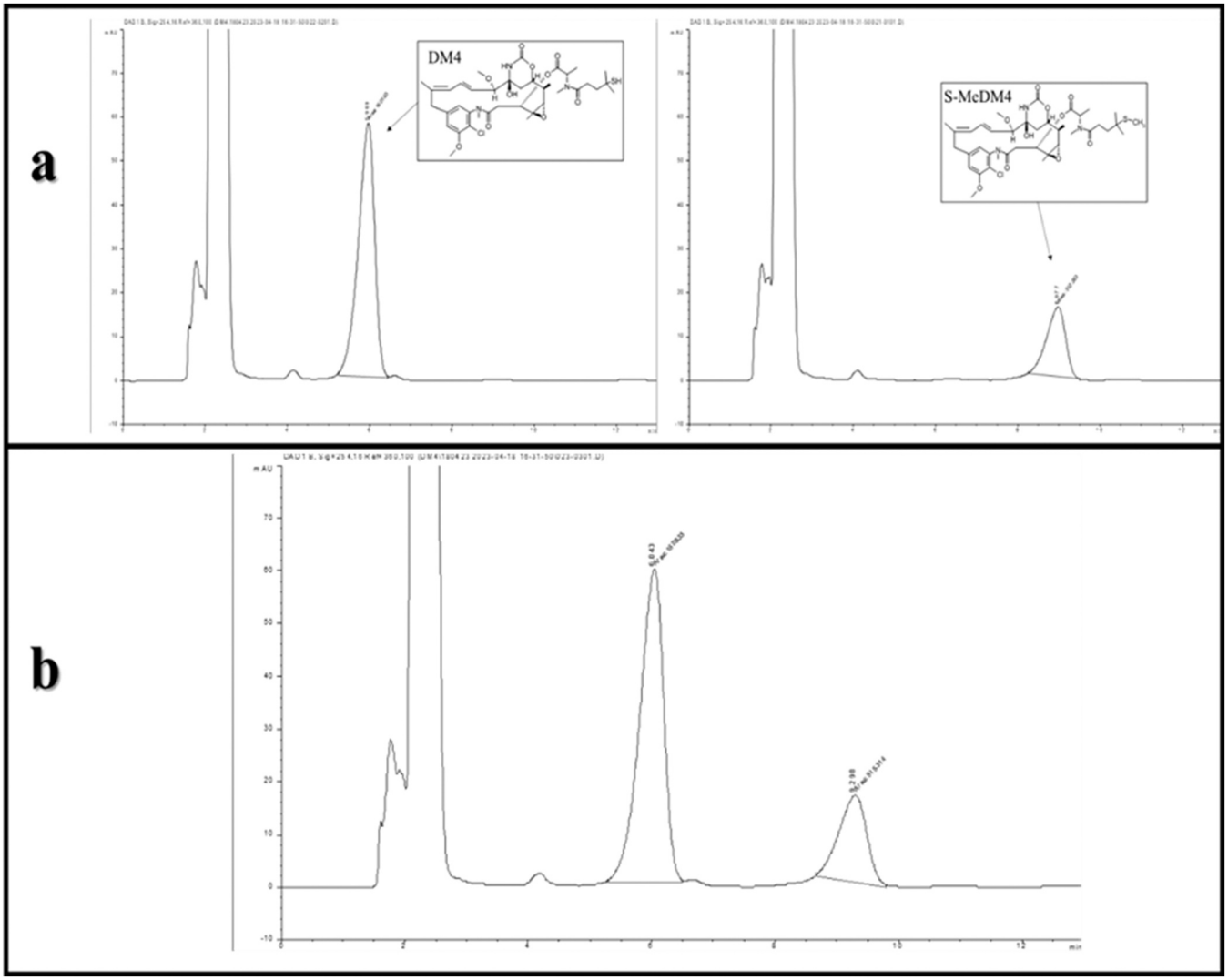

Fig. 2. Reference chromatograms obtained at 254 nm of the analytes in solvent DMA (a, left DM4 and right S-Me-DM4), the mix of the two analytes (b).

ruggedness, limit of detection (LOD), and limit of quantification (LOQ) [18,20]. By optimizing the instrumental setup and developing a comprehensive method validation, reliable quantification of DM4 and S-Me-DM4 was achieved, ensuring the precision and trueness necessary for further analyses and stability studies.

