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Short communication

Development and validation of a liquid chromatography-tandem mass spectrometry assay for the quantification of lurbinectedin in human plasma and urine

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ABSTRACT

Lurbinectedin is a novel highly selective inhibitor of RNA polymerase II triggering caspase-dependent apoptosis of cancerous cells. This article describes the development and validation of a liquid chromatography-tandem mass spectrometry (LC–MS/MS) assay to quantify lurbinectedin in human plasma and urine. Plasma samples were pre-treated with 1 M aqueous ammonia after which they were brought onto supported liquid extraction (SLE) columns. Lurbinectedin was eluted from the columns using *tert*-butyl methyl ether (TBME). Urine was first diluted in plasma and lurbinectedin was extracted from this matrix by liquid-liquid extraction using TBME. Samples were measured by LC–MS/MS in the positive electron ion spray mode. The method was linear over 0.1–100 ng/mL and 1–1000 ng/mL in plasma and urine, respectively, with accuracies and precisions within ±15% (20% for LLOQ) and below 15% (20% for LLOQ), respectively. The method was developed to support a mass balance study in which patients received a dose of 5 mg lurbinectedin.

1. Introduction

Lurbinectedin (PM01183, Zepsyre®), a new synthetic tetrahydroiso-quinoline alkaloid, is currently under investigation in a pivotal phase III study in patients with small-cell lung cancer (NCT02566993). It exerts its antitumor activity by inhibition of RNA polymerase II triggering caspase-dependent apoptosis [1,2].

To enable support in pharmacokinetic analysis of lurbinectedin in cancer patients, validated bioanalytical assays are vital. One such method has been described which allows for analysis of lurbinectedin in non-human plasma [3]. This method has now been adapted and made suitable for human samples. It is the first reported method to quantify lurbinectedin in human matrices addressing the analyte's tendency to adsorb to container walls when present in aqueous matrices. The reliable quantification of lurbinectedin in human plasma and urine was demonstrated by applying the method to samples collected in a mass balance clinical trial. Method validation was performed according to the latest guidelines on bioanalytical method development as described by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA) [4,5]. The analysis was performed according to Good Clinical Practice (GCP) and Good Laboratory Practice (GLP) regulations [6,7].

Abbreviations: CV, coefficient of variation; CS, calibration standard; DMSO, dimethylsulfoxide; EMA, European Medicine Agency; FDA, Food and Drug Administration; GCP, Good Clinical Practice; GLP, Good Laboratory Practice; IS, internal standard; K_3EDTA, tripotassium ethylenediaminetetraacetic acid; LC–MS/MS, liquid chromatography – tandem mass spectrometry; LLE, liquid liquid extraction; LLOQ, lower limit of quantification; MF, matrix factor; MRM, multiple reaction monitoring; QC, quality control; rpm, rotations per minute; SLE, supported liquid extraction; ULOQ, upper limit of quantification; TBME, tert-butyl methyl ether.

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2. Material and methods

2.1. Chemicals

Reference standards of lurbinectedin ($C_{41}H_{44}N_4O_{10}S$) and its stable isotopically labelled internal standard PM040038 ($C_{41}H_{40}D_4N_4O_{10}S$; IS) were kindly provided by Pharma Mar, S.A (Colmenar Viejo, Madrid, Spain). Water, acetonitrile and formic acid (>98%) were purchased from Biosolve (Valkenswaard, The Netherlands). *Tert*-butyl methyl ether (TBME), dimethylsulfoxide (DMSO) and ammonia 25% were obtained from Merck (Darmstadt, Germany). K_3EDTA was purchased from Bioreclamation (Hicksville, New York, United States) and urine was obtained from healthy volunteers.

2.2. Stock solutions, calibration standards and quality control samples

Stock solutions and working solutions (WS) of lurbinectedin and PM040038 were prepared as follows: 1 mg of lurbinectedin was dissolved in 1 mL of DMSO in duplicate. PM040038 was provided in glass vials containing approximately 1 mg, hence 1 mL of DMSO was added directly into the vial. Stock solutions were divided into 100 μL aliquots and stored at −70 °C prior to use. From the two lurbinectedin stock solutions, two separate sets of WS were prepared freshly for each validation run by diluting the stock solutions with 0.1% formic acid in acetonitrile-water (30:70, v/v). A PM040038 WS of 20 ng/mL was also made by diluting the stock solution with 0.1% formic acid in acetonitrile-water (30:70, v/v). Plasma calibration standards were prepared by diluting the WS 20 times in control human K_3EDTA plasma, obtaining concentrations of 0.1, 0.2, 0.5, 1, 10, 25, 80 and 100 ng/mL. Quality control (QC) samples were prepared by spiking the other set of WS (obtained from a separate stock solution) to K_3EDTA plasma, yielding concentrations of 0.1, 0.3, 3 and 75 ng/mL (lower limit of quantification [LLOQ], QC Low, QC Mid and QC High concentrations, respectively).

