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Quantitative analysis of trace-level bleomycin in complex matrices: Application to in vitro electrochemotherapy

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ABSTRACT

Bleomycin, a cytotoxic antibiotic, poses substantial challenges for mass spectrometry-based analysis due to its extreme polarity, chelating properties, heterogeneity of fractions, and propensity to form multiple charged species during electrospray ionization. As one of the few effective drugs used in electrochemotherapy, the ability to quantify trace levels of bleomycin is critical for evaluating treatment efficacy, often requiring sensitivity beyond the capabilities of existing analytical methods. Such precise quantification would facilitate the evaluation of electrochemotherapy efficacy, such as comparing the in vitro effects of nanosecond electric pulses with conventional microsecond pulses. To address these challenges, we integrated cell viability assays with a robust chemical analytical approach. This approach employed solid-phase extraction for sample preparation, combined with HILIC-LC-MS/MS, achieving exceptional sensitivity with LLOQ of 0.075 μg/L and overcoming analyte and matrix complexity. Although a significant reduction in cell survival was confirmed when combining nanosecond pulses (25 × 400 ns) with bleomycin, chemical analysis revealed discrepancies, underscoring the complex interaction between electric pulse parameters and drug action. These findings highlight the need for further refinement of treatment protocols and the development of advanced analytical techniques.

1. Introduction

Bleomycin (BLM) represents a group of structurally related metallo-glycopeptide compounds with both antimicrobial and cytotoxic properties. Clinically used in chemotherapy for treating lymphomas as well as squamous and testicular carcinomas, its application has more recently been expanded to sclerotherapy, proving effective in the treatment of vascular malformations [1,2]. BLM's cytotoxic effect is attributed to its ability to cleave DNA, which is driven by both intracellularly generated activated BLM species and by free radicals formed upon their interaction with molecular oxygen [3]. Reaching the DNA inside the cell nucleus requires transition of BLM molecules through cell membrane, a process, severely hindered by its size and considerable hydrophilicity (logP: −7.5; computed by XLogP3 3.2.2) [4]. Achieving a sufficient concentration of the drug in the nucleus, which is critical for its cytotoxic effect, is therefore a challenge that cannot be tackled by increasing the administered dose as doing so would exacerbate the adverse side effects. Expressed due to BLM's lack of specificity towards tumor cells, these are already quite prevalent. Of particular concern is pulmonary toxicity with reported incidence rates as high as 46 % [5].

To address the challenge of membrane impermeability for intracellularly acting therapeutics, considerable advancements have been achieved using electroporation. This innovative procedure relies on facilitating mass transport across the plasma membrane by electroporation. Delivery of short electric pulses to cells, either in vitro or in vivo in tissue causes transient membrane permeabilization [6]. By appropriately adjusting the parameters, electroporation can be conducted in a reversible manner for applications such as electrochemotherapy or irreversibly for applications such as tissue ablation. Utilized in oncological treatments, they both induce cell death reducing tumor burden and are locally very efficient [7].

In electrochemotherapy (ECT), reversible electroporation acts as a physical delivery method for chemotherapeutic agents, exploiting the transient increase in membrane permeability to facilitate

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transmembrane transport of otherwise impermeant cytotoxic molecules [8]. Other than membrane permeability increase and intracellular drug accumulation, effects such as vascular disruption, vascular lock and immune response triggering have been observed to arise when applying electrical pulses and are considered to enhance the efficacy of electrochemotherapy [9].

The most common chemotherapeutic agents used in ECT, BLM and cisplatin [10–13], both show significantly potentiated cytotoxicity when combined with electroporation. This has been demonstrated in vitro as well as in vivo and led to the implementation of ECT into clinical practice in 2006, its use steeply rising since [14]. However, nerve stimulation, muscle contractions, and pain experienced in patients remains a significant limiting factor in an otherwise effective therapy. Exploring innovative therapeutic approaches to mitigate these side effects, conventionally employed ECT protocols have been modified by shortening the applied pulses significantly; from micro- to nanosecond range. Nanosecond pulsed electric field as a novel therapeutic strategy indeed promises to induce less contractions explained by the fact that electroporation by nanosecond pulses often occurs below the excitation threshold [15–17]. The effectiveness of these protocols, extensively evaluated using BLM and cisplatin, has confirmed their efficacy to be comparable to longer pulses [12,18,19]. However, Vižintin et al. demonstrated that higher concentrations of BLM were necessary to attain equivalent decreases in cell survival when employing nanosecond pulses, as opposed to the conventional 8 × 100 μs pulses [12]. They suggest this to be due to the smaller membrane pores formed and the resulting diminished cellular BLM uptake when applying nanosecond pulses.

To support the research of these new treatment regimens reliable quantitative analytical methods are of crucial importance. There are but few published methods for the determination of BLM, which is likely a result of the numerous challenges, accompanying its analysis. Two most recent LC-MS-based methods, published by our group, addressed determination of BLM in biological samples, including plasma, serum and tumor tissue [20,21] and present straightforward sample preparation protocols, suitable for simpler matrices, focusing on the applicability in clinical environment with sizeable sample batches. These methods provide a practical approach, emphasizing simplicity and time efficiency, which are critical for clinical settings. Lower limit of quantification (LLOQ) values for BLM in blood-derived samples reach 15 μg/L [20,21]. Studies such as electroporation-mediated transition of BLM into cells however require a different approach, mainly on the account of a different, more complex matrix as well as the extremely low analyte concentrations utilized in the experiments, which fall well below the LLOQ of the said methods.

The objective of this study was to investigate the effect of pulse duration and BLM concentration on cell survival after electrochemotherapy, using murine melanoma (B16F1) cell lines as the experimental model. The efficiency of applied conditions was assessed by both monitoring cell death rates along with the quantification of the intracellular BLM in B16F1 and Chinese hamster ovary (CHO) cells via LC-MS-based analysis. The developed sample preparation included solid-phase extraction-based purification with prior cell lysis to release BLM from the cells. The instrumental analytical method was developed on an ultra-high-performance liquid chromatograph coupled with a hybrid quadrupole-linear ion trap mass spectrometry analyzer QTRAP 7500.