2.5. Real samples analysis

Following the successful completion of the method validation process, such protocol was applied to investigate the behavior of DM4 and S-Me-DM4 in human plasma. Samples were subjected to the previously described preparation procedure and then analyzed. In this study, various time points were considered, namely t_0, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h. For each time point, 270 µL of plasma was spiked with 30 µL of a DM4/S-Me-DM4 mixture (100 µg/mL) to achieve a final analyte concentration of 10 µg/mL. The samples were then subjected to incubation under stirring conditions at 37 °C, or keeping in an ice water bath temperature of 2–4 °C.

In addition, to assess the applicability of the method, were examined the presence of free payload following the incubation, along the time points, of an aliquot of 1959-sss/DM4 antibody-drug conjugate (ADC) in plasma; 270 µL of human plasma and 30 µL of the ADC solution in phosphate buffer (1.25 mg/mL) were combined. This concentration reflects the dosage used in *in vivo* mouse experiments, where a dose of 10 mg/kg was reported [5]. The incubation of the ADC samples was carried out at 37 °C to simulate physiological conditions.

3. Results and discussion

3.1. Sample preparation

DM4, a lipophilic drug with a LogP value of 5.39, exhibits significant hydrophobic characteristics. Its main metabolite, S-Me-DM4, undergoes intracellular enzymatic S-methylation, potentially increasing its lipophilicity [21,22]. To establish the linearity of the method, working solutions were prepared by diluting a stock solution of DM4 and S-Me-DM4 (1 mg/mL in DMA) with various solvents, including water, phosphate buffer solution (PBS) at pH 7.4, H_2O:MeOH (25:75, *v:v*), and DMA.

Analysis of samples prepared in water and phosphate buffer yielded incongruent results, showing inadequate linearity across different concentrations. This can be attributed to incomplete solubilization of the analytes or their tendency to form aggregates, as discussed in a study by Mohamed H.E. et al., which characterized emtansine (DM1), a precursor of DM4, with similar physicochemical properties [23]. A mixture with a prevalent organic phase (H_2O:MeOH, 25:75, *v:v*) increased solubility,

but the optimal chromatographic profile was obtained when the samples were prepared in DMA. In the evaluation of the best solvent to use in the preparation of the working solutions it was observed that passing from a solvent such as water which shows a polarity index equal to 10.2 with a dielectric constant of 80.1, to a solvent such as DMA (where these parameters are clearly lower and equal to 6.5 and 37.78, respectively) a complete solubilization of the analytes is obtained. This allow avoiding the possible formation of aggregates (due to the lipophilicity of the molecules) and on the one hand improving the instrumental response not only in terms of signal-to-noise ratio (and also peak symmetry), but above all in terms of reproducibility of the analyses. This last point, extremely important in quantitative analysis, made it possible to validate the precision and trueness based on the international guidelines. In this specific case, the formation of any aggregates (a phenomenon that is not very reproducible and not very controllable) must be avoided in order to obtain solutions (stock and working) with known concentration not only for the matrix validation process, but also for the *in vitro* study herein reported.

Notably, it was also observed that the addition of a small amount of DMA did not induce plasma protein precipitation or cause other modifications in the matrix during the fortification step. Consequently, DMA was selected as the ideal solvent for effective solubilization of the analytes in human plasma citrate.

Subsequent sample preparation involved protein precipitation (PP) using a 1:3 ratio of AcN to sample (*v:v*). The samples were vortexed and centrifuged at 12,000 *x g* for 15 min. The resulting supernatant (1 mL) was carefully transferred to a new Eppendorf tube and dried under vacuum using a SpeedVac system. Each dried sample was reconstituted with 300 μL of DMA, subjected to ultrasound bath treatment (2 min), followed by centrifugation (3 min). After supernatant recovery, and a simple vortex step, samples were transferred to HPLC vials for subsequent HPLC-DAD analysis.

3.2. Method optimization

During method optimization, various HPLC variables were investigated, including the composition of the mobile phase and the column temperature. Initially, different ratios of H_2O:MeOH (30:70, 25:75, and 20:80, *v:v*) were tested, followed by H_2O:AcN (25:75, 35:65, 40:60, *v:v*) and ammonium acetate (10 mM and 20 mM, pH 7):MeOH (25:75, *v:v*). The best performance in terms of resolution and peak intensity was achieved with H_2O:MeOH (25:75, *v:v*). The other tested conditions resulted in asymmetrical peaks that were either too close to the void time or had excessively long elution times, leading to peak broadening.

