Journal of Chromatography B, 879 (2011) 1149–1155

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Validation of high-performance liquid chromatography–tandem mass spectrometry assays for the quantification of eribulin (E7389) in various biological matrices

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ARTICLE INFO

Article history:
Received 25 November 2010
Accepted 9 March 2011
Available online 17 March 2011

Keywords:
Eribulin
E7389
LC–MS/MS
Human mass balance study

ABSTRACT

This paper presents specific and sensitive high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) assays for the quantification of the novel anticancer agent eribulin in human plasma, whole blood, urine and faeces. These assays, developed to support clinical pharmacological studies with the drug, quantify eribulin concentration ranges of 0.2–100 ng/mL for plasma, 0.5–100 ng/mL for whole blood and urine and 0.1–25 μg/g for faeces, using sample volumes of 500 μL or 250 μg (faeces). Samples were prepared with liquid–liquid extraction, separated on a C18 column with gradient elution and analysed with a triple quadrupole MS, in positive ion mode. A structural analogue of eribulin was used as internal standard for the quantification. The assays were linear with correlation coefficients (r^2) of 0.99 and better, whereby the deviation from nominal concentrations ranged from −8.2 to 8.9% with CV values of maximally 14.2%. Stability assessments demonstrated that eribulin is stable at −20 °C in plasma, whole blood, urine and faeces for at least 38, 4, 10.5 and 5 months, respectively. In conclusion, the validation results show that the assays are specific and accurate and can therefore adequately be applied to support clinical studies of eribulin.

1. Introduction

Eribulin mesylate (E7389) (Fig. 1A) is a nontaxane microtubule dynamics inhibitor with a distinct mode of action. While it is still being investigated in clinical trials, eribulin has recently been approved by the United States Food and Drug Administration (FDA) for treatment of patients with metastatic breast cancer, who have previously received at least two chemotherapeutic regimens, including an anthracycline and a taxane [1].

Like most anti-mitotic drugs, eribulin affects the microtubule dynamics, resulting in a cell cycle block, leading to apoptosis [2]. Unlike other tubulin-targeted agents, eribulin only inhibits the growth and not the shortening of microtubules and it induces the formation of tubulin aggregates [3]. These distinct modes of action may contribute to the results of phase II studies wherein eribulin shows activity in patients who had received previous therapy with taxanes and anthracyclines [4].

To support clinical pharmacological studies of eribulin, and especially mass balance studies, it was essential to develop and validate quantitative bioanalytical assays of eribulin in plasma, whole blood, urine and faeces. The quantification of eribulin in human plasma and urine described by Desjardins et al. [5] served as a starting point for the development of the plasma assay in this paper. As our attempts to reproduce their method resulted in an insufficient separation between eribulin and the internal standard, we further optimized the chromatographic conditions. Additionally, we present methods for quantification of eribulin in whole blood and faeces, for which thus far no methods have been published. The described validations were performed according to the FDA guidelines for bioanalytical method validation [6,7].

2. Experimental

2.1. Chemicals and reagents

Eribulin methanesulfonic acid salt and its internal standard ER-076349 (Fig. 1B) were provided by Eisai Co., Ltd, Japan. Methanol

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doi:10.1016/j.jchromb.2011.03.021