2. Experimental

2.1. Chemicals and materials

Reference standard: Bleomycin sulfate salt (CAS No.: 9041–93–4, cat. no. sc-200134) was purchased in a metal-free form with a declared purity of 95.7 % (C55H84N17O21S3 × H2SO4) from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Bleomycin A5 hydrochloride (C57H89N19O21S2 × HCl, CAS: 55658–47–4, cat. no. B4517) used as an internal standard was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA) in a metal-free form and had a declared purity of ≥ 90 %. Agent for control of metal complex formation was CuSO4 × 5H2O, purchased from Alkaloid AD Skopje (Skopje, North Macedonia). Mobile phase additives ammonium formate (≥99.0 %) and formic acid (99 %) were purchased at Sigma-Aldrich Corp. (St. Louis, MO, USA) and Carlo Erba Reagents S.r.l. (Val de Reuil, France), respectively, both were of LC-MS purity grade. Buffers for cell lysis were prepared using the following buffering agents: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; cat. no. 1101100250), purchased from Merck & Co., Inc. (Rahway, NJ, USA), 3-(N-morpholino) propanesulfonic acid (MOPS), purchased at ≥ 99.5 % purity from Sigma-Aldrich Corp., and tromethamine (TRIS; cat. no. 5429.1) purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Sodium hydroxide for pH adjustment was purchased at Merck & Co., Inc. (Rahway, NJ, USA) and sodium dodecyl sulfate (SDS; cat. no. L3771) detergent from Sigma-Aldrich Corp. Acetonitrile (MeCN) and water used as the mobile phases were of LC-MS purity, and all solvents used in sample prep (methanol, water, MeCN) were of analytical grade purity.

The chromatographic column used was ACQUITY PRM BEH Amide (1.7 μm, 2.1 mm×50 mm) (Waters Corp., Milford, MA, USA). For solid-phase extraction, Oasis HLB 1cc (30 mg) extraction cartridges (Waters Corp., Milford, MA, USA) were used. Filtering of the samples was performed on GVS Centrex 0.45 μm cellulose acetate centrifuge filters (Centrex, Oilville, VA, USA) and on 0.2 μm PhenexTM regenerated cellulose membrane syringe filters (Phenomenex Inc., Torrance, CA, USA). The software used included Sciex OS 3.3.1 (Sciex, Framingham, MA, USA), ChemDraw 14.0 (Perkin-Elmer Cp., Norwalk, CT, USA), Microsoft Excel (Microsoft Corp., WA, USA), MATLAB R2020a (MathWorks, MA, USA) and Python programming language (v3.10.0), utilizing the Pingouin (v0.5.3) [22] and the StatsModels library (v0.14.0) [23].

For cell culture, CHO-K1 Chinese hamster ovary cell line, obtained directly from the European Collection of Authenticated Cell Cultures (ECACC, cat. no. 85051005, mycoplasma free), mouse skin melanoma cell line B16F1 (ECACC, cat. no. 92101203, mycoplasma free), Nutrient Mixture F-12 Ham (cat. no. N6658, Sigma-Aldrich), Dulbecco's Modified Eagle Medium (DMEM, cat. no. D5671, Sigma-Aldrich), fetal bovine serum (cat. no. F9665, Sigma-Aldrich), L-glutamine (cat. no. G7513, Sigma-Aldrich), penicillin/streptomycin (cat. no. P0781, Sigma-Aldrich), gentamycin (cat. no. G1397, Sigma-Aldrich), trypsin-EDTA (cat. no T4174, Sigma-Aldrich), Hank's basal salt solution (cat. no. H4641, Sigma-Aldrich) and bleomycin sulphate (Medac, Germany) were used.

2.2. Preparation of standard and working solutions

The lysis buffers HEPES (50 mM HEPES with 0.5 % SDS, pH 7.4), TRIS (50 mM Tris-HCl, 0.5 % SDS, pH 8) and MOPS (50 mM MOPS, 0.5 % SDS, pH 7.4) were freshly prepared prior to analyses by first diluting 1.1915 g of HEPES, 0.6055 g of TRIS or 1.046 g of MOPS in water, respectively, adjusting the pH to 7.4 with 1 M NaOH solution (prepared from NaOH pellets purchased at Merck & Co., Inc., cat. no. 1.06498.1000), adding 5 mL 10 % SDS solution and finally MilliQ water to 100 mL.

The stock solution of BLM was prepared by dissolving 10 mg of BLM sulfate reference standard in 10 mL of methanol and MilliQ water mixture (8:2) and was stored at –18 °C. The working solution at BLM concentrations 0.25, 2 and 20 μg/L were freshly prepared before the analysis by diluting the BLM stock solution with 0.1 % formic acid in MilliQ water. The internal standard (bleomycin A5) stock solution was prepared by dissolving 5 mg of bleomycin A5 in in 5 mL of methanol and MilliQ water mixture (8:2) and was stored at –18 °C. Each time before analysis, a fresh working standard was prepared by diluting the internal standard stock solution to 500 μg/L. This prepared standard was then

added to the samples, resulting in a final concentration of 10 μg/L.

Preparation of calibration and quality control standards:

Calibration standards were prepared as matrix-matched standards by spiking BLM working solutions (concentration range: 0.05 μg/L – 2.5 μg/L; 8 concentration points) into the cell matrix (blank CHO and B16F1 cell pellets in MeOH) prior to the addition of lysis buffer, IS at 10 μg/L and CuSO4 at 3.5 μg/L.

Quality control samples were prepared at low (0.075 μg/L) and high (7.5 μg/L) concentrations in two aliquots, by spiking BLM working solutions into the cell matrix in the same way as calibration standards. Both calibration and quality control standards were prepared like the actual samples (see 2.4 Sample preparation).

$CuSO_4$ was used to drive metal complex formation and was prepared by dissolving 1.22 mg of $CuSO_4$ 5H2O in 20 mL of MilliQ water and was further diluted to 3.05 μg/mL. This solution was then added in excess to the calibration standards, quality control and real samples.

The aqueous mobile phase for the LC-MS method (20 mM ammonium formate with 0.1 % formic acid) was prepared by dissolving 1.261 g of ammonium formate in 100 mL of MilliQ water, and then by 10-times further diluting it. Finally, 0.1 % formic acid was added before degassing.

2.3. Electroporation

Cell culture and measurement of cell survival after electroporation and electrochemotherapy were described previously [12,24]. Briefly, cell survival of B16F1 cells after ECT with BLM was measured by the clonogenic assay. Cells were exposed either to 0, 1, 20, 40 nM BLM and/or 8 × 100 μs electric pulses at 0.9 kV/cm, 1 Hz pulse repetition rate were delivered by a laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275–102N06A, IXYS, Milpitas, California, USA) or 0, 60, 100 and 140 nM BLM and/or 25 × 400 ns electric pulses at 3.9 kV/cm, 10 Hz repetition rate delivered by the CellFX System (Pulse Biosciences, Hayward, California, USA).