To further enhance peak shape, the addition of varying percentages of formic acid (0.05 %, 0.10 %, 0.15 %, and 0.20 % *v:v*) was evaluated in both the aqueous and organic phases. Although only minor differences were observed, the final composition selected was H_2O:MeOH (25:75, *v:v*) acidified with 0.10 % *v:v* of formic acid. Changes in column temperature (ranging from 40 °C to 50 °C) did not yield significant improvements, thus the lower temperature of 40 °C was chosen for method validation.

Under these optimized conditions, the chromatographic profiles of blank human plasma and fortified human plasma samples are reported in Fig. 3. As illustrated, at 254 nm, no interfering peaks were observed, and the two analytes, DM4 and S-Me-DM4, were fully resolved without noticeable peak asymmetry or fronting/tailing issues. The retention times for DM4 and S-Me-DM4 under the optimized conditions were determined to be 5.6 ± 0.5 and 9.2 ± 1.2, respectively.

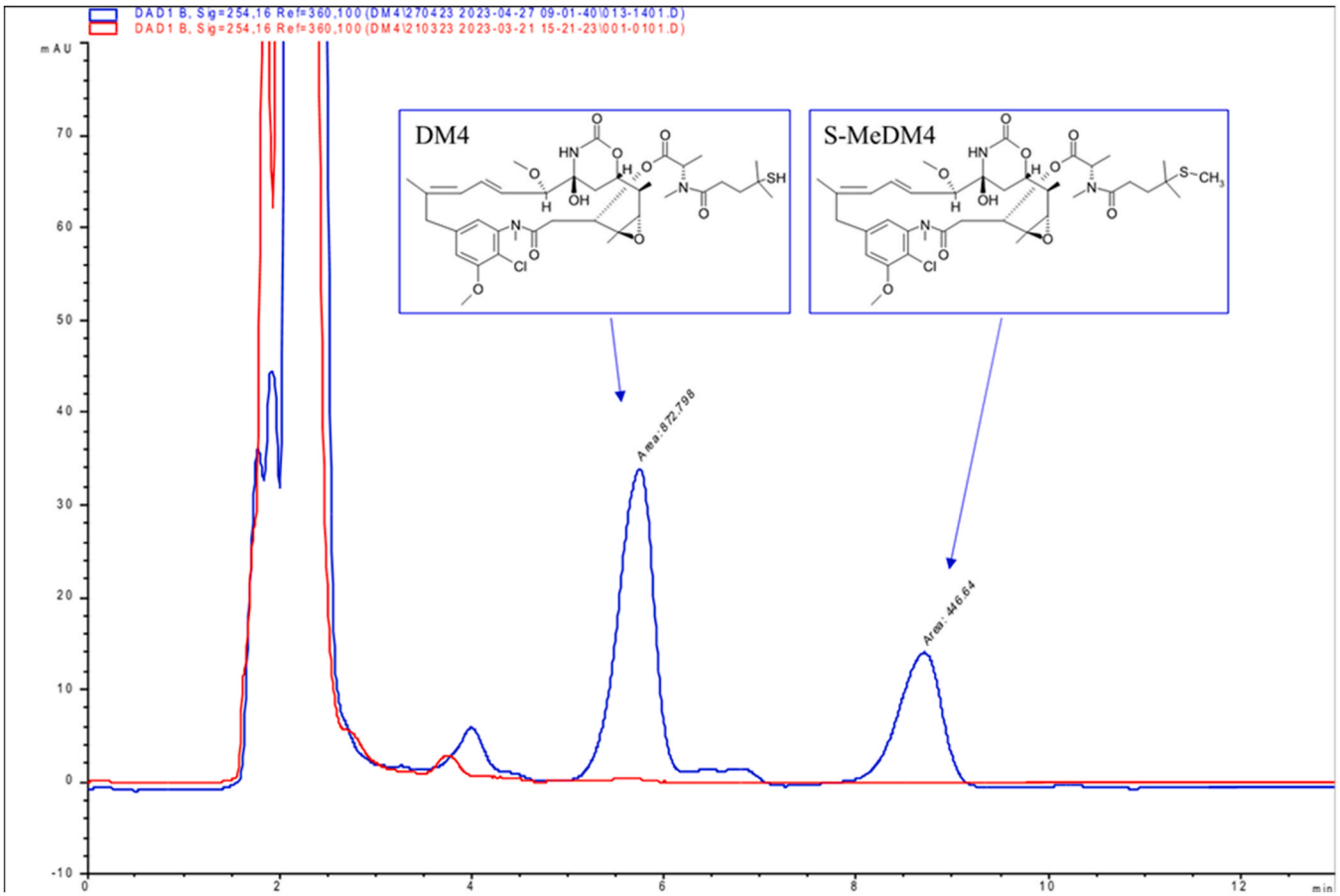

Fig. 3. Chromatograms obtained at 254 nm for the blank human plasma (red trace), and the blank human plasma fortified with the mix (DM4/S-Me-DM4) (blue trace).

3.3. Method validation

The method was validated under the optimized conditions according to the International Guidelines. The validation parameters assessed included linearity, selectivity, precision and trueness (both intraday and interday), ruggedness, LOD, and LOQ [18–20]. Additionally, a stability study of the processed samples was conducted at 4 °C.

The results obtained indicated that the method exhibited linearity within the concentration range of 0.06–20 μg/mL, with an R^2 value equal to or greater than 0.9881. The calibration curves were plotted using a weighting factor of $1/x^2$, as recommended by the International Guidelines. The LOD was determined to be 0.025 μg/mL, corresponding to a signal-to-noise (S/N) ratio of 3. The LOQ was established at 0.06 μg/mL for both analytes, based on a signal-to-noise ratio of 10 and the BIAS % value for the back-calculated concentration. At the LOQ level, the intraday precision and trueness for DM4 were 5.4 % and −1.1 %, respectively. The interday precision and trueness were 10.5 % and 9.2 %, respectively. At the same concentration level, the intraday precision and trueness for S-Me-DM4 were 6.3 % and −2.5 %, respectively. The interday precision and trueness were 8.5 % and 10.3 %, respectively.

The precision and trueness, evaluated both intraday and interday, were found to be below ± 15 %, as presented in Table 1. The selectivity of the method was assessed by analysing blank human plasma samples to confirm the absence of interfering substances. In Table 2 were reported the main figure of merits related to the calibrations during intra- and interday data elaboration.