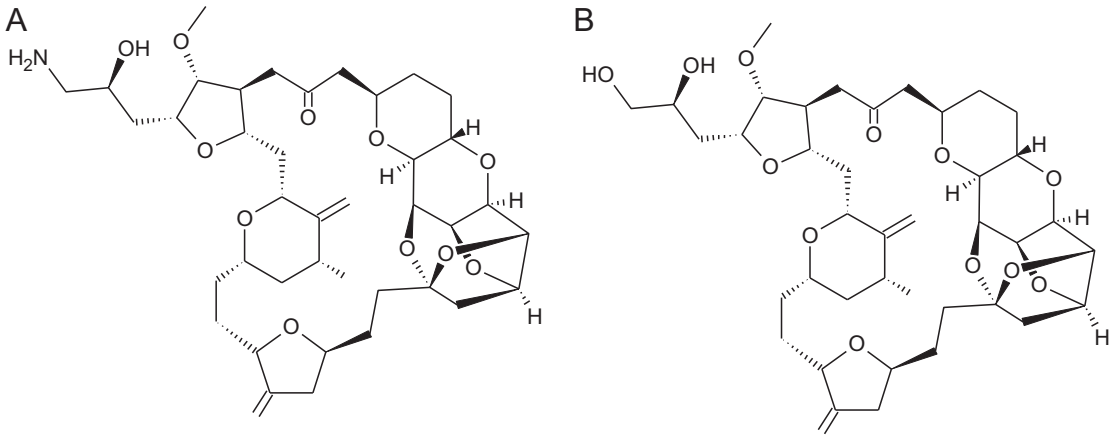

Fig. 1. Chemical structures of eribulin (A) and the internal standard ER-076349 (B).

(Supra-Gradient grade), ethanol absolute (HPLC-grade) and acetonitrile (ACN, Supra-Gradient grade) were obtained from Biosolve Ltd, Valkenswaard, The Netherlands. Ethyl acetate (LiChrosolv), sodium hydroxide (NaOH) (>99%) and formic acid (98%) were purchased from Merck, Darmstadt, Germany. Water (distilled) used for sample preparation originated from B. Braun Medical, Melsungen, Germany and water (LiChrosolv) used to prepare eluentia from Merck. Drug-free control human lithium heparinized plasma was obtained from Sanquin, Amsterdam, the Netherlands and Bioreclamations, Hicksville, USA. Control human lithium heparinized whole blood, urine and faeces originated from healthy volunteers.

2.2. Preparation of stock solutions, calibration standard (CS) and quality control (QC) samples

Stock solutions of eribulin (1 mg/mL) and the internal standard (IS) (0.1 mg/mL) were prepared in methanol and diluted in methanol:water (1:1, v/v) to obtain CS and QC or IS working solutions. IS working solutions of 500 ng/mL, 2.5 μg/mL and 10 μg/mL were used for the plasma, urine and whole blood and faeces assay, respectively.

Calibration standard and QC samples were prepared by diluting the corresponding working solutions with control human plasma, whole blood (with at least 1 freeze–thaw cycle at −20 °C), urine or faecal homogenate in water (1:3, w/v). The final concentrations of the CS samples were 0.2, 0.5, 1, 2, 5, 10, 50 and 100 ng/mL for plasma, 0.5, 1, 2.5, 10, 25, 50, 75 and 100 ng/mL for whole blood and urine and 0.1, 0.25, 1, 2.5, 5, 12.5 and 25 μg/g (undiluted faeces) for faeces. Final concentration of the QC samples at the lower limit of quantification (LLOQ), QC low, QC mid, QC high and QC above the upper limit of quantification (>ULOQ, for plasma only) were 0.2, 0.6, 5, 80 and 500 ng/mL for plasma, 0.5, 1.5, 10 and 80 for whole blood and urine and 0.1, 0.25, 2.5 and 20 μg/g for faeces.

Stock and working solutions were stored at nominally 4 °C, QC samples were stored at nominally −20 °C and CS samples were freshly prepared before each validation run.

2.3. Processing of samples

Plasma, whole blood and urine samples were prepared following the procedure of [5], with the modification that urine samples were diluted with 500 μL control human plasma instead of water. After liquid–liquid extraction (LLE), followed by evaporation of the organic layer, reconstitution and filtration, 20 μL of the final extract were injected.

Faecal samples were homogenized with water (1:3, w/v). Since CS and QC samples for faeces were prepared by adding 50 μL working solution to 1.00 mL of faecal homogenate, 50 μL of MeOH:water (1:1, v/v) was added to patient samples of 1.00 mL of faecal homogenate.

Subsequently, 50 μL IS working solution and 2 mL acetonitrile were added. Samples were vortex mixed for 30 s, shaken at 1250 rpm for 10 min and centrifuged for 5 min at around 2250 g. The supernatant was filtered using 0.2 μm micro-spin filters (Alltech, Deerfield, IL, USA) and after centrifuging for 5 min at 10,300 *g*, 3 μL of the filtrate was injected.