For determination of intracellular BLM concentration, CHO cells were treated with 140 nM BLM alone, 25 × 400 ns pulses (at 3.9 kV/cm, 10 Hz repetition rate) alone, the combination of 60, 100 or 140 nM BLM and 25 × 400 ns pulses or the combination of 5, 10 or 40 nM BLM and 8 × 100 μs pulses (at 1.1 kV/cm, 1 Hz pulse repetition rate), while B16F1 cells were treated with 140 nM BLM alone, 25 × 400 ns pulses (at 3.9 kV/cm, 10 Hz repetition rate) alone, the combination of 60, 100 or 140 nM BLM and 25 × 400 ns pulses or the combination of 1, 20 or 40 nM BLM and 8 × 100 μs pulses (at 0.9 kV/cm, 1 Hz pulse repetition rate) following the same protocol as in the previous electrochemotherapy experiments. After treatment, the cell suspension was transferred from the electroporation cuvette to a 1.5 mL centrifuge tube. 25 min after electroporation (or addition of BLM for non-electroporated controls), 530–570 μL of the cell suspension was diluted in 15 mL of complete growth medium in 15 mL centrifuge tubes purchased from TPP Techno Plastic Products AG (Trasadingen, Switzerland) and centrifuged for 5 min at 900 rcf at room temperature. The number of cells was in the range of approximately 1.5 – 3.5 × 106 cells; it was determined with Countess Automated Cell Counter (Invitrogen, Thermo Fisher Scientific) following manufacturer's instructions. The supernatant was discarded and the pellet was washed with 4 mL saline and then centrifuged again for 5 min at 900 rcf at room temperature. The supernatant was discarded and 100 μL of methanol purchased from Sigma-Aldrich Corp. was added to the pellet. The pellets were stored at −20 °C until sample analysis. The experiments were repeated 3 – 4 times per treatment.

Samples for calibration curve and blanks were prepared by putting the cell suspension of CHO or B16F1 in their respective growth medium in a 15 mL centrifuge tube so that the number of cells was in the same range as of the samples for measurements of intracellular BLM concentration. Then, they were processed the same way as the samples for measurements of intracellular BLM concentration.

Stringent measures were implemented to prevent contamination of samples not treated with BLM during the in vitro electrochemotherapy procedure. Fresh electroporation cuvettes, pipette tips, tubes, and other cell culture consumables were utilized for each individual sample.

2.4. Sample preparation

To the cell pellet in methanol, lysis buffer was added in two steps; each time 150 μL. Upon adding the buffer, the cell aggregate was broken up by aspirating it into a pipette. Samples were then sonicated for 15 minutes to aid the cell lysis. 20 μL of internal standard (IS) (500 μg/L) and 600 μL of $CuSO_4$ working solutions (3.05 μg/L) were added to each sample and mixed thoroughly. This was followed by centrifugation at 12900 × g for 15 minutes. Supernatants were then transferred into centrifuge filters (CENTREX, cellulose acetate membrane filters, 0.45 μm pores) and filtered by centrifugation at 1500 x g for 15 minutes. The subsequent solid-phase extraction was carried out on HLB cartridges (Oasis HLB, 30 mg, 1cc), using the following procedure: sorbent was first conditioned with 1 mL of methanol and 0.1 % formic acid in MilliQ water, respectively. The samples were transferred onto the preconditioned cartridges to pass the sorbent under vacuum, which was then dried under vacuum for five minutes. Elution was performed with 2 × 0.5 mL MeCN and MQ water mixture (7:3). Eluates were filtered (regenerated cellulose membrane, 0.2 μm pores) and transferred into vials. Measures have been taken during all sample preparation steps to avoid direct light exposure as BLM has been shown to be light-sensitive [25].

2.5. Instrumental analysis

Instrumental analysis was conducted using an ultra-high-performance liquid chromatograph (UHPLC, Waters, Milford, MA, USA) coupled with a hybrid quadrupole-linear ion trap mass spectrometry analyzer SCIEX 7500 system (Sciex, Framingham, MA, USA) employing positive electrospray ionization (ESI+). Separation was achieved with an ACQUITY PRM BEH Amide (1.7 μm, 2.1 ×50 mm) column. The chromatographic approach was adapted from our group's previously published method, with the only modification being the concentration of the mobile phase modifier. Mobile phase A consisted of MeCN, while mobile phase B comprised 20 mM ammonium formate with 0.1 % formic acid. The gradient program initiated at 5 % B, increased to 50 % at 2 min, further elevated to 60 % at 4 min, and then reverted to 5 %, maintaining this composition until 6 min. The total mobile phase flow rate was 0.3 mL/min, and the column temperature was held at 40 °C. An injection volume of 1.0 μL was used.

The ion source parameters were maintained as follows: ion spray voltage + 2500 V; source temperature 350 °C; Collision Gas (CAD) 15 psi, curtain gas (CUR) 40 psi; ion source gas 1 (GS1) 40 psi; ion source gas 2 (GS2) 50 psi. Ions were acquired in multiple reaction monitoring (MRM) mode; the transitions are together with the compound-specific parameters presented in Table 1. Molecular weight of the BLM-A2-Cu complex being 1475.4, the monitored transitions were from double charged parent ions; two isotopic ions of the analyte BLM-A2-Cu (BLM-

Table 1
MRM parameters for the LC-MS/MS method.

	Q1 *m/z* (Da)	Q3 *m/z* (Da)	Dwell time (ms)	EP (V)	CE (V)	CXP (V)
BLM-A2-Cu	738.4	707.3	75.0	10.0	24.0	14.0
BLM-A2-Cu	739.4	708.2	75.0	10.0	23.0	4.0
BLM-A5-Cu	751.2	706.2	75.0	10.0	40.0	21.0
BLM-A5-Cu	751.2	714.7	75.0	10.0	34.0	24.0

A2-^{63}Cu and BLM-A2-^{65}Cu) and two for the internal standard BLM-A5-Cu.