CS and QC samples for the urine assay were produced by spiking WS to plasma-urine (90:10 v/v), yielding concentrations in the plasma concentration range. The theoretical urine concentrations were therefore 10-fold higher than the actual concentration in the plasma-urine matrix (1–1000 ng/mL).

2.3. Sample preparation

2.3.1. Plasma

Lurbinectedin was extracted from plasma by supported liquid extraction (SLE) using TBME as described previously [3]. An Isolute SLE 96 well plate and a positive pressure manifold were not available, so instead Isolute SLE+ 400 μL columns (Biotage, Charlotte, NC, USA) were used and TBME was eluted under gravity only. First, 25 μL of IS (PM040038) working solution was added to 100 μL sample, except for double blanks. Subsequently, 200 μL of 1 M aqueous ammonia was added to all samples before being transferred to Isolute+ SLE columns. Samples were loaded on the column under a slight vacuum. TBME (750 μL) was used twice to elute the analytes from the column, after which the samples were evaporated to dryness under a gentle nitrogen flow (25 °C). Samples were redissolved in 100 μL 0.1% formic acid in acetonitrile-water (30:70, v/v). Finally, 10 μL of each sample was loaded onto an analytical ACE 3 C18 column (Achrom, Zulte, Belgium).

2.3.2. Urine

Control human K_3EDTA plasma (90 μL) and 25 μL of IS (PM040038) working solution were added to 10 μL urine. Lurbinectedin was extracted from the sample by liquid-liquid extraction using TBME. The supernatant was transferred to a clean tube and was subsequently dried under a nitrogen flow (25 °C). Samples were reconstituted as described above.

Table 1
Mass spectrometry settings.

Parameter	Value
Run time (min)	5
Scan type	MRM
Polarity	Positive
Ion source	Turbo spray
Gas 1 (au) nebulizer gas	30
Gas 2 (au) turbo gas	40
Curtain gas (au)	30
Collision gas (au)	9
Ion spray voltage (V)	4000
Temperature (°C)	650 °C
Dwell time (msec)	200
Declustering potential	181
Collision energy	51
Collision cell exit potential	32
Entrance potential	10

2.4. Instrumentation and operating conditions

2.4.1. Liquid chromatography

An Acquity Waters (Milford, MA, USA) HPLC was used, equipped with an ACE C18 column (2.1 × 30 mm, 3 μm) (Achrom). The autosampler was thermostated at 8 °C and the column oven was raised to 50 °C. The flow was 600 μL/min and gradient elution was applied using 0.1% formic acid in water-acetonitrile (90:10, v/v; mobile phase A) and 0.1% formic acid in water-acetonitrile (10:90, v/v; mobile phase B). The following gradient was applied: from 0 to 100% B in 2.5 min, 100% B for 1 min, back to initial conditions in 0.1 min and re-conditioning the column from 3.6 to 5 min.

2.4.2. Mass spectrometry

A Sciex (Framingham, MA, USA) API5500 triple quadrupole mass spectrometer equipped with a turbo ion spray (TIS) operated in the positive ion mode was used. The transition m/z 767 → 273 was observed for lurbinectedin and m/z 771 → 277 for the internal standard. The m/z value of 767, which corresponds to a loss of water from the parent compound, was more pronounced than the $[M+H]^+$ of m/z 785. The product ion reflects the fragment with the highest intensity [3]. Detailed mass spectrometry settings are displayed in Table 1. Analyst software version 1.6.2. (Sciex) was used for data acquisition.

2.5. Validation procedures

A complete validation of the bioanalytical assay for plasma and urine was performed according to the regulatory guidelines [4,5], and included calibration curve, accuracy and precision, carry-over, selectivity, dilution integrity, matrix effect, recovery and stability.