In Fig. S1 (supplementary material) was reported an example of calibration curves obtained during the intraday validation.

3.4. Ruggedness and system suitability test

To assess the ruggedness of the validated method, a standard sample containing DM4 and S-Me-DM4 was utilized to evaluate the performance of the method under intentionally varied instrumental parameters. Small but deliberate changes were made to parameters such as flow rate, temperature, mobile phase percentage, and composition. The results obtained from these experiments, as summarized in Table S1 (supplementary material), indicate that the procedure is robust and reliable, making it suitable for routine clinical laboratory applications. The method demonstrates simplicity, speed, and the ability to withstand minor deviations in instrumental parameters without compromising its performance.

3.5. Real sample study

This experiment was taken to investigate the temperature dependent behavior of DM4 and S-Me-DM4 in human plasma, aiming to provide indications into their *in vitro* characteristics such as plasma protein binding, aggregate formation, and metabolite formation. The concentration changes of the analytes were monitored over various incubation times. Samples corresponding to each time point, both for 37 °C and 4 °C incubations, were prepared as described (Section **2.5**) and analyzed using the validated method (Table 3, Fig. 4).

Concurrently, a similar experiment was conducted on 1959-sss/DM4 ADC to assess the presence of free DM4 at each time point. After the designated incubation time, all samples were treated and analyzed as outlined (Table 4).

The results depicted in Table 3 provide information into the recovery of DM4, approximately 93 % at t_0 (0 h) 37 °C. The concentration of DM4 decreases rapidly up to 2 h of incubation, followed by a relatively constant fraction of approximately 3 % from 4 to 24 h. These findings suggest a time-dependent binding of DM4 to plasma proteins until a dynamic equilibrium is achieved. In contrast, the stability observed for S-Me-DM4 (approximately 99 % at t_0 and 37 °C, decreasing to approximately 15 % at 24 h) follows a linear trend over time. The presence of an S-methyl group instead of an -SH group significantly reduces interactions related to time-dependent binding to plasma proteins, resulting in improved stability in human plasma. These values align closely with the free DM4 detected on average over 24 h, previously described, designed to assess the release of free payload from the ADC.