2.4. Liquid chromatography

The assay in plasma was conducted on a HPLC system comprising a LC-20AD Prominence binary solvent delivery system with a column oven, a SiL-HTc autosampler and a DGU-20A3 online degasser (Shimadzu, Kyoto, Japan). The HPLC system used for the other assays consisted of a HP1100 binary pump, a degasser and a HP1100 autosampler (Agilent technologies, Palo Alto, CA, USA).

For all four assays, separation was achieved within 10 min on a Polaris® C18-A column (30 mm × 2.0 mm, particle size 3.0 μm; Varian Inc., Palo Alto, CA, USA), thermostatted at 30 °C, like in [5], but with different mobile phases. Eluent A consisted of 0.1% formic acid in (Lichrosolv) water and B of 0.1% formic acid in acetonitril at a flow rate of 0.3 mL/min. For the plasma assay, the mobile phase composition was as follows: mobile phase B: 20% (0–1 min), from 20 to 40% (1–5.5 min), from 40 to 80% (5.5–6 min), 80% (6–7 min), from 80 to 20% (7–7.5 min) and 20% (7.5–10 min). For the other assays, it consisted of: mobile phase B: 18% (0–1.7 min), 33% (1.7–6.5 min), 80% (6.5–7.5 min) and 18% (7.5–10 min). The autosampler temperature was kept at 4 °C for the plasma assay and at room temperature for the other assays.

2.5. Mass spectrometry

For all four assays, a triple quadrupole mass spectrometer was used, operating with an electrospray ionization (ESI) source in positive mode and configured in multiple reaction monitoring (MRM). Mass spectrometric parameters were optimized for the transitions of m/z 730.5 → 712.5 (eribulin) and 731.5 → 681 (IS).

A Finnigan TSQ Quantum Ultra (Thermo Fisher Scientific, Waltham MA, USA) was used for the assay in plasma. The optimized instrument parameters were as follows: capillary temperature, 375 °C; ion spray voltage, 4.75 kV; sheath gas, 47 mTorr; auxiliary gas, 16 mTorr; ion sweep gas, 1.5 mTorr; source CID collision energy, 8 V; Q2 collision gas pressure, 1.5 mTorr; collision energy, 32 V (eribulin) and 25 V (IS); tube lens voltage, 137 V (eribulin) and 90 V (IS) and scan time, 100 ms.

The whole blood, urine and faeces assays were performed on an API 3000 triple quadrupole with a turbo ion spray interface (AB Sciex, Thornhill, ON, Canada) using the following settings: turbo ionspray temperature, 550 °C; ionspray voltage, 2 kV; turbo gas flow, 7 L/min; nebulizer gas; 13 psi; curtain gas, 6 psi; collision gas, 8 psi; dwell time 250 ms (eribulin) and 100 ms (IS).

2.6. Validation procedures

A full validation according to the FDA guidelines [6,7] was performed for the quantification of eribulin in human heparinized plasma, including linearity, intra-assay and inter-assay accuracy and precision, dilution test, specificity and selectivity, recovery and matrix effect, carry-over and stability.

The other assays are modifications of the plasma assay and validation of these assays covered linearity, intra-assay accuracy and precision, carry-over and stability. Additionally, specificity and selectivity were assessed in whole blood and urine, matrix effect was determined in urine and recovery was tested in both urine and faeces.

3. Results and discussion

3.1. Method development

3.1.1. Sample pretreatment

The apolar nature of eribulin theoretically makes it a good substrate for LLE with an organic solvent. Ethyl acetate only or in combination with methanol and ethanol (90:5:5, v/v/v) and tert-butyl methyl ether were tested. Extraction with the ethyl acetate, methanol and ethanol combination resulted in clean extracts and reproducible recoveries and was chosen as pretreatment method.

Because whole blood study samples collected from patients are stored frozen, and thereby haemolysed, it was decided to use only control whole blood that had undergone at least 1 freeze–thaw cycle for the preparation of CS and QC samples.

Application of the plasma sample processing method to urine resulted in diverging calibration curves and QC samples being incompatible with the calibration curves. High pH variation between individual urine batches, even after the addition of NaOH, and a varying matrix effect were suspected to cause the observed problems. Therefore, to neutralize the effect of urine as a matrix, the 500 μL of water – that was added to the plasma samples before LLE – was for the urine samples replaced with control human plasma.