3. Results and discussion

3.1. Method development

The challenge during method development was to anticipate the needs for the upcoming clinical trials. Therefore, it was necessary to understand the practicalities in the clinic (e.g. sampling, sample handling and storage) before starting the validation procedures. Initially, the intention was to follow the bioanalytical method for animal samples as published by Pernice et al. [3] Besides the low sample pre-treatment recovery no relevant problems were encountered. However, the method had to be adjusted considerably for the urine matrix. First of all, lurbinectedin, a hydrophobic com-

pound, tends to adhere to the polypropylene surfaces of centrifuge tubes. This problem was solved by using glass tubes rather than plastic ones for preparation and storage of urine samples. Secondly, despite the concentrations being 10-fold higher than the plasma concentrations (1–1000 ng/mL versus 0.1–100 ng/mL), the signal, expressed in cps, was 10-fold lower than that of the plasma samples. It was hypothesised that this was due to ion suppression effects in the MS source, an effect which was not observed during analysis of plasma samples. Therefore, the plasma-urine (90:10, v/v) matrix was prepared, by mixing 18 mL of blank human K_3EDTA plasma with 2 mL blank human urine. The ion suppression of lurbinectedin in urine was indeed minimised by dilution in plasma. Very low concentrations of lurbinectedin were expected to be excreted from the human body via the urine. Therefore, in order to ensure that even small amounts of lurbinectedin could accurately be quantified by LC–MS/MS, a different sample pre-treatment method was developed to increase the recovery from the matrix. Ultimately, LLE using TBME was found to yield high recoveries (>90%), which was then chosen as the pre-treatment method. The addition of 1 M aqueous ammonia did not increase the signal-to-noise and was therefore omitted from the sample preparation.

3.2. Method validation

3.2.1. Calibration curve

Six non-zero CS samples were freshly prepared in duplicate for each validation run. The run was acceptable when at least 75% of the CS fell within ±15% (or ±20% for the LLOQ) of the nominal concentrations. Table 1 shows the calibration parameters of the validation runs.

3.2.2. Accuracy and precision

Five replicates of QC LLOQ (0.1 ng/mL), QC Low (0.3 ng/mL), QC Mid (3 ng/mL) and QC High (75 ng/mL) were analysed in 5 and 4 analytical runs for plasma and urine validation, respectively. To assess accuracy and precision, expressed as the bias and the coefficient of variation (CV), respectively, the following equations were used:

$$Intra-run\ bias\,(\%) = 100\% \cdot \frac{(mean\ \ measured\ \ conc.\ per\ run - nominal\ conc.)}{(nominal\ \ conc.)} \quad (1)$$

$$Overall\ \ bias\,(\%) = 100\% \cdot \frac{(overall\ \ mean\ \ measured\ \ conc. - nominal\ \ conc.)}{(nominal\ \ conc.)} \quad (2)$$

$$Intra-run\ \ CV\,(\%) = 100\% \cdot \frac{(SD\ \ of\ \ the\ \ measured\ \ conc.\ perrun)}{(mean\ \ measured\ \ conc.\ perrun)} \quad (3)$$

$$Inter-run\ \ CV\,(\%) = 100\% \cdot \frac{\left(\frac{Mean\ \ square\ \ between\ \ groups - mean\ \ square\ \ within\ \ groups}{n}\right)^{1/2}}{(over\ all\ mean\ \ measured\ \ conc.)} \quad (4)$$

SD: standard deviation

n = total number of replicates

The acceptance criteria were met if the accuracy was within ±20% for the LLOQ level and within ±15% at the other QC levels, and precision was ≤20% for the LLOQ and ≤15% at the other QC levels. Table 2 shows that all acceptance criteria were met.

3.2.3. Selectivity

Six different batches of plasma and urine were prepared and were spiked at LLOQ level. HPLC–MS/MS chromatograms of the blanks and LLOQ samples were monitored and compared for chromatographic integrity and potential interferences. The LLOQ samples were within ±20% of their nominal concentrations. No interferences from endogenous material at the retention time of the analyte with areas >20% (or >5% for the internal standards) of the LLOQ areas were observed in the blanks.