The same experiment conducted in an ice water bath (4 °C) showed higher recovery for both DM4 and S-Me-DM4, indicating a temperature-dependent effect. Specifically, for DM4, the average recovery between 1 and 8 h was approximately 60 %, which further decreased to 30 % at 24 h. In the case of S-Me-DM4, considering the same time points, the average recovery was approximately 85 %, significantly higher than that of DM4 due to the exclusion of disulfide binding with proteins. Notably, the recovery values for t_0 and 30 min for both analytes were lower compared to those obtained at 37 °C. These values are likely influenced by the lower solubility of DM4 and its metabolite in human plasma at low temperatures, resulting in incomplete homogeneity of the solution at these time points. Therefore, the observed trend for both analytes suggest an initial increase in recovery within the first 30 min due to

Table 2
Main figure of merits related to the calibrations during intra- and interday data elaboration.

Analyte	Parameter	Intraday values	Interday values
DM4	Intercept	-0.8153 ± 0.1605	-0.5572 ± 0.1617
	Slope	70.35 ± 1.248	43.69 ± 1.163
	R^2 (unweighted)	0.9881	0.9975
S-Me-DM4	Intercept	0.7770 ± 0.08226	0.8417 ± 0.1061
	Slope	24.03 ± 0.4980	25.62 ± 0.4422
	R^2 (unweighted)	0.9956	0.9960

Table 1
Figure of merits of the validated HPLC-DAD method for DM4 and its metabolite S-Me-DM4 quantification in human plasma citrate matrix.

Analyte	Linearity (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)		Intraday		Interday	
					Precision (Std. Dev.)	Trueness (BIAS %)	Precision (Std. Dev.)	Trueness (BIAS %)
DM4	0.06–20	0.025	0.06	QC low (0.25 μg/mL)	8.2	-11.0	10.1	-10.4
				QC med (2 μg/mL)	6.4	-7.7	1.7	-6.2
				QC high (10 μg/mL)	2.3	3.1	0.7	-4.4
S-Me-DM4	0.06–20	0.025	0.06	QC low (0.25 μg/mL)	5.2	-9.4	4.7	-1.0
				QC med (2 μg/mL)	2.4	-4.8	5.8	-4.3
				QC high (10 μg/mL)	3.4	-6.0	8.6	7.5

Table 3
DM4 and S-Me-DM4 at 5 μg/mL in human plasma at 37 °C and in ice bath (2–4 °C).

	Time (h)	Incubation at 37 °C			Incubation in ice bath (2–4 °C)		
		Concentration (μg/mL)	%	Loss	Concentration (μg/mL)	%	Loss
DM4	**0**	4.64 ± 0.52	92.72 ± 9.30	7.28 ± 0.89	2.24 ± 0.26	44.85 ± 4.45	55.15 ± 5.15
	0.5	2.29 ± 0.28	45.89 ± 5.60	54.11 ± 6.84	2.27 ± 0.28	45.57 ± 4.56	54.43 ± 5.60
	1	0.86 ± 0.32	17.18 ± 2.40	82.82 ± 8.85	3.36 ± 0.36	67.37 ± 6.65	32.63 ± 3.60
	2	0.21 ± 0.06	4.21 ± 0.63	95.79 ± 10.3	3.43 ± 0.32	68.70 ± 7.10	31.30 ± 2.90
	4	0.19 ± 0.08	3.84 ± 0.28	96.16 ± 9.91	2.50 ± 0.23	50.05 ± 5.23	49.96 ± 5.01
	8	0.14 ± 0.05	2.74 ± 0.22	97.26 ± 9.80	2.43 ± 0.26	48.68 ± 5.10	51.32 ± 5.20
	24	0.10 ± 0.06	2.02 ± 0.18	97.98 ± 10.2	1.54 ± 0.14	30.90 ± 2.90	69.10 ± 7.05
S-Me-DM4	**0**	4.994 ± 0.56	99.87 ± 8.54	0.13 ± 0.05	3.88 ± 0.35	77.63 ± 7.80	22.37 ± 2.34
	0.5	4.878 ± 0.68	97.56 ± 5.24	2.44 ± 0.26	4.06 ± 0.41	81.30 ± 7.98	18.70 ± 1.94
	1	4.471 ± 0.54	93.41 ± 7.98	6.59 ± 0.74	4.28 ± 0.42	85.77 ± 8.45	14.23 ± 1.43
	2	4.126 ± 0.53	82.53 ± 8.20	17.47 ± 1.82	4.60 ± 0.48	92.16 ± 9.34	7.84 ± 0.78
	4	3.244 ± 0.35	64.89 ± 4.65	35.11 ± 4.51	4.40 ± 0.45	88.02 ± 8.96	11.98 ± 1.25
	8	2.621 ± 0.31	52.42 ± 5.82	47.58 ± 4.20	4.35 ± 0.41	87.06 ± 8.65	12.94 ± 1.34
	24	0.730 ± 0.09	14.60 ± 1.87	85.40 ± 7.96	3.93 ± 0.38	78.72 ± 7.89	21.28 ± 2.15