A simple extraction method was applied on faecal samples, excluding the snap-freezing and concentration step of the other assays. The disadvantage of this method is that the samples are being diluted; on the other hand, the expected concentrations in faeces are much higher than in the other matrices. Thus, sensitivity was not critical for this assay.

3.1.2. Liquid chromatography

Starting point of the development of the chromatographic system of the plasma assay were the conditions used by DesJardins et al. [5]. However, this system resulted in an insufficient separation between eribulin and the internal standard. Although full separation between analytes is generally not required for LC–MS/MS quantification in MRM mode, in this case, with an IS molecular mass differing only 1 Da from eribulin itself, cross-interference between the analytes would arise. Namely, the transition of the ^{13}C-isotope peak of the internal standard and its unspecific loss of water would be indistinguishable from the transition of eribulin. Furthermore, a significant amount of noise dominated the chromatograms obtained using chromatographic system of [5]. As THF is generally not recommended for mass spectrometry [8], the first step was to eliminate this solvent from the eluent. With the alternative eluentia 0.1% formic acid in water and 0.1% formic acid in ACN, the gradient was optimized until acceptable baseline separation between eribulin and the IS was established, without prolonging the total runtime. The final chromatographic system for the plasma assay was also tested for the urine assay, however, for this matrix, a stable chromatography could not be accomplished. Consequently, other gradients were tested, resulting in the selected step-wise gradient, which turned out to be equally applicable for the whole blood and faeces assay. Fig. 2 shows the MRM chromatograms of eribulin and the IS of a LLOQ and a blank sample of each matrix.

3.1.3. Mass spectrometry

Mass spectrometric parameters were optimized with direct infusion and flow injection analysis. The positive ionization mode resulted in higher ion counts than the negative mode and was therefore selected. The full-scan mass spectrum of eribulin (Fig. 3A) showed the protonated ions $[M+H]^+$ and $[M+Na]^+$ at m/z 730 and 752, respectively. The product ion (PI) spectrum (Fig. 3B) demonstrated that eribulin is hardly fragmented. Product ions were observed at m/z 712 (loss of H_2O), 698 (loss of the methoxy group, CH_3OH) and 680 (loss of both CH_3OH and H_2O). Since maximum sensitivity was aimed for, the most abundant product ion was selected for quantification. Therefore, although loss of water is generally an unspecific and variable transition, this particular transition of m/z 730.5 → 712.5 was used.

The full-scan spectrum of the IS (Fig. 3C) showed protonated ions $[M+H]^+$ and $[M+Na]^+$ with nominal m/z values of 731 and 753, respectively. Again, the most abundant product ion from the PI spectrum (Fig. 3D), m/z 681, was monitored for quantification.

3.2. Validation procedures

3.2.1. Linearity, accuracy and precision

Linearity in plasma and whole blood was assessed in three analytical runs and in urine and faeces in two runs. Using a $1/x^2$ weighting factor, differences between back-calculated and nominal concentrations were minimized. The assays were linear over concentration ranges of 0.2–100 ng/mL for plasma, 0.5–100 ng/mL for urine and whole blood and 0.1–25 μg/g for faeces with correlation coefficients (r^2) of 0.99 or better. Deviations from the nominal concentrations were −5.29 to 4.81% for all concentrations in plasma, −1.72 to 4.03% in whole blood, −8.25 to 3.92% in urine and −4.23 to 8.87% in faeces, with CV values less than 10.7, 14.2, 8.14 and 9.12%, respectively. Since also the signal to noise ratios at the LLOQ were above 5, the selected ranges comply with the criteria for linearity. Moreover, plasma samples can be diluted 10 times, as the accuracy and precision of six analysed diluted replicates were 5.15 and 8.78%, respectively.

Also the accuracies and precisions met the criteria of the FDA guidelines [6]. Table 1 summarizes the intra-assay accuracies and precisions. The plasma inter-assay accuracy over three runs ranged from −3.18 to 2.77% with an inter-assay precision ≤13.0%.

