Mass balance study of ^{14}C-eribulin in patients with advanced solid tumours

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List of abbreviations:

ACN: acetonitrile; AE: adverse event; AUC: area under the concentration –time curve; ECOG-PS Eastern Cooperative Oncology Group performance status; FDA: U.S. Food and Drug Administration; HPLC: high performance liquid chromatography; ICH: International Conference on Harmonisation; LC-MS/MS: liquid chromatography tandem mass spectrometry; LLOQ: lower limit of quantitation; LOD: limit of detection; LSC: liquid scintillation counting; MBC: metastatic breast cancer; MeOH: methanol; MIST: Metabolites in Safety Testing; PP: polypropylene; RECIST: response evaluation criteria in solid tumours; TRA: total radioactivity.

Abstract

This mass balance study investigated the metabolism and excretion of eribulin, a non-taxane microtubule dynamics inhibitor with a novel mechanism of action, in patients with advanced solid tumours. A single ~2 mg (~80 µCi) dose of ^{14}C-eribulin acetate was administered as a 2–5 min bolus injection to 6 patients on day 1. Blood, urine, and faecal samples were collected at specified time points on days 1–8 or until sample radioactivity was ≤1% of the administered dose. Mean plasma eribulin exposure (627 ng.h/mL) was comparable to that of total radioactivity (568 ng eq.h/mL). Time-matched concentration ratios of eribulin to total radioactivity approached unity in blood and plasma indicating that unchanged parent compound constituted almost all eribulin-derived radioactivity. Only minor metabolites were detected in plasma samples up to 60 min post-dose, pooled across patients, each metabolite representing ≤0.6% of eribulin. Elimination half-lives for eribulin (45.6 h) and total radioactivity (42.3 h) were comparable. Eribulin-derived radioactivity excreted in faeces was 81.5% and that of unchanged eribulin was 61.9%. Renal clearance (0.301 L/h) was a minor component of total eribulin clearance (3.93 L/h). Eribulin-derived radioactivity excreted in urine was comparable (8.9%) to that of unchanged eribulin (8.1%) indicating minimal excretion of metabolite(s) in urine. Total recovery of the radioactive dose was 90.4% in urine and faeces. Overall, no major metabolites of eribulin were detected in plasma. Eribulin is primarily eliminated unchanged in faeces, while urine constitutes a minor route of elimination.

Introduction

Eribulin (Fig. 1) is a synthetic analogue of Halichondrin B, a complex polyether macrolide, first isolated from the marine sponge *Halichondria okadai* Kadota by Hirata and Uemura (Hirata and Uemura, 1986). In human tumour cell lines, Halichondrin B proved to be highly cytotoxic, causing mitotic cell cycle arrest by inhibiting polymerization of tubulin and microtubule assembly (Bai et al., 1991). The naturally low abundance of the molecule led to the development of complex methods for total synthesis and eventually to the discovery of eribulin (Jackson et al., 2009), a structurally simplified Halichondrin B analogue with similar or identical anti-cancer properties in pre-clinical models (Towle et al., 2001). In clinical studies, eribulin has demonstrated anti-tumour activity in extensively pre-treated patients who had advanced or metastatic breast cancer (MBC), with manageable tolerability (Cortes et al., 2010; Vahdat et al., 2008; Vahdat et al., 2009). A multicentre, randomised, Phase III study involving heavily pre-treated patients with metastatic breast cancer (MBC) showed a significant improvement in median overall survival by 2.5 months for patients treated with eribulin (n=508) compared with patients who received treatment of physician's choice (n=254) (Cortes et al., 2011). Eribulin mesylate was recently approved in the United States for the treatment of patients with metastatic breast cancer who have previously received at least two chemotherapeutic regimens for the treatment of metastatic disease. Prior therapy should have included an anthracycline and a taxane in either the adjuvant or metastatic setting.