To assess the cross-analyte interference of the method, plasma and urine samples were separately spiked with lurbinectedin at the upper limit of quantitation (ULOQ) and PM040038 at the assay concentration. The interference of PM040038 at the mass transition of lurbinectedin, expressed as the percentage of the mean area of the LLOQ, was not detected in plasma and less than 8% in urine. The interference of lurbinectedin at the mass transition of PM040038 was 2.5% and 1.4% of the LLOQ in plasma and urine, respectively. These values were within the requirements and were therefore found to be acceptable.

3.2.4. Dilution integrity

Dilution integrity was assessed by spiking lurbinectedin at 2000 ng/mL in control human K_3EDTA plasma and 1600 ng/mL in control human urine. Ten μL of these samples were diluted with 190 μL control human K_3EDTA and control human urine, after which 100 μL was processed. The bias and precision were −3.8% and 3.2%, respectively for plasma and −7.6% and 1.4% for urine. Hence, samples containing concentrations well above the ULOQ can be diluted 25/20 times with acceptable accuracy and precision values.

3.2.5. Lower limit of quantification

The analyte response at the LLOQ level was compared to the double blank response in three validation runs. The mean signal to noise was 9 and 19, for plasma and urine, respectively and was deemed sufficient as it was above 5.

3.2.6. Matrix effect

The matrix effect was tested by spiking lurbinectedin to six different batches of plasma and six different batches of urine QC Low and QC High concentrations. The matrix factor (MF) was calculated for both lurbinectedin and IS (PM040038) by calculating the ratio of the peak area in the presence of matrix, to the peak area in absence of matrix. Furthermore, the internal standard normalised (IS-normalised) MF was calculated using the following equation:

$$IS\text{-}\ normalised\ MF = \frac{MF\ \text{of the}\ \ \text{analyte}}{MF\ \text{of the}\ \ \text{internal standard}}$$

The coefficient of variation of the IS-normalised MF was <3.5% in plasma and <3.2% in urine. This was deemed sufficient as it is ≤15%.

3.2.7. Recovery

The overall recovery of lurbinectedin (remaining relative concentration after sample pre-treatment and the effect of the matrix) from the plasma and the urine matrix was calculated by dividing the peak area of a processed sample over the peak area in absence of matrix. The recovery from the plasma matrix using SLE was 15.4–19.9%, while the recovery from urine using LLE was 90.9–93.1%.

3.2.8. Carry-over

Carry-over was tested by injecting a double blank sample after a ULOQ sample. It was considered acceptable once the area of the carry-over sample was ≤20% of the LLOQ. This was the case in both the plasma and the urine assay, with mean carry-over, expressed as the percentage of the LLOQ, of 13.8% and 17.8%, for plasma and urine, respectively. The carry-over of PM040038 was 0.1% in the first plasma blank and 0% for the urine blank.

3.2.9. Stability

Various storage and processing conditions were tested for plasma, plasma-urine, urine, stock and WS. Stability was assessed at QC High and QC Low concentrations by comparing the measured concentration to the nominal concentration, except for short-term stability of lurbinectedin in urine, which was determined by

Table 2
Assay performance.

		Intra-run		Inter-run	
	Nominal concentration (ng/mL)	Bias[a] (%)	CV (%)	Bias[a] (%)	CV (%)
Plasma	0.1	-17.4–0.5	5.2–15.7	−5.7	10.1
	0.3	6.4–14.7	4–7.9	9.4	3.4
	3	0.7–5	2.6–5.8	2.9	0.5
	75	−3.8–0.9	2.5–2.9	−1.6	2
Urine	0.1	−3.3–14.3	6.2–8	4.5	7.6
	0.3	2.6–14.8	1.5–5.2	8.8	5.3
	3	3.2–12.2	1.2–4	8.2	4.1
	75	4.4–11.7	0.7–2.6	7.4	3.5

[a] If multiple validation runs were performed, the range of bias' and imprecisions are listed.