2.6. Validation protocols

In adherence to the European Medicines Agency (EMA) guidelines [26], we assessed the performance parameters of the method. Selectivity was evaluated by examining blanks (matrix spiked with the internal standard but lacking the analyte), required to show analyte peak areas $\leq$ 20 % of the mean peak area at the LLOQ level. Carryover, defined as the residual signal in solvent blanks obtained immediately after the highest calibrator (2.5 μg/L), needed to be less than 20 % of the peak area in the LLOQ for the analyte and less than 5 % of the mean peak area of the internal standard. Linearity was assessed using calibration curves prepared from matrix-matched calibration standards that consisted of seven points within the range of 0.075 μg/L – 2.5 μg/L in the final extract, which corresponds to 0.075 ng – 2.5 ng of the analyte per one cell pellet. The relationship between the peak area ratio and BLM concentration was determined through linear regression, taking into account that EMA criteria require at least 75 % of the calibrators to fall within ± 15 % (or 20 % for the LLOQ) of their expected concentrations. The determined LLOQ concentration was examined by analyzing multiple (n = 3) replicates of the estimated LLOQ concentration with the calibration curve. The average of these replicates was expected to fall within ± 20 % of the nominal concentration for a minimum of 50 % of the replicates. QC standards were prepared along with each batch of samples in duplicates at 0.075 μg/L to determine accuracy of the method, defined by trueness and precision. Precision was described by assessing injection (n = 3) and method repeatability (n = 3). Extraction efficiency (excluding the lysis efficiency) was evaluated by comparing the analyte peak areas of pre-spiked (spiked with the analyte before the sample preparation process) against the post-spiked (spiked prior to the instrumental analysis) calibration standards, without IS normalization.

2.7. Statistical analysis

Cell survival of B16F1 cells after electrochemotherapy measured by the clonogenic assay was compared to the untreated control with Welch's *t*-test ($\alpha = 0.05$). Non-detects in the measured BLM concentrations were replaced by 0, then the measured BLM concentrations were normalized by dividing them by the number of cells in the sample. Subsequently, samples below the method detection limit and outliers identified by the interquartile range method were excluded. A significance level of $p < 0.05$ was set. The Shapiro-Wilk test assessed normal distribution, with only the CHO cells electroporated with nanosecond pulses showing a violation of this assumption. For these samples, log-transformed normalized BLM concentrations, which demonstrated a normal distribution, were utilized. The Brown-Forsythe test examined the assumption of equal variances for the one-way analysis of variance (ANOVA) and independent *t*-test used to compare differences between the mean measured BLM concentrations among sample groups. In cases of violation, Welch's ANOVA or Welch's *t*-test was employed. Upon detecting a statistically significant difference with the (Welch's) ANOVA, Tukey post hoc test was used to determine which specific groups exhibited significant differences.

3. Results and discussion

3.1. Cell survival

As expected, cell survival of B16F1 was mostly unaffected by the electric pulses or BLM alone, while their combination drastically reduced cell survival (Fig. 1). In accordance with the observations by Vižintin et al. [12] on CHO cells, the decrease in cell survival of B16F1 following electrochemotherapy (i.e., the concurrent application of electric pulses and BLM) was notably more significant with the standard

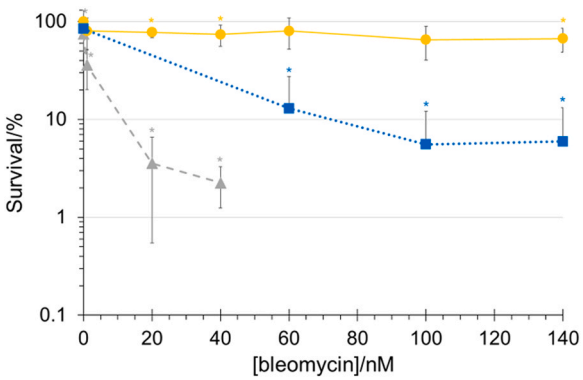

Fig. 1. Cell survival of B16F1 cells measured by the clonogenic assay 7 days after treatment with different bleomycin (BLM) concentrations and/or electroporation. Cells were exposed to either 8 × 100 μs pulses (grey triangles), 25 × 400 ns pulses (blue squares) or no pulses (yellow circles). Asterisks (*) represent a statistically significant difference ($p < 0.05$) in cell survival compared to control cells that were not exposed to either BLM or electric pulses. Note that the y-axis is on a logarithmic scale.

8 × 100 μs pulses than with the 25 × 400 ns pulses. Moreover, cell survival was more reduced when cells were subjected to 8 × 100 μs pulses at lower BLM concentrations compared to treatment with 25 × 400 ns pulses at higher BLM concentrations. However, also the BLM concentrations at which reduction of cell survival was achieved with ns pulses are significantly lower than the therapeutic doses used in clinical practice and from the measured BLM amounts in clinical samples after ECT [20,27]. In contrast, when cisplatin was employed as the chemotherapeutic agent in in vitro electrochemotherapy, both the standard 8 × 100 μs pulses and the 25 × 400 ns pulses demonstrated equal efficacy in reducing cell survival for both CHO and B16F1 cells. Additionally, they both led to a comparable increase in cellular cisplatin uptake [12,24]. The observed discrepancy in the effectiveness of electrochemotherapy with nanosecond pulses when using these two drugs may stem from differences in molecular size. BLM (approximately 1500 Da) is larger than cisplatin (approximately 300 Da), and it is suggested that the pores created by nanosecond pulses are smaller compared to those produced by longer electroporation pulses [28–30]. Therefore, at the same extracellular BLM concentration, more BLM molecules might have penetrated cells through the pores generated by the 8 × 100 μs pulses than through nanopulse-induced pores, as the micro-second pulses might have created more pores of sufficient size to allow BLM entry.