Values were reported as mean ± standard deviation (n = 3)

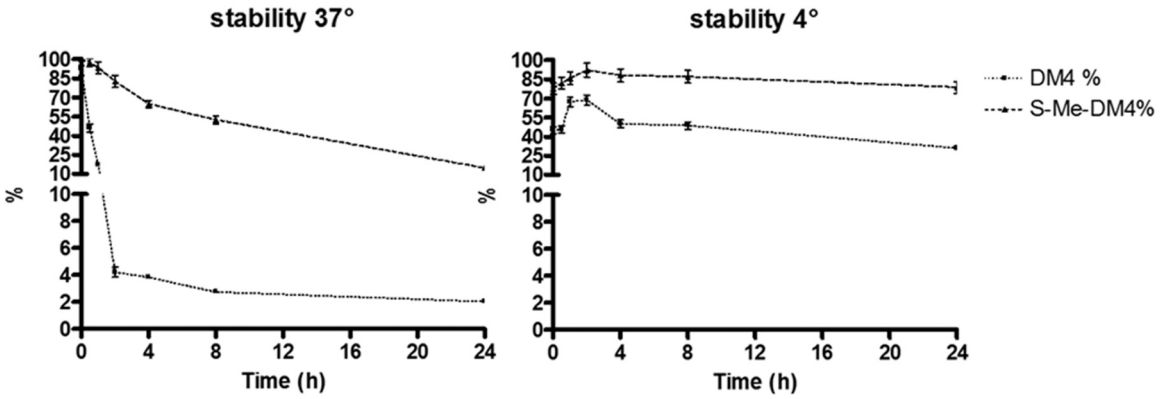

Fig. 4. Time course profiles for DM4, and S-Me-DM4 in human plasma incubated at 37 °C (left) and + 4 °C (ice bath, right). Values are referred to Table 4.

Table 4
ADC 125 μg/mL in human plasma at 37 °C.

Time (h)	Incubation at 37 °C	
	Concentration (μg/mL)	% DM4
0	-	-
0.5	-	-
1	0.05 ± 0.02	1.83 ± 0.2
2	0.06 ± 0.02	2.10 ± 0.2
4	0.05 ± 0.02	1.96 ± 0.2
8	0.10 ± 0.03	3.72 ± 0.4
24	0.08 ± 0.02	2.98 ± 0.3

Values were reported as mean ± standard deviation (n = 3)

improved solubility, reaching its maximum at 1 h, followed by a decrease over 24 h due to protein interactions.

As highlighted in Table 4, the percentage of free DM4 derived from the ADC in human plasma at 37 °C remained relatively stable, at approximately 3 % both after 30 min and within 24 h (free fraction). These results collectively prove the applicability of the developed method to detect free DM4 and its metabolite S-Me-DM4.