3.2.2. Specificity, selectivity and carry-over

Six individual batches, spiked with eribulin at the LLOQ level showed deviations between −18.9 and 18.0% for plasma, between 3.72 and 19.0% for whole blood and between −7.23 and 12.4% for urine. Co-eluting peaks with areas >20% of the LLOQ were not observed in the MRM chromatograms of 6 individual batches of blank controls of the tested matrices, neither were peaks >5% of and co-eluting with the IS. The same applied for the blank samples injected after ULOQ samples for the carry-over test. Thus, the specified criteria for specificity and carry-over were fulfilled.

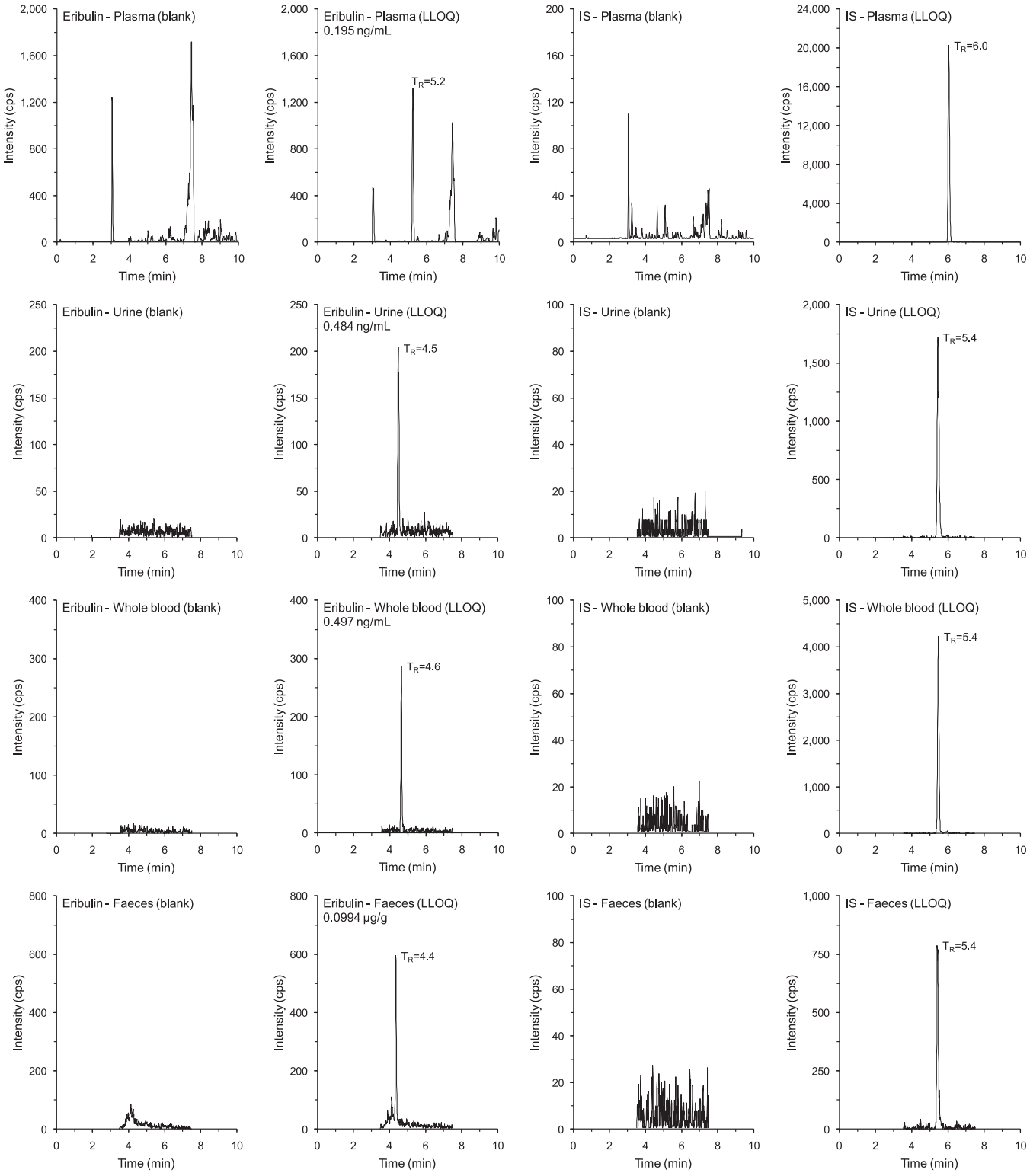

Fig. 2. MRM chromatograms of eribulin (left, m/z 730.5 → 712.5) and the internal standard (right, m/z 731.5 → 681) of blank samples and QC LLOQ samples, in plasma (upper row), urine (second row), whole blood (third row) and faeces (lower row).

3.2.3. Matrix effect and total recovery

The total recoveries (including sample pretreatment and matrix effect) of eribulin were 63.7 ± 3.8% (mean ± SD) in plasma, 68.8 ± 3.4% in urine and 114 ± 1.0% in faeces. The matrix effect of plasma ranged from −8.4 to 2.9%, for urine this was between 13.3 and 18.1% (ion enhancement). The recovery of eribulin from the LLE of plasma and urine samples was 65.0 ± 0.7% and 59.2 ± 2.1%, respectively.

The total recovery of the IS was 79.4% in plasma, 90.0% in urine and 111% in faeces, with a matrix effect of 2.2% in plasma and 20.2% in urine and a LLE recovery of 77.7% in plasma and 74.8% in urine.