3.2. Chemical analysis of BLM

3.2.1. Cell lysis: procedure development and modifications

Cell lysis constitutes a pivotal step in the sample preparation procedure aimed at quantifying intracellular BLM to evaluate electroporation efficiency. The lysis procedure involved chemical disruption of membranes through the addition of a pH-adjusted solution of a detergent in a buffer, followed by sonication. A buffer was only considered suitable if it supported effective lysis and did not negatively affect the subsequent analysis. Evaluation of the efficacy of three commonly utilized buffers (TRIS, HEPES, and MOPS) was conducted through a two-part experimental approach, aiming to measure the efficiency of cell lysis but also to investigate any potential effects or interferences these buffers might have on the analyte. Blank CHO cell pellets in methanol were spiked with BLM (5 μg/L) and were prepared in triplicates using each of the buffers (pH-adjusted, with the addition of SDS) with the same protocol from this point onwards. While sufficient cell lysis (evaluated by observation under an inverted microscope) was demonstrated regardless of the buffer used, some notable differences were observed in the analyte recovery rates. Results, outlined in Fig. 2 show

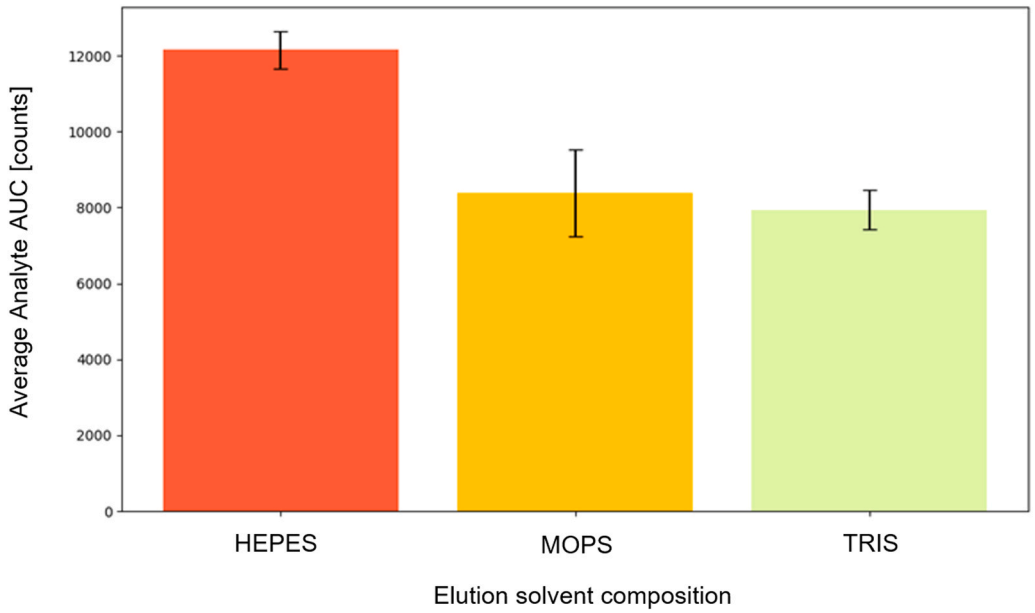

Fig. 2. Abundance of the analyte at 5 μg/L using different lysis buffers (n = 3).

that the recoveries for samples treated with TRIS exhibit lower recoveries compared to the rest, as indicated by lower analyte peak areas. This was interpreted to likely happen due to TRIS' metal-complexing properties [31]. The formation of BLM-Cu complex (the analyte) is in this case constrained due to limited availability of Cu ions in the presence of TRIS, causing an apparent reduction of the analyte inevitably leading to lower signals. HEPES and MOPS, both exhibiting weaker complexing properties [31], proved more suitable, with HEPES showcasing superior peak shapes as well as higher signals, solidifying its selection as the optimal choice for the method.

In the final protocol, the cell aggregate is disrupted by repeated pipette aspiration after introducing the buffer. An additional practical modification of the protocol included adding the lysis buffer in two steps (2 × 150 μL) rather than the entire volume at once, to enhance the efficiency of the cell pellet break-up and subsequently the lysis.

3.2.2. *Solid-phase extraction*

Sample clean-up was performed using solid-phase extraction, with the final method being an adaptation of a previously developed in-house protocol aimed at quantifying BLM in tissue and blood-derived matrices [20,21]. This adaptation was driven by the need for substantial reduction of LLOQ to make it suitable for trace level analysis of BLM in electroporated cell samples. In pursuit of optimizing the protocol, efforts were focused on enhancing the efficiency of sample preparation. This involved evaluating the impact of various parameters, including the mass of HLB sorbent per cartridge (30 mg and 60 mg), the choice of elution solvents, and the possible post-extraction drying and reconstitution.

The initial method employed 30 mg of HLB sorbent per sample. A comparative study was performed to determine if using a larger amount of sorbent (60 mg per cartridge) could improve recovery rates, given the extremely weak interactions between BLM and HLB sorbent which can be disrupted already by water itself, where the increase of the sorbent mass theoretically counteracts for these losses during extraction [32]. For this purpose, blank CHO cell pellets were spiked with 5 ng of BLM per sample and processed in triplicate using each cartridge type, adjusting solvent volumes as necessary. A statistical evaluation employing a *t*-test to compare the analyte response areas derived from each sample revealed no statistically significant difference in recovery rates between the two sorbent amounts. This indicates that an increase in the sorbent mass does not confer a performance advantage and was therefore not adopted.

Suitability of various elution solvents was assessed with respect to extraction recovery as well as the similarity to the initial LC mobile phase composition, finding that MeCN:water (7:3) was the optimum one (Fig. 3). Building upon prior research [20,27] that established a solid-phase extraction protocol using HLB 96-well plates for blood-derived samples, this study navigates a more complex matrix and lower analyte concentrations, which is the reason for further solvent optimization. Elution solvent selection was guided by stringent criteria, considering that complete drying and reconstitution post-SPE, driven by analyte losses due to vial surface adsorption, should be avoided. This selection was corroborated by an extraction efficiency of 52 % at 2.5 μg/L and 73 % at 7.5 μg/L, determined through a comparison between the peak areas of the analytes before and after extraction. Despite extensive optimization efforts, it is important to acknowledge that the improvements achievable through modifications to the solvent composition are inherently limited and though the extraction efficiency remains suboptimal, it represents the best-case scenario achievable by modifying the elution solvents.

3.2.3. *Instrumental analysis*

The optimization of chromatographic conditions was conducted through experiments, focusing on the evaluation of mobile phase composition. The combination exhibiting optimal peak shapes and responses involved MeCN as the organic and ammonium formate as the aqueous mobile phase. Notably, detector responses were maximized at the salt concentration of 20 mM, exhibiting a 20 % improvement over the responses observed at 10 mM. Conversely, the introduction of ammonium formate to the MeCN mobile phase was observed to negatively impact peak shape and response.