The recovery of DM4 and its metabolite are mainly linked to the phenomenon of protein binding (99 %, [25]) which varies in entity as the temperature varies. The main problem observed in the present work is that one could work at lower temperatures (better stability profile in the matrix, Fig. 4), but this leads to an increase in the formation of possible aggregates (with a reduction in solubility) which reduce the recovery of the analytes, in particular of DM4 as it has the free terminal -SH group. Another important element is linked to the fact that DM4 (as well as DM1 studied by Heudi et al. [24]) along with reacting with other -SH groups present in proteins, could form dimers by S-S bond. From the present study it emerges at the moment that the recovery problem is mainly linked to protein binding, even if it is not excluded that other factors (chemical breakdown or enzymatic reactions) can modify the profiles (DM4 *in vivo* is rapidly methylated by an endogenous S-methyl transferase to form S-Me-DM4, [26]).

3.6. Comparison with literature

The validated method presented in this study offers significant advantages compared to other methods reported in the literature. A comprehensive analysis of previously published procedures reveals various drawbacks and limitations (Table 5).

For instance, Heudi et al. focused only on DM1 as the target analyte, employing a complex sample preparation and instrumentation. The use of gradient elution and incomplete validation procedures, omitting crucial aspects such as optimization of injection volume to enhance ionization efficiency and sensitivity, may restrict its practical applicability [24]. Similarly, Wei et al. exclusively considered DM4 as the target analyte, using gradient elution in LC-MS/MS analysis [27], limiting the method transferability. Fu et al. investigated both DM4 and its S-Me-DM4 metabolite but employed complex sample preparation, complex instrumentation, and gradient elution, posing potential challenges for method transferability [28]. Lazar et al. presented an unvalidated procedure with a complex instrument configuration and focused on standard solution analysis, employing gradient elution. While their method was applied to determine optimal conditions for the analysis of Antibody-maytansinoid conjugates, its suitability for broader applications remains uncertain [29]. Widdison et al. employed a complex instrument configuration (LC-MS/MS) with gradient elution and an unvalidated procedure, resulting in a lengthy LC runtime of 60 min [22]

Table 5
Comparison with other published methods.

Analyte	Matrix	Extraction	System	Elution	Column	Runtime (min)	LOD (ng/mL)	LOQ (ng/mL)	Ref.
DM1	Human serum	*On line* SPE	LC-MS/MS	Gradient	C18	10	-	0.2	[24]
DM4	Monkey plasma Rat plasma	LLE	LC-MS/MS	Isocratic	PFP	2	-	0.5	[27]
DM4 S-Me-DM4	Human plasma	PP followed by SPE	LC-MS/MS	Gradient	C18	3.1	-	0.1	[28]
Antibody-maytansinoid conjugates	Standard solution	Deglycosylation followed by desalting	HPLC-MS	Gradient	C18 and SEC	-	-	-	[29]
Primary antibody−maytansinoid conjugates metabolites	Human liver microsomes	-	LC-MS/MS	Gradient	C8	60	-	-	[22]
Three antibody-maytansinoid conjugates	Mouse tumour tissue	Tissue homogenate followed by LLE	HPLC-DAD-β-counter	Gradient	C18	-	-	-	[30]
DM4 S-Me-DM4	Human plasma	PP	HPLC-DAD	Isocratic	C18	13	25	60	Current work

PP: protein precipitation; LLE: liquid-liquid extraction; PFP: pentafluorophenyl; LC-MS/MS: liquid chromatography tandem mass spectrometry; HPLC-DAD: high performance liquid chromatography diode array detector; SPE: solid-phase extraction; SEC: size exclusion chromatography.

without exploiting the potential of the LC-MS/MS configuration in quantifying analytes that are even slightly resolved but show different multiple reaction transitions (parent ion → daughter ion). This allow reducing the analysis time and, consequently, increasing the throughput (an important element in the pharmaceutical industry). Similarly, Erickson et al. described an unvalidated procedure utilizing a complex instrument configuration and gradient elution, with the additional use of scintillation reagents for analyte detection [30].

In contrast, the current study represents a significant advancement as it is the first to simultaneously determine DM4 and S-Me-DM4 in human plasma using a well-established HPLC-DAD instrument. The method employs a rapid, easy, and robust sample preparation procedure. Furthermore, it has been fully validated in the biological matrix of human plasma, demonstrating performance characteristics suitable for clinical and pre-clinical studies. While the sensitivity of this method may not match that of LC-MS/MS setups, it is sufficient for clinical and pre-clinical applications. Moreover, the use of fully compatible mobile phases with MS instrumentation, isocratic elution, fast HPLC analysis (achieving resolution within 13 min), and the absence of the need for highly specialized personnel facilitate whole method transfer to other configurations. This opens possibilities for enhanced sensitivity without sacrificing transferability, which is of greatest importance in the clinical and pharmaceutical fields.