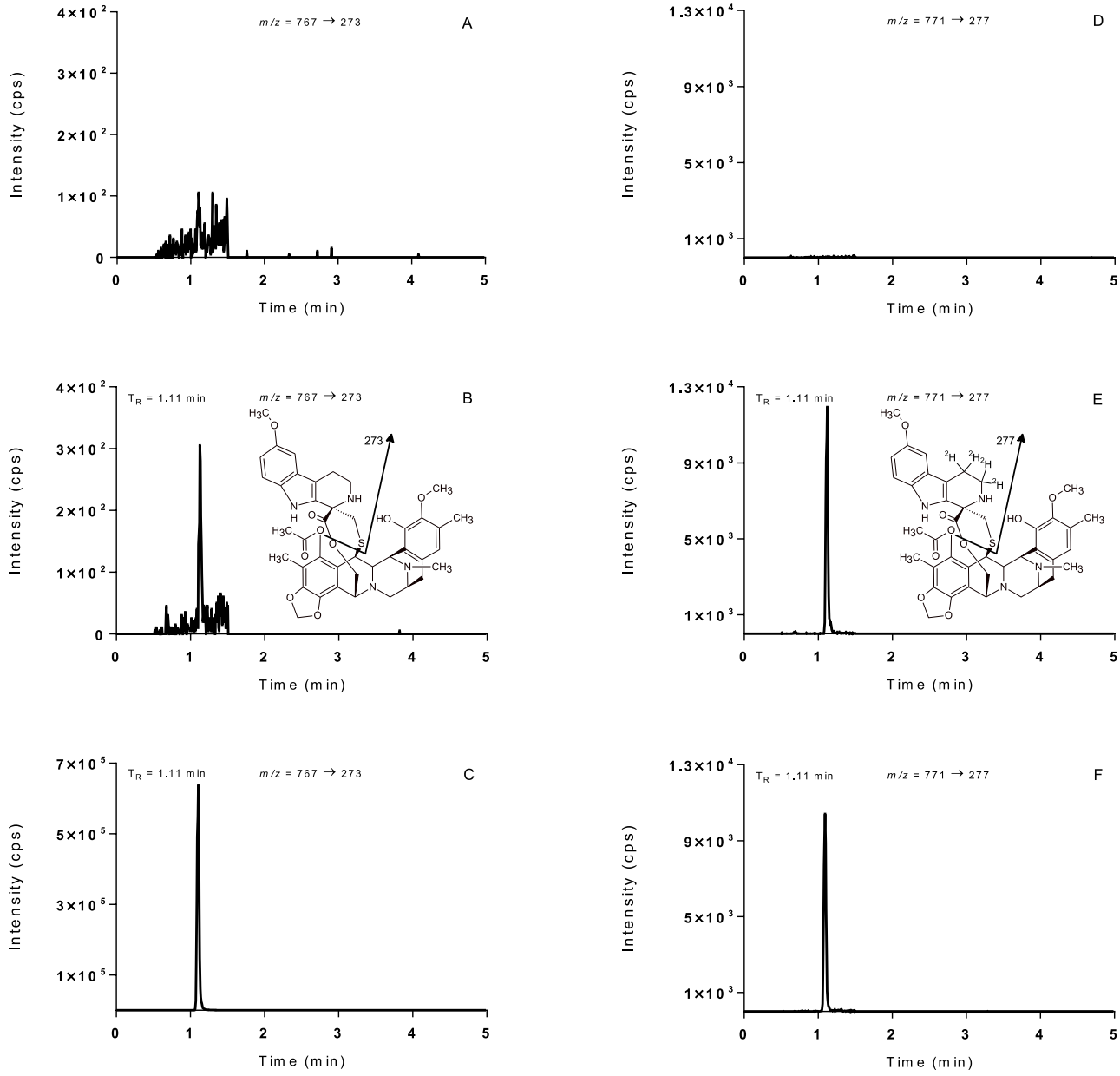

Fig. 1. Representative MRM chromatograms: (A) a blank plasma sample, (B) lurbinectedin in a plasma sample spiked at LLOQ level (0.1 ng/mL), (C) lurbinectedin in a patient plasma sample drawn at the end of infusion, and the corresponding MRM chromatograms of IS PM040038 (D) in a blank plasma sample, (E) in a plasma sample spiked at LLOQ level (20 ng/mL) and (F) spiked to a patient plasma sample drawn at the end of infusion.