The SCIEX 7500 mass spectrometer was employed aiming to reach the sensitivity required for determining extremely low concentrations in complex matrices. Detection was carried out in Multiple Reaction Monitoring (MRM) mode with MS operating parameters (electrospray voltage, source temperature, gases, collision energies and exit potentials for individual transitions, etc., see Table 1) automatically optimized. We selected the transitions for BLM-A2-Cu (analyte) based on the understanding that our analyte tends to form chelate with copper, and that copper has two stable isotopes (^{63}Cu and ^{65}Cu), which are clearly distinguishable in mass spectrum. Consequently, two distinct complexes with BLM-A2 are identified, i.e. BLM-A2-^{63}Cu and BLM-A2-^{65}Cu. M^{2+}

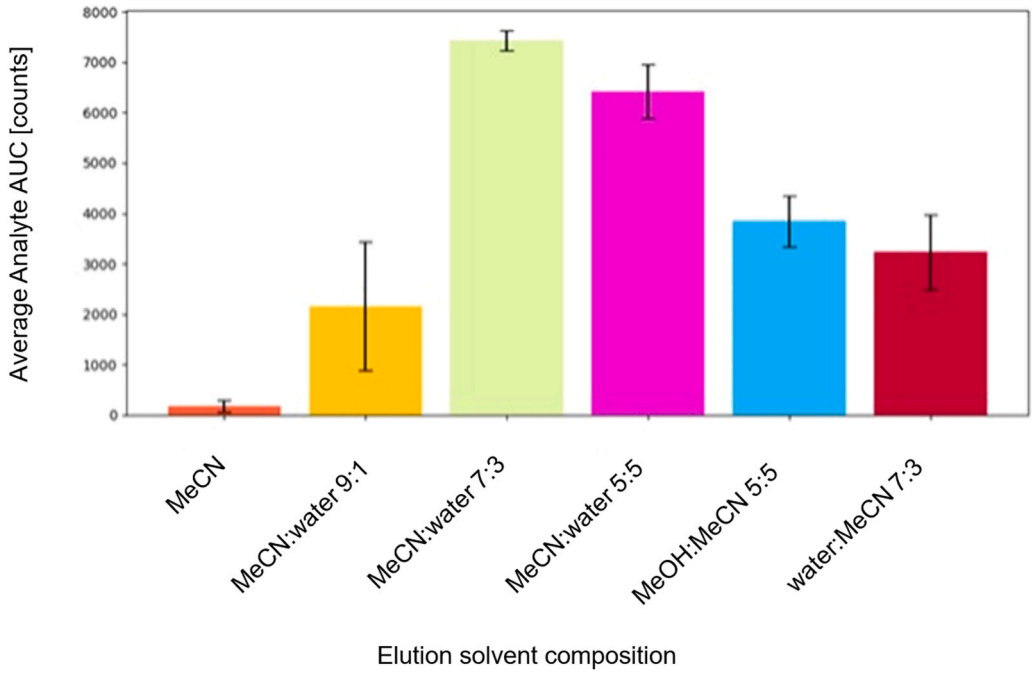

Fig. 3. Comparison of the analyte signal after modifying elution solvent composition (c(BLM) = 5 μg/L; n = 2).

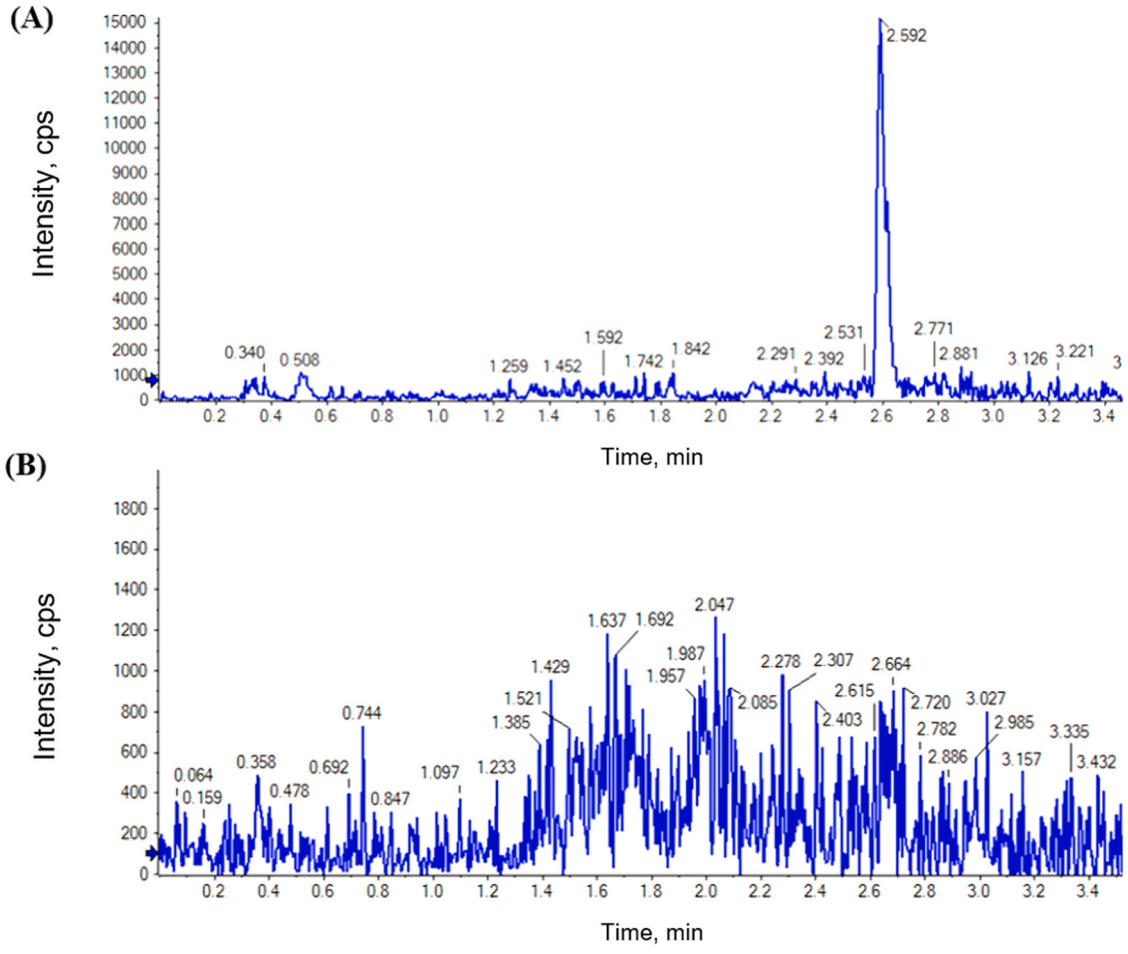

Fig. 4. Extracted ion chromatogram of BLM-A2-Cu at LLOQ concentration (A) and the chromatogram of the same species in a blank matrix (B).

ions of these isotopes correspond to *m/z* 738.7 and *m/z* 739.7, respectively. The ratio between ^{63}Cu and ^{65}Cu is 69.17–30.83 %, which leaves the less abundant *m/z* corresponding to BLM-A2-^{65}Cu complex as a confirming transition, increasing its analytical reliability.