Knowledge about the metabolism and excretion of a new drug are essential for its approval. Over the past few years, the interest in drug metabolism has increased rapidly due to discussion on the potential contribution of drug metabolites to toxicity. Within this scope, the U.S. Food and Drug Administration (FDA) and the International Conference on Harmonisation (ICH) recently issued guidances for Metabolites in Safety Testing (MIST), recommending additional safety assessments for major metabolites (FDA, 2008; ICH, 2009). Major metabolites are herein defined as metabolites with a systemic exposure greater than 10% of the parent (FDA) or the total drug-related (ICH) exposure that are either identified only in human plasma or present at disproportionally higher levels in humans than in any preclinical test species.

Knowledge about drug excretion is particularly important in treatment of patients with impaired renal or liver function, as clinical implications of urinary or biliary excreted drugs may be more pronounced for these patients, with potential need for dosage adjustments.

Pre-clinical studies showed that eribulin is predominantly metabolized by CYP3A4 (Zhang et al., 2008). In clinical studies, eribulin was found to be minimally eliminated as unchanged form in urine (Goel et al., 2009; Tan et al., 2009). The objective of this study was to determine the metabolism and excretion of eribulin after a single dose of ^{14}C-eribulin in patients with advanced solid tumours. Pharmacokinetics and excretion of both unchanged drug and total radioactivity (comprising parent-drug and metabolites) were determined using validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assays and liquid scintillation counting (LSC), respectively. Radiochromatography and high-resolution mass spectrometry were combined to detect and identify eribulin metabolites.

Methods

Compounds and reagents

^{14}C-eribulin acetate was manufactured by GE Healthcare UK limited (Chalfont St Giles, UK) and provided by Eisai Ltd. (Hatfield, Hertfordshire, UK). Sterile water for injection, normal saline (0.9% NaCl) and distilled water for sample preparation originated from B. Braun (Melsungen, Germany). All solvents for sample preparation and high performance liquid chromatography (HPLC) were analytical or HPLC grade. Methanol (MeOH) and acetonitrile (ACN) were obtained from Biosolve Ltd. (Valkenswaard, the Netherlands). Ammonium acetate was purchased from Fluka (Zwijndrecht, the Netherlands) and water for mobile phase preparation (LiChrosolv), isopropanol, EDTA, 30% hydrogen peroxide (w/w) and acetic acid originated from Merck (Darmstadt, Germany). Solvable and Ultima Gold liquid scintillation cocktail were obtained from Perkin Elmer (Waltham, MA, USA).

Patients

Patients aged at least 18 years with a histologically or cytologically confirmed advanced solid tumour, that had progressed following standard therapy or for which no standard therapy existed, were eligible for this study. Prior chemotherapy other than eribulin, mitomycin C or nitrosourea was allowed, as was radiation or biological therapy, provided that the last treatment was at least 3 weeks prior to study entry (4 weeks for investigational drugs and alternative therapies). Other eligibility criteria included: Eastern Cooperative Oncology Group performance status (ECOG-PS) ≤ 2, adequate renal function (serum creatinine ≤ 135 µmol/L (≤ 1.5 mg/dL) or creatinine clearance ≥40 mL/min), adequate bone marrow function (absolute neutrophil count ≥1.5 x 10^9/L and platelet count ≥100 x 10^9/L), adequate hepatic function (bilirubin ≤1.5 times the upper limit of normal (ULN) and alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase ≤3 x ULN or ≤5 x ULN in the case of liver metastases) and resolution of all chemotherapy or radiotherapy related toxicities to ≤ grade 1, except for stable sensory neuropathy ≤ grade 2 and alopecia.

Exclusion criteria included presence of severe intercurrent illness or infection, significant cardiovascular impairment, known positive human immunodeficiency virus status, major surgery within 4 weeks before treatment start, pulmonary dysfunction requiring active treatment, treatment with warfarin or related compounds other than for line patency, pregnancy and breast-feeding, meningeal

carcinomatosis, pre-existing neuropathy > grade 2, organ allografts requiring immunosuppression, radiation therapy that encompassed > 30% of marrow and active brain metastases.