Moreover, in comparison to the work presented by Wei et al. the current study offers notable advantages, even though with a slightly longer and less sensitive analysis due to the employed instrumental configuration [27]. Notably, the present study encompasses both DM4 and its metabolite, addresses the issue of thiol reactivity specific to DM4, and includes experiments conducted at physiological temperatures, rather than solely stability tests at low temperatures as conducted by Wei et al. [27].

4. Future perspective

The successful application of this methodology in assessing the presence of the free amount of this analyte in ADC 1959-sss/DM4-treated plasma samples demonstrates its utility in future ADC pharmacokinetic studies. In particular, in the future, the present method paves the way for interesting developments for the pharmaceutical industry and for pharmaceutical quality control.

This method represents an important element in support of studies on ADCs involving the use of DM4 as payload by the Pharmaceutical industry, as it does not present problems related to its transferability (isocratic elution) and/or interfacing with more sensitive detection systems (such as MS and MS/MS) thanks to the use of mobile phases totally compatible with this instrumentation. It can also be easily transferred to chromatographic instruments, which are currently starting to be present in pharmaceutical laboratories such as UPLC (or UHPLC, Ultra High Performance Liquid Chromatography) without having problems relating to chromatographic performances. Furthermore, at present it is a method that exploits a simple, well-known instrumental configuration, widespread in all laboratories, robust, and which does not require trained personnel.

These key-elements make it a highly valuable method for its use and/or implementation to develop new protocols for quality control and characterization of new DM4-based ADCs. The short duration of the analysis also makes it possible to respond to another very important element towards which the pharmaceutical industry is particularly "sensitive", the high productivity (or high-throughput), which allows processing a large number of samples per unit of time.

The simplified and efficient sample preparation procedure allows a rapid analysis, making it particularly suitable for *in vitro* and *in vivo* clinical and preclinical applications, since no matrix interferents were found to affect the analytical performances.

5. Conclusion

The herein validated HPLC-DAD methodology enables accurate detection and quantification of Maytansinoid DM4 and its metabolite S-Me-DM4 in human plasma samples. The concurrent determination of DM4 and S-Me-DM4 provides valuable information regarding their behaviour's profiles. This method represents a significant advancement in the field and holds great potential for facilitating clinical trials characterized by large sample sizes. The developed and validated method employed in this study offers significant advancements compared to existing literature. Previous procedures encountered limitations such as complex sample preparation, gradient elution, and incomplete validation. In contrast, the herein streamlined and robust HPLC-DAD method allows the simultaneous determination of DM4 and S-Me-DM4 in human plasma, providing accurate and reliable results. Comparative analysis with existing literature highlights the valuable advantages of this procedure. While LC-MS/MS approaches offer enhanced sensitivity, the HPLC-DAD method maintains compatibility with MS instrumentation, utilizes isocratic elution, and ensures straightforward method transferability without compromising analytical performances.

Notably, with our findings it can be supposed a time-dependent bond between DM4 and plasma proteins, reaching a dynamic equilibrium. In contrast, the presence of an S-methyl group in S-Me-DM4 enhances its stability, as demonstrated by a linear concentration decrease over time. This observation highlight the significance of payload structure in antibody-drug conjugates.

Furthermore, temperature-dependent behaviour was elucidated, with higher recovery observed at lower temperatures. The impact of temperature on solubility and protein interactions is evident,

emphasizing the importance of precise temperature control during experimental procedures.

In conclusion, this study provides comprehensive insights into the behaviour of DM4 and S-Me-DM4 in human plasma, facilitated by a robust and validated HPLC-DAD method. The obtained results contribute to a partial and initial understanding of antibody-drug conjugate stability and pave the way for further investigations in clinical and pre-clinical studies. The applicability of this method, along with its potential for sensitivity enhancements and optimization, positions it as a valuable tool for studying the behaviour of antibody-drug conjugates and their payloads in biological matrices.

Author statement

The authors declare that does not exist any economic interest or any conflict of interest.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115642.

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