3.2.4. Method validation

The method's performance was evaluated through partial validation procedures aligned with EMA guidelines [26] to ensure the reliability of the obtained results. This validation encompassed an assessment of selectivity, linearity, lower limit of quantification (LLOQ), and accuracy.

Selectivity was ascertained via the two transitions of BLM-A2-^{63}Cu and BLM-A2-^{65}Cu species at the chromatographic retention time of 2.6 min, along with the check-up of a blank matrix extract, which displayed noise intensities significantly lower than 20 % of those for the lowest calibrator (0.05 μg/L). Fig. 4 (top) illustrates the LLOQ signal for BLM-A2-^{63}Cu and the absence of the signal in a blank cell matrix (bottom).

Linearity was investigated across the concentration range of 0.075 μg/L – 2.5 μg/L for both CHO and B16F1 tumor cells to evaluate the method's ability to produce results, directly proportional to the analyte concentration, while also examining matrix effects. The calibration curve for CHO cell matrix indicated satisfactory linearity, with a correlation coefficient R of 0.994. Conversely, the calibration with B16F1 cells exhibited a dispersed profile with a lower R of 0.907, suggesting notable variances that could be attributed to the intrinsic differences in cell pellet structure, constraining the sample preparation. Despite these variances, the matrix effect was found to be consistent in both cell types, as demonstrated by the near-identical linear regression formulae, justifying the use of the CHO cell-derived calibration curve for subsequent sample measurements to maintain analytical consistency. To accommodate samples with concentrations below the LLOQ of the initially prepared calibration curve, an additional curve spanning from 0.005 μg/L to 2.5 μg/L was established post-extraction. This curve demonstrated a commendable linear response with an R of 0.993, extending the (instrumental) method's applicability to lower concentrations.

Accuracy was assessed using calibration samples prepared from CHO cells. The guidelines permit a maximum deviation of 15 % from the nominal value for more than 75 % of the calibration standards above the LLOQ. In our study, we achieved this criterion for 60 % of the calibrators, indicating a performance that, while not fully aligning with the standards, we determined as acceptable considering the constraints encountered. The LLOQ was determined to be 0.075 μg/L, a level at which the EMA guidelines [26] allow for a permissible error margin of up to 20 %. QC samples were prepared at this concentration in duplicate for each batch. They were used to evaluate the method's repeatability (values up to 25 % RSD) and accuracy with 43 % samples at LLOQ concentration falling within ± 20 % of the nominal concentration (while the required limit is 50 %). Additionally, a concentration of 7.5 μg/L was tested, despite being above the highest calibrator used in the calibration curve, to provide supplementary data on method performance. The method validation results are summarized in Table 2.

It is acknowledged that these results, though not fully meeting the EMA guidelines [26] criteria, represent the optimum achievable performance under the existing methodological conditions. This deviation from the guidelines, albeit suboptimal, does not substantially compromise the method's overall utility and reliability. Our findings underscore the method's consistent performance and its potential applicability in the field, highlighting the balance struck between methodological rigor and the practical limitations inherent in such analytical endeavors.

Table 2
Analytical method validation results.

	N = 3 at 0.075 μg/L BLM (LLOQ)	N = 3 at 2.5 μg/L BLM (highest cal. measured with samples)	N = 3 at 7.5 μg/L BLM
Extraction efficiency (%)	117	52	73
Method repeatability (% RSD)	24	5.0	9.0
Inj. repeatability (% RSD)	19	3.8	4.0
Accuracy (all data) in %	88.9, 137, 95.9	80.4, 76.3, 72.8	112, 112, 95.4

3.3. Results of EP cell samples analyses

In the absence of electric pulses, BLM exhibits limited permeability across the cell membrane, primarily entering cells via receptor-mediated endocytosis mechanisms [33]. However, the cytotoxicity of BLM undergoes a potent increase of several hundred to thousandfold with electrochemotherapy [9]. Consequently, we anticipated that non-electroporated cells would exhibit minimal BLM uptake. Therefore, we measured intracellular BLM concentrations in non-electroporated cells solely at the highest concentration utilized in this study (i.e., 140 nM).

Contrary to our expectations, (Welch's) t-tests revealed no significant differences in BLM uptake between CHO cells or B16F1 cells exposed to 140 nM BLM with or without electroporation using 25 × 400 ns pulses (Fig. 5 (B) and Fig. 6 (B)). While ANOVA indicated a statistically significant difference only in B16F1 cells electroporated with 25 × 400 ns pulses between those not exposed to BLM and those incubated with 140 nM of BLM, a trend can be observed that a higher BLM concentration was measured for cells that were incubated with higher BLM concentrations, regardless of the electroporation protocol (Fig. 5 (A) and Fig. 6 (A)). These observations may be attributed to the detection of residual BLM from the treatment, which was not internalized by the cells, thereby potentially masking the actual signal originating from internalized BLM.

The detection of signals in the non-spiked samples is not entirely unexpected in a highly sensitive method. Such low concentrations, as they fall below the determined LLOQ, would normally be disregarded. However, given the extremely low concentrations observed in many samples, it was deemed reasonable to also report these sub-LLOQ values to avoid excluding a substantial portion of the data, which explains the traces of BLM, detected (and reported) in non-spiked cell samples.

4. Conclusions

The primary objective of this study was developing an LC-MS-based analytical method for quantifying bleomycin at ultra-trace levels in electroporated cells. This effort was driven by the need to support findings from cell survival assays, which suggest that nanosecond pulses can permeabilize cell membranes similarly to traditional microsecond pulses, albeit at higher concentrations. The optimization and application of this analytical method are shown in the study.