Study design

Six patients with advanced solid tumours were enrolled into this Phase I, open-label, non-randomised, single centre (the Netherlands Cancer Institute, Amsterdam, the Netherlands) study. Patients received a 2 mg flat dose of ^{14}C-eribulin acetate (approximately 80 to 90 µCi) on Day 1, Cycle 1 after pre-dose evaluations (medical/surgical history, tumour assessment, complete physical examination, vital signs, ECG and clinical laboratory tests for safety) were completed. During the study phase (day 1-8), the patients remained hospitalised for collection of blood samples and excreta. Collection of urine and faeces was continued until the radioactivity levels of 24 h collections were below 1% of the administered dose. Patients received 1.4 mg/m^2 unlabelled eribulin mesylate on Day 8 of Cycle 1. If, however, sample collection was continued beyond day 8, the Day 8 dose was delayed until completion of the sample collection. From cycle 2 onwards, patients could enter the extension phase of the trial and receive 1.4 mg/m^2 unlabelled eribulin mesylate on Days 1 and 8 of each subsequent 21-day cycle. Patients were permitted to continue their participation in the study and receive eribulin treatment if they had stable disease, partial or complete response to treatment, assessed according to response evaluation criteria in solid tumours (RECIST).

The safety of eribulin was assessed by measuring vital signs, laboratory testing, physical examinations and by documenting concomitant medication and adverse events.

The study was conducted in accordance with the ICH guidelines for Good Clinical Practice (CPMP/ICH/135/95), the European Clinical Trials Directive (2001/20/EC) and the Declaration of Helsinki. The protocol was approved by the Netherlands Cancer Institute Independent Ethics Committee.

^{14}C-Eribulin drug formulation and administration

Individual aseptic preparations of ^{14}C-eribulin infusions were prepared in a laminar flow cabinet, situated in a laboratory suited for handling radio-labelled drugs. ^{14}C-eribulin acetate was provided in vials containing 0.5 mL ethanol with 10 mg/mL eribulin acetate, at a specific activity of 40 µCi/mg (chemical and radiochemical purity >99.6%). A volume of 0.2 mL was extracted from the vial, added to 5 mL of sterile water for injection and combined with 45 mL normal saline (0.9% NaCl). The solution

was filtered through a 0.22 µm syringe filter (Millipore Millex, Billerica, MA, USA). A 0.5 mL aliquot was separated from the final dosing solution for analysis, and two 200 µL aliquots thereof were mixed with 10 mL liquid scintillation cocktail, analysed using liquid scintillation counting and used to calculate the total radioactivity of the final dosing solution. The final dosing solution was administered to the patient by a syringe pump as a 2-5 min bolus infusion, which was followed by administration of normal saline to flush the lines. Remaining radioactivity was determined in both tubing and syringe. This was done by flushing them with a known volume of normal saline and analysis of a 1 mL aliquot for total radioactivity. The actual administered activity was calculated by subtracting the radioactivity remaining in the dosing system from the radioactivity in the final solution.

Sample collection

Venous blood samples (6 mL) were collected pre-dose, at the end of the 2-5 min ^{14}C-eribulin infusion (EoI) and at 5 min, 15 min, 30 min, 1, 2, 4, 6, 8, 10, 24, 48, 72, 96, 120, 144 and 168 h after EoI. The blood samples were collected in lithium heparinised tubes, inverted gently and centrifuged at 2,000 g and 4ºC for 15 min. Two 0.2 mL aliquots of the plasma layer were transferred to scintillation vials for total radioactivity (TRA) determination and the remainder was divided over 2 labelled polypropylene (PP) tubes for quantitative bioanalysis and for metabolite profiling and identification.