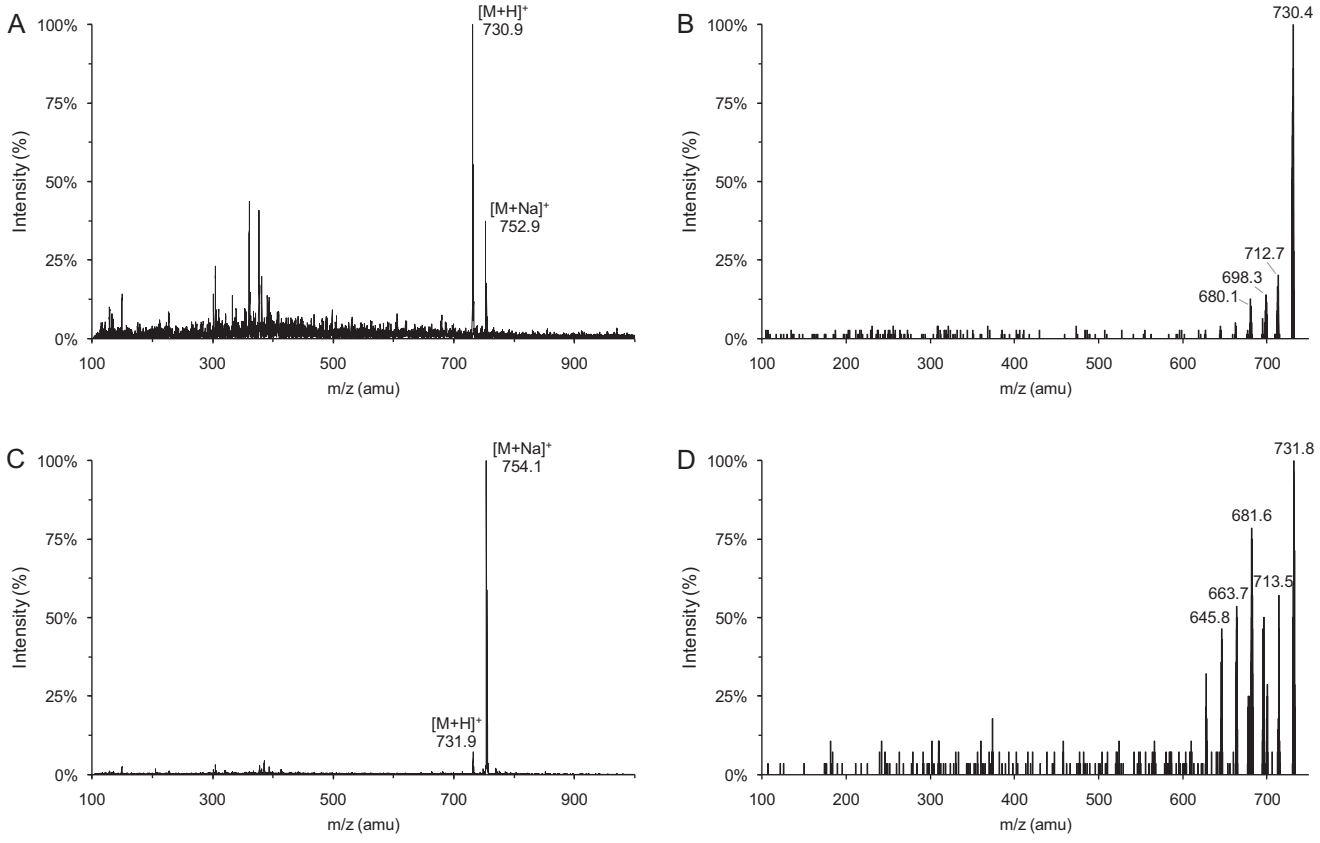

Fig. 3. Full-scan positive ion mass spectrum of eribulin (A) and the internal standard ER-076349 (C) and the corresponding product ion spectra (B, D).

Table 1
Assay performance data for eribulin in plasma, whole blood, urine and faeces.

Matrix	Nominal conc.[a]	Measured conc.[a]	Intra-assay accuracy (%)	Intra-assay precision (%)	Number of replicates
Plasma	Run 1				
	0.195	0.180	−7.95	9.38	6
	0.586	0.604	3.10	6.73	6
	4.88	5.14	5.40	2.73	6
	78.1	76.6	−1.98	4.97	6
	Run 2				
	0.195	0.218	11.6	8.25	6
	0.586	0.647	10.4	11.1	6
	4.88	5.41	10.8	4.55	6
	78.1	80.1	2.54	2.69	6
	Run 3				
	0.195	0.174	−10.7	6.17	6
	0.586	0.535	−8.65	8.85	6
	4.88	4.49	−7.92	4.03	6
	78.1	70.2	−10.1	3.02	6
Whole blood	0.484	0.497	2.77	7.93	5
	1.45	1.61	11.2	2.46	5
	9.68	10.4	7.85	2.21	5
	77.5	85.1	9.86	2.28	5
Urine	0.484	0.470	−3.00	9.35	6
	1.45	1.51	4.37	4.99	6
	9.68	9.34	−3.51	4.13	6
	77.5	77.0	−0.688	6.00	6
Faeces	0.0968	0.0963	−0.475	5.11	5
	0.242	0.248	2.48	3.28	5
	2.42	2.55	5.45	4.13	5
	19.4	20.2	4.02	3.33	5

Conc.: concentration.
[a] Concentrations are in ng/mL for plasma, whole blood and urine and in μg/g for faeces.

Table 2
Stability data for eribulin. All experiments were performed in triplicate.