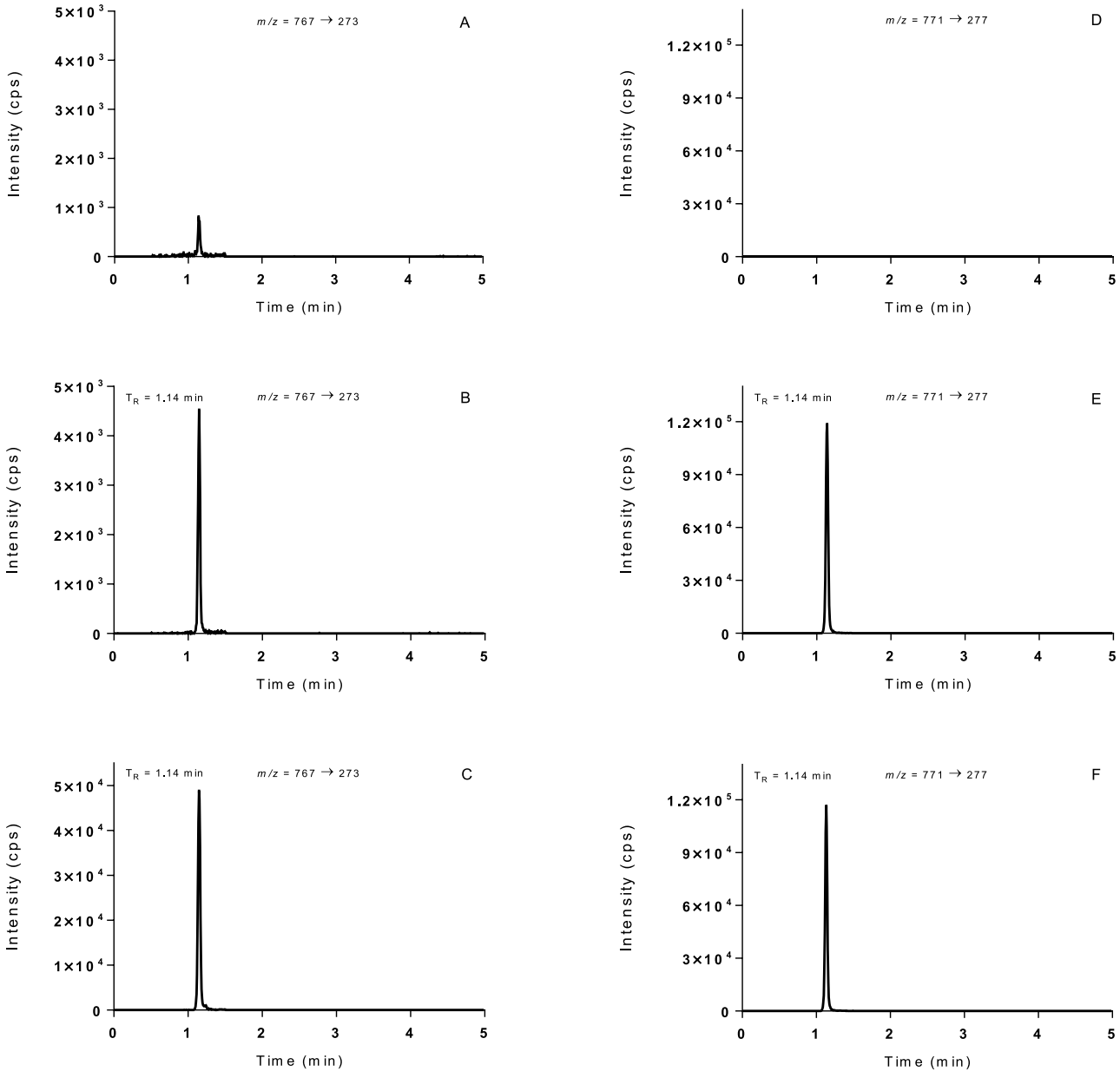

Fig. 2. Representative MRM chromatograms: (A) a blank urine sample, (B) lurbinectedin in a urine sample spiked at LLOQ level (1 ng/mL), (C) lurbinectedin in patient urine collected in the first 12 h after dosing, and the corresponding MRM chromatograms of IS PM040038 (D) in a blank urine sample, (E) in a urine sample spiked at LLOQ level (20 ng/mL) and (F) spiked to patient urine collected in the first 12 h after dosing.

comparing the measured concentration to the concentration determined at time zero. Stability criteria of ±15% for the biomatrices and ±5% for the stock- and working solutions was applied.

The most important findings include stability in urine at 2–8 °C for at least 72 h, final extract stability at 2–8 °C of at least 15 days, and stability in ice-water for at least 1 and 4 h, for plasma and plasma-urine, respectively. WS and stock solutions were stable for at least 4 h at room temperature, and additionally, the stock solutions were stable for at least 17 days at −70 °C. WS were not stable at −70 °C, hence these should be prepared freshly before preparing new CS and QC samples.