Whole blood samples (4 mL) were collected pre-dose, at EoI and 5 min, 15 min, 30 min, 1, 2, 4, 8, 24, 72 and 168 h after EoI. Two 0.2 mL aliquots were transferred to scintillation vials to measure TRA and the remainder was divided over 2 labelled PP tubes for quantitative bioanalysis.

Urine samples were collected before ^{14}C-eribulin administration, over 6 h periods for the first 48 h after administration and then over 24 h periods until day 8 or longer, i.e. until the daily urinary recovery was <1% of the administered activity. Total mass and collection time were recorded, a 1 mL aliquot was transferred to a scintillation vial to determine TRA and 5 and 25 mL aliquots were stored for quantitative bioanalysis and for metabolite profiling and identification.

Pre-dose faecal samples were collected and all faecal portions produced after administration were individually collected in pre-weighed containers until day 8 or longer, i.e. until the daily faecal recovery was <1% of the administered activity. Samples were stored refrigerated until homogenisation with water in a 1:3 (w/v) ratio. Three aliquots of 0.2 mL were used to determine TRA, 1, 5 and 25 mL aliquots were stored for quantitative bioanalysis and for metabolite profiling and identification.

All samples were stored at nominally -70ºC. The samples were collected for: (i) measurement of TRA with LSC (to determine concentrations of all eribulin-related ^{14}C-labelled compounds combined), (ii) quantitative bioanalysis with LC-MS/MS (to determine concentrations of unchanged eribulin), (iii) metabolite profiling with LC-LSC-MS/MS (to detect and quantitate eribulin metabolites) and (iv) metabolite identification with LC-LTQ Orbitrap MS.

Total radioactivity

After recording the exact sample sizes by mass, radioactivity was determined by liquid scintillation counting. Before adding scintillation cocktail, whole blood and faecal homogenate samples were prepared (dissolved and decolourized) similar to the procedures described in (van den Bongard et al., 2002). One mL Solvable^{TM}, 1 mL isopropanol and 0.4 mL 30% hydrogen peroxide were added to faecal homogenates (triplicate) and 1 mL Solvable^{TM}, 100 µL 0.1 M EDTA and 0.5 mL 30% hydrogen peroxide were added to whole blood samples (duplicate). Subsequently, both faecal homogenates and whole blood samples, the latter only after at least 1 h storage in the dark at room temperature, were gently shaken in a water bath (Salm en Kipp, Breukelen, the Netherlands) at 40-45ºC until decolourization. After cooling down, these samples were treated identically to plasma (duplicate) and urine (singular) samples: 10 mL liquid scintillation cocktail was added and samples were stored in the dark for at least 1 h.

Counting was then performed on a Tri-Carb 2800TR liquid scintillation counter (Perkin Elmer), employing a ^{14}C counting protocol with automatic quench correction and a maximum counting time of 60 min per sample.

Quantitative bioanalysis

Concentrations of unchanged eribulin in plasma, whole blood, urine and faeces were measured using validated LC-MS/MS assays as described elsewhere (Dubbelman et al., 2011). Briefly, liquid-liquid extraction was used for sample clean-up. Supernatants of plasma, whole blood and urine were concentrated and extracts of all matrices were filtered. Final extracts were injected onto a C18 column and eluted using a gradient with 0.1% formic acid in water and ACN. An electrospray ionisation source produced positive ions that were detected with a triple quadrupole MS. Using multiple reaction monitoring, the mass transition of *m/z* 730 → 712 was recorded for eribulin and 731 → 681 for the internal standard ER-076349. The validated ranges were 0.2 – 100 ng/mL for plasma, 0.5 – 100

ng/mL for urine and whole blood and 0.1 – 25 µg/g (undiluted faeces) for faeces. Quality control samples were prepared and assayed together with the study samples. Criteria for the acceptance of bioanalytical data during routine drug analysis, as described in the FDA guidelines (FDA, 2001), were applied.