We demonstrated the capability of state-of-art analytical equipment to determine trace levels of highly complex analytes. However, the limitation is still the significant divergency between the capacity of state-of-art equipment, i.e. what can be measured, and on the other edge, the trace level concentration that can kill the cell, which is a few hundred BLM molecules per cell (translating to 1×10^{-6} ng per 1000 cells). Despite advancements, this low level is still unachievable and demonstrates the need for further improving the equipment and analytical methods sensitivity. One important constraint is also the losses during sample preparation and an immense impact of matrix on electrospray ionization, which requires further innovations in analytical practices, especially when it comes to such demanding and thus important analytical challenges. Another solely analytical and interesting finding was the choice of lysis buffer to enable the analysis of intracellular chelating agents.

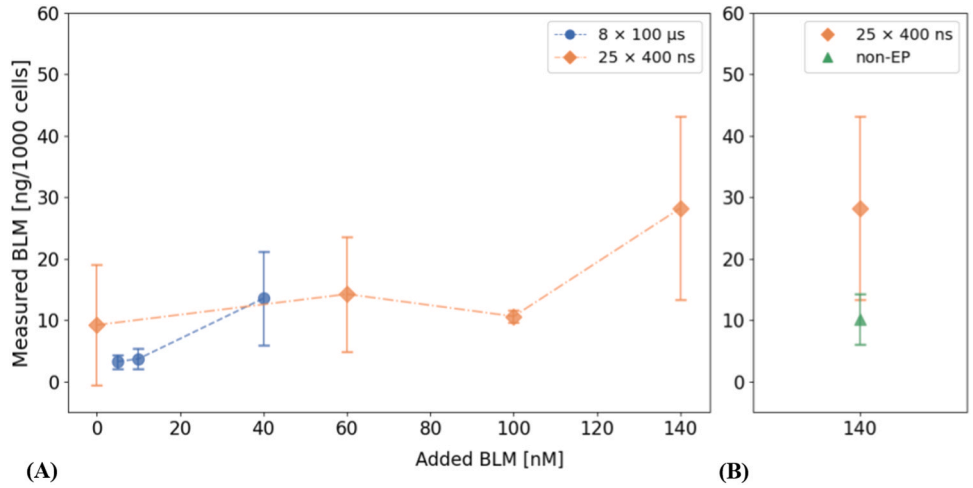

Fig. 5. The arithmetic mean and standard deviation of bleomycin (BLM) concentration normalized by the number of cells in the samples plotted against the initial BLM concentration applied to CHO cells prior to electric pulse delivery. CHO cells were exposed to 8 × 100 µs pulses (blue circles) or 25 × 400 ns pulses (orange diamonds) (A). The measured BLM concentration in cells exposed to 140 nM BLM, comparing results between cells electroporated with 25 × 400 ns pulses (orange diamond) and non-electroporated CHO cells (green triangle) is shown on (B).

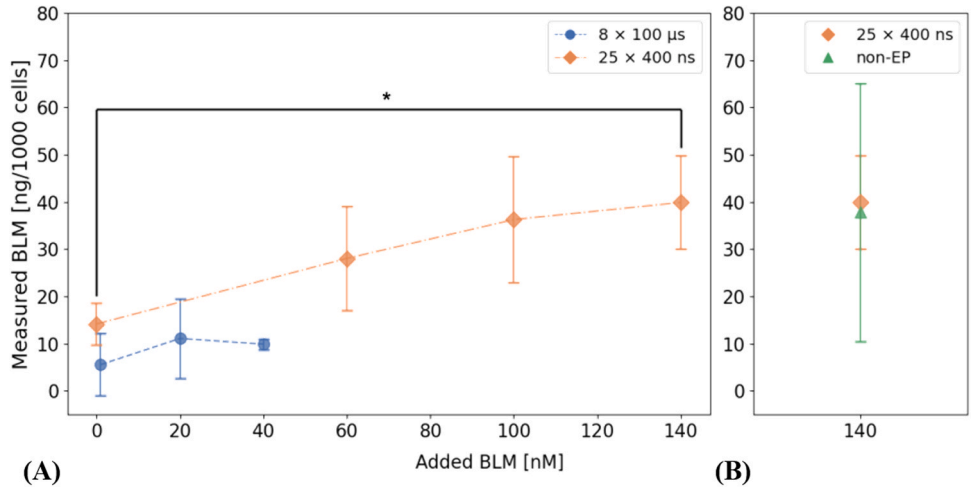

Fig. 6. The arithmetic mean and standard deviation of bleomycin (BLM) concentration normalized by the number of cells in the samples plotted against the initial BLM concentration applied to B16F1 cells prior to electric pulse delivery. B16F1 cells were exposed to 8 × 100 µs pulses (blue circles) or 25 × 400 ns pulses (orange diamonds) (A). The measured BLM concentration in B16F1 cells exposed to 140 nM BLM, comparing results between cells electroporated with 25 × 400 ns pulses (orange diamond) and non-electroporated cells (green triangle) is shown on (B). Asterisk (*) represents a statistically significant difference ($p < 0.05$) in the measured BLM concentration.

We found contradictory results for nonpermeabilized B16F1 cells showing highest concentration of BLM, however this is not in line with the finding for CHO cells that in nonpermeabilized form exhibited lowest concentration, which may be explained by externally deposited BLM that was insufficiently washed off during the pellet washing. Previous studies have demonstrated that BLM binds to and interacts with cell membranes [34,35] suggesting that this washing step might be more critical than previously thought. However, further intensification of the washing procedure could potentially lead to cell lysis and compromise subsequent analyses. Therefore, our approach maintains a careful balance between efficient washing and preserving cell integrity.

Further research should explore the mechanistic differences in membrane permeabilization between pulse durations and investigate the potential for combining nanosecond pulses with other therapeutic agents to enhance clinical outcomes. The utility of our developed analytical method, however, reaching extremely low LLOQs compared to existing methods, could be extended to various targeted delivery studies beyond electroporation, including alternative delivery methods like nanoparticles and liposomes, and can guide other chemotherapeutic quantifications, offering valuable insights for pharmacokinetic and cellular uptake studies. This versatility underscores the method's broader utility in advancing targeted cancer therapy research.

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CRediT authorship contribution statement

Vižintin Angelika: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Plešnik Helena:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Miklavčič Damijan:** Writing – review & editing, Supervision, Funding acquisition. **Stahl-Zeng Jianru:** Writing –

review & editing. **Steed Jack:** Supervision, Investigation. **Kosjek Tina:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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.

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