Condition	Sample type	Nominal conc.[a]	Initial conc.[a]	Measured conc.[a]	CV (%)	Dev.[b] (%)
Stock/working solutions in MeOH						
Ambient, 6 h	Eribulin solution	–	1.00E+06	1.04E+06	1.24	4.05
Ambient, 6 h	IS solution	–	1.00E+05	1.03E+05	1.00	2.88
2–8 °C, 7.5 m	IS solution	–	9.96E+04	1.04E+05	8.29	4.64
2–8 °C, 8 m	IS solution	–	473	530	5.54	12.0
2–8 °C, 18.5 m	IS solution	–	498	558	4.79	12.0
Plasma						
3 Freeze (−20 °C)–thaw cycles	Biomatrix	0.616	0.561	0.639	7.96	13.8
		82.1	75.5	74.5	3.56	−1.32
Ambient, 24 h	Biomatrix	0.586	0.574	0.571	5.89	−0.465
		78.1	71.3	70.6[c]	4.51	−1.10
2–8 °C, 7 days	Dried extract	0.586	0.553	0.553	8.06	−0.120
		78.1	77.6	75.5	10.9	−2.71
Ambient, 5 days	Processed	0.586	0.583	0.529	11.3	−9.27
		4.88	5.05	5.08	4.95	0.462
		78.1	77.4	79.5	0.856	2.80
2–8 °C, 7 days	Processed	0.598	0.592	0.604	7.37	1.97
		79.8	72.8	79.1	7.73	8.66
−20 °C, 38.5 m	Biomatrix	0.616	0.561	0.623	2.58	11.1
		82.1	75.5	69.2	3.01	−8.35
Whole blood						
3 Freeze (−20 °C)–thaw cycles	Biomatrix	1.45	NA	1.58	12.8	8.74
		77.5	NA	85.1	9.29	9.81
Ambient, 24 h	Biomatrix	1.45	NA	1.44	3.82	−0.460
		77.5	NA	68.8	4.71	−11.2
2–8 °C, 9 days	Dried extract	1.45	NA	1.39	9.13	−4.37
		77.5	NA	74.0	7.43	−4.52
Ambient, 9 days	Processed	1.45	NA	1.60	0.720	10.6
		77.5	NA	87.1	3.17	12.3
−20 °C, 4 m	Biomatrix	1.45	NA	1.59	11.4	9.89
		77.5	NA	74.2	2.09	−4.30
Urine						
Ambient, 16 h	Biomatrix	1.45	1.50	1.57	5.21	4.67
		77.5	74.4	82.4	7.70	10.7
2–8 °C, 28 days	Dried extract	1.45	1.40	1.19	4.13	−14.8
		77.5	66.2	57.0	2.62	−13.9
Ambient, 28 days	Processed	1.45	1.40	1.20	3.63	−14.3
		77.5	66.2	63.8	3.68	−3.63
−20 °C, 10.5 m	Biomatrix	1.50	NA	1.54	5.77	2.67
		79.8	NA	75.9	1.79	−4.93
Faeces						
3 Freeze (−20 °C)–thaw cycles	Biomatrix	0.242	0.245	0.273	5.22	11.1
		19.4	19.9	19.7	7.05	−1.17
Ambient, 20 h	Biomatrix	0.242	0.254	0.277	2.41	8.92
		19.4	19.8	22.5	14.8	13.8
Ambient, 8 days	Processed	0.242	0.245	0.281	8.00	14.7
		19.4	19.9	18.9	3.61	−5.2
−20 °C, 5 m	Biomatrix	0.242	NA	0.269	13.5	11.0
		19.4	NA	19.2	5.46	−0.859

Conc.: concentration; CV: coefficient of variation; Dev.: deviation; MeOH: methanol; h: hours; m: months; NA: not available.
[a] Concentrations are in ng/mL for MeOH, plasma, whole blood and urine and in μg/g for faeces.
[b] Deviation is calculated against the initial concentration if available, otherwise against the nominal concentration.
[c] Experiment was performed in duplicate instead of triplicate.

A total recovery of >100%, as observed for eribulin and its IS in faeces, may be due to ion enhancement, which was also demonstrated for urine, or can be caused by analyte loss in the absence of matrix during analysis [9].

3.2.4. Stability

Results of the stability experiments are displayed in Table 2. Eribulin is a relatively stable compound, with a demonstrated stability at −20 °C in plasma, whole blood, urine and faeces of at least 38, 4, 10.5 and 5 months, respectively. In whole blood and plasma eribulin is stable for at least 24 h at ambient temperature, indicating that no specific stability precautions are required during sample handling at the clinical site.

Reinjection reproducibility experiments demonstrated that runs with plasma and urine can be reinjected after 24 h and with faeces after 5 days residence in the autosampler at ambient temperature.

4. Conclusion

For the quantification of eribulin in human plasma, whole blood, urine and faeces, sensitive and accurate LC–MS/MS assays are presented. Using sample volumes of 500 μL of plasma, whole blood and urine and 250 μg faeces, linear ranges from 0.2 to 100 ng/mL for plasma, 0.5 to 100 ng/mL for whole blood and urine and 0.1 to 25 μg/g for faeces were validated. The assays have successfully been used to support clinical studies. Especially in mass balance studies they can play a major role, making it possible to quantify concentrations and excreted amounts of unchanged drugs.

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