The ratio of ^{14}C-eribulin to ^{12}C-eribulin in the dosing solution, as determined by LC-MS, was 1.03. As only ^{12}C-eribulin was quantitated with these bioanalytical assays, the concentrations were multiplied by 2.03 to obtain the total eribulin concentrations.

Metabolite profiling and metabolite identification

Sample preparation

To check for the presence of major metabolites, plasma samples collected up to 60 min after EoI were pooled across patients, to obtain a single 300 µL plasma sample for each time point, containing equal volumes from each patient.

Additionally, plasma samples of individual patients from EoI and 5 min, 15 min, 60 min, 2, 6, 24, 72 and 144 h after EoI were processed and analysed if the radioactivity in the extracts was considered sufficient for radiochromatography. Both urine and faecal samples were pooled within patients over from 0-24 h, 24-48 h, 48-72 h and 72-168 h, in proportion to the total mass of the excreta.

To process plasma samples for metabolite profiling, proteins were precipitated by adding 900 µL of MeOH:ACN (50:50, v/v) to 300 µL plasma. The mixture was vortex mixed for 30 s and centrifuged for 10 min at 23,100 g. The clear supernatant was transferred to a clean 1.5 mL vial, evaporated to dryness under a gentle nitrogen stream at 40ºC and reconstituted in 100 µL of 20 mM ammonium acetate pH 5:ACN (70:30, v/v) by vortex mixing for 10 s, followed by sonication for 1 h. After centrifuging for 10 min at 23,100 g, a 10 µL aliquot of the final supernatant was mixed with 4 mL scintillation cocktail and assayed for radioactivity using LSC, to calculate the recovery of the sample preparation. The remaining supernatant was transferred to an autosampler vial and used for LC-LSC-MS/MS analysis (described later). Additionally, for each sample, the pellets formed after the first and second centrifugation step were mixed with 1 mL Solvable^{TM}, dissolved by sonication for 1 h and analysed for TRA.

For metabolite profiling, pooled urine samples were injected directly without sample preparation. For metabolite identification, a single urine sample, collected 0-6 h after infusion, was lyophilised. To this

end, a 25 mL sample of frozen urine was transferred to a freeze-dryer (Snijders, Tilburg, The Netherlands) operating at -80ºC. Vacuum was applied until the sample was totally dehydrated. The dried sample was reconstituted in 1 mL water. After centrifugation for 10 min at 23,100 g, the supernatant was used for LC-LTQ Orbitrap MS.

Faecal homogenates were prepared for metabolite profiling by extracting 100 µL aliquots of faecal homogenates with 100 µL ACN. The mixtures were then vortex mixed for 30 s, shaken for 10 min at 1,250 rpm on an automatic shaker (Labinco, Breda, the Netherlands), vortex mixed another 30 s and centrifuged for 10 min at 23,100 g. This process was repeated with the pellet remaining after transfer of the clean supernatants to empty vials. After the second extraction, the pellet was dissolved and analyzed for radioactivity, identically to the plasma pellets. The supernatants of the first and second extraction were combined and vortex mixed after addition of 300 µL water. An aliquot of 50 µL was mixed with 4 mL scintillation cocktail and radioactivity was determined to calculate the recovery of the sample preparation. The remainder was distributed over autosampler vials for metabolite profiling. For metabolite identification, the faecal homogenate with the highest radioactive concentration was further concentrated by evaporation. Two 500 µL aliquots of faecal homogenate were mixed with 500 µL ACN and shaken and centrifuged identically to the other faecal homogenates. In 4 h, the supernatants were partially evaporated at 45ºC in a SpeedVac (SPD1010, Thermo Scientific, Waltham, MA, USA). After centrifuging for 5 min at 23,100 g, the clear supernatants were combined and the volume was adjusted to 500 µL with ACN.