4. Clinical application

Six patients were enrolled in a mass balance clinical trial and were given a dose of 5 mg ^{14}C-lurbinectedin (approximately 3.5 mg cold lurbinectedin and 1.5 mg hot lurbinectedin) through a 1 h intravenous (i.v.) infusion. Blood samples were taken up to 168 h post-infusion and were collected in vacutainer tubes with K_3EDTA anticoagulant (Becton Dickenson, Etten-Leur, the Netherlands). Samples were centrifuged at 2000 rpm for 10 min (4 °C) and the obtained plasma was stored in polypropylene tubes at −70 °C until analysis. Urine was collected in glass Schott-Duran bottles at predose, in 12-h periods on day 1 and over 24-h intervals on the following days. Figs. 1 and 2 show representative plasma and urine chromatograms. The concentration-time curve in Fig. 3 shows that lurbinectedin levels were measureable up to 7 days after the start of infusion. Urine data revealed that lurbinectedin concentrations were low and the maximum lurbinectedin concentrations (up to 24.1 ng/mL) were measured in the first 24 h after dosing.

Despite analysing samples which contain both ^{12}C and ^{14}C, no interferences from naturally occurring ^{14}C with regards to the ^{14}C-isotope are expected, since quantification is solely based on the ^{12}C-

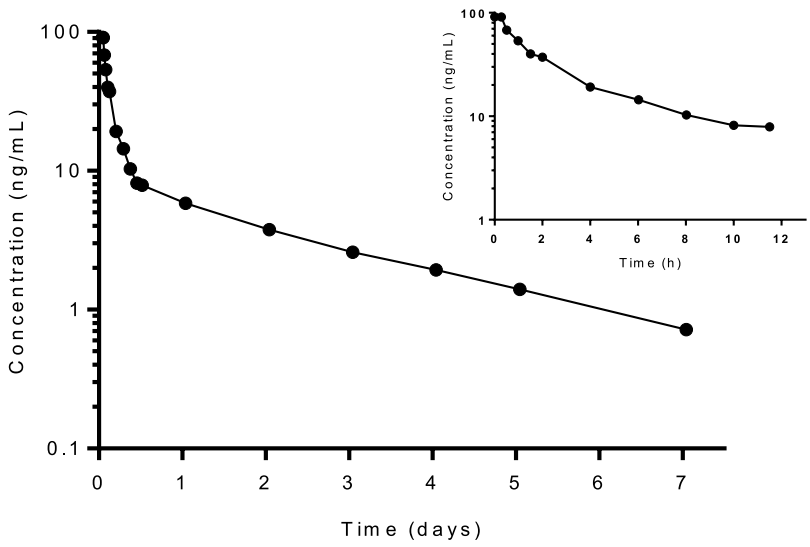

Fig. 3. Representative plasma-concentration time curve after administration of 5 mg lurbinectedin to a patient with advanced cancer. The dose contained a mixture of ^{12}C-lurbinectedin and ^{14}C-lurbinectedin, but only the ^{12}C-lurbinectedin concentrations are represented here. The time on the x-axis is relative to the end of infusion. Insert: first part of the curve from time zero to 12 h.

isotope transition. To calculate the pharmacokinetic parameters, total concentrations (^{12}C and ^{14}C) will be used.

5. Conclusion

We developed a reliable and sensitive method to quantify lurbinectedin in human biological matrices. This is the first reported method to quantify lurbinectedin in human samples. TBME was used to extract lurbinectedin from plasma by SLE and from urine by LLE. While SLE resulted in very clean samples, with satisfactory signal-to-noise-ratio, the recovery was low, but all analytical results were well within the pre-set requirements. LLE improved the recovery of lurbinectedin from urine significantly, nearing 100%. It was shown that dilution of urine in control human plasma before processing reduced the ion suppression effect and furthermore the loss of lurbinectedin by adsorption to the wall of the container was eliminated. Extensive stability testing was executed and revealed that urine samples need to be collected in glass and are stable for at least 72 h at 2–8 °C. Due to instability in plasma and plasma-urine at room temperature, sample processing needs to take place on ice-water. To conclude, the presented lurbinectedin LC–MS/MS method has been validated according to the latest guidelines and meets its requirements, and its clinical application has been demonstrated.

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