LC-LSC-MS/MS and LC-LTQ Orbitrap MS

For both metabolite profiling and metabolite identification, chromatography was performed using a 1 h gradient containing 2 mobile phases (A and B) on a Synergi Polar RP column (150 x 4.6 mm, particle size 4 µm) (Phenomenex, Torrance, CA, USA) preceded by an in-line filter (0.2 µm, Upchurch scientific, Oak Harbor, WA, USA). Mobile phase A consisted of 20 mM ammonium acetate at pH 5 and mobile phase B of 100% ACN. Starting at 3% B, the percentage B linearly increased to reach 5% at 5.0 min, 10% at 15.0 min, 20% at 25 min, 40% at 35 min, 60% at 45 min, and 80% at 50 min, where it was maintained until 54.9 min. From 55 to 60 min, the column was re-equilibrated at 3% B. The flow rate was set to 1.0 mL/min, the injection volume to 50 µL (20 µL for the high resolution mass spectrometry) and during the first 4 minutes the eluent was directed to waste.

The system used for metabolite profiling consisted of an Accela HPLC pump coupled to a LTQ XL linear ion trap mass spectrometer equipped with an electrospray ionisation probe (Thermo Fisher Scientific, Waltham, MA, USA). The mass spectrometer operated in positive ion mode, with a spray voltage of 5.4 kV, a capillary temperature of 300ºC and a capillary voltage of 6.5 V. The sheath, auxiliary and sweep gas flow were optimised to 60, 10 and 5 arbitrary units, respectively. Wideband activation was enabled, the scan range was 100-1100 amu, the isolation width 2.0 and the normalised collision energy used for collision induced dissociation was 35%. To collect MS^2 and MS^3 spectra, data dependent acquisition was performed based on a parent list. This list contained masses of eribulin and hypothetical metabolites (e.g. products of single or multiple hydroxylation) and was expanded when additional potential metabolites were found.

Radiochromatograms were generated using HPLC coupled to a post-column accurate flow splitter (LC Packings, Sunnyvale, CA, USA), directing ¼ of the flow to a linear ion trap MS and ¾ to a fraction collector (LKB-FRAC-100, Pharmacia, Amersham Biosciences, Uppsala, Sweden) to collect fractions for radioactivity measurement using LSC. The fraction collector collected the eluent in 6 mL plastic LSC vials, at a rate of 1 min/vial (or in 20 mL vials at a rate of 5 min/vial in regions containing low radioactivity levels). After addition of 4 mL (or 10 mL) liquid scintillation cocktail, the fractions were assayed for TRA for 20 min per vial with LSC. Radiochromatographic profiles were prepared by plotting the net disintegrations per minute against the time after injection.

For metabolite identification, high-resolution mass spectrometry was applied to the concentrated urine and faecal samples. The equipment used for these samples consisted of a Finnigan Surveyor MS pump Plus which was coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Pump and mass spectrometer settings were identical to those described above, except for the normalised collision energy, which was set at 40%. Additionally, the resolution was set on 60,000.

Calculations

The metabolite concentrations in plasma and total amounts excreted in urine and faeces were derived from the radiochromatograms. Concentrations were calculated by dividing the activity of the fraction presenting the metabolite (in disintegrations per minute, dpm) by the injection volume (50×10^{-3} mL), the split ratio (¾), the specific activity (88.8 dpm/ng eribulin, corresponding with 40 µCi/mg eribulin), the sample preparation recovery (only for plasma and faeces, 100% for urine) and the concentration

factor (3 for plasma, 1 for urine and 1/6 for faecal homogenates). To obtain the total amounts, the concentrations in urine and faecal homogenates were multiplied with the total volume of the pooled urine and pooled faecal homogenate, respectively.

The limit of detection (LOD) and lower limit of quantitation (LLOQ) of the LSC results were based on the formulas described by Currie (Currie, 1968; Zhu et al., 2005), using a maximum counting error of 5% for total radioactivity measurements and 20% for metabolite profiling. The LLOQ for TRA determination was 0.304 ng eq/mL in urine and 1.52 ng eq/mL in plasma, whole blood and faeces. The LOD and LLOQ for metabolite profiling were 4 and 14 dpm per collected fraction, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameters of eribulin and total radioactivity were calculated using non-compartmental analysis with WinNonlin™ Professional (version 5.1.1, Pharsight Corp, Mountain View, CA, USA). For plasma radioactivity, the pharmacokinetic parameters determined included maximum concentration (C_{max}), the terminal phase half-life ($t_{½}$) and the area under the plasma concentration time curve (AUC). For eribulin in plasma also the clearance (CL), the renal clearance (CL_r) and the apparent volume of distribution (V_z) were determined.

Results

Patients

Four male and two female patients were enrolled in this study. All patients were Caucasian, had an ECOG-PS of 0 or 1, a median age of 60.5 years (range 34 - 70), a median weight of 70.5 kg (range 55.0 - 175), a median height of 172.5 cm (range 162.0 - 210.0) and a median body surface area of 1.9 m^2 (range 1.6 - 3.2). The patients had metastatic cancer with the primary tumour in one of the following locations: lung, testis, nasopharynx, ovaries, esophagus and prostate.

Total radioactivity and quantitative bioanalysis

Pharmacokinetics

Plasma concentration/time curves of unchanged eribulin and TRA are presented in Figure 2 and Table 1 summarises the pharmacokinetic parameters. Since the actual administered dose ranged from 1.75 to 3.01 mg, the dose-normalised values for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were also calculated and presented in Table 1.

To obtain a standard infusion time of 5 min, the dosing solution was administered at a flow of 10 mL/min. T_{max} was typically observed at the end of infusion. The values for dose-normalised C_{max} and exposure ($AUC_{0-\infty}$) to unchanged eribulin (222 ng/mL/mg and 328 ng.h/mL/mg, respectively) were comparable to those of TRA (224 ng eq/mL/mg and 357 ng eq.h/mL/mg, respectively). The $t_{½}$ value for eribulin (45.6 h) was similar to that of the total radioactivity (42.3 h). Figure 3 shows the time courses of eribulin and TRA distribution as the whole blood to plasma ratios. For eribulin as well as TRA, the whole blood to plasma ratio approximated unity.

Excretion

For all six patients, urine and faeces was collected as planned during the first 168 h after administration of ^{14}C-eribulin. Thereafter, four of the patients continued collecting faeces for up to a maximum of 312 h, until the daily recovery of radioactivity in faeces was <1% of the administered radioactivity.

Figure 4 shows the cumulative excretion of unchanged eribulin and TRA in urine and faeces. The mean (± SD) recovery of total radioactivity in the combined excreta (faeces and urine) after 312 h was 90.4 ± 11.7% of the administered activity (81.5 ± 13.4% in faeces and 8.9 ± 4.0% in urine). The total contribution of unchanged eribulin was 68.6 ± 14.1% of the administered dose.

Within 168 h, the minimal excreta collection period for all patients, 86.5 ± 16.4% of the administered activity was excreted, with unchanged eribulin accounting for 68.0 ± 13.1%. Most of the administered radioactivity (77.6 ± 19.0%) was excreted via faeces and a minor part (8.9 ± 4.0%) via urine.

Metabolite profiling

Plasma

The mean sample pre-treatment recovery of radioactivity in pooled and individual plasma extracts used for metabolite profiling was 87.7 ± 4.6%. Similar amounts of non-extractable radioactivity were found in the first (4.3 ± 1.8%) and second (4.1 ± 1.9%) pellet that were formed during sample preparation.

Radiochromatograms of the pooled plasma samples collected up to 1 h after EoI revealed the presence of a single major radioactive component, accounting for over 90% of the injected radioactivity (data not shown). The mass spectrum at this retention time showed a protonated molecular ion at m/z 730 with a ^{14}C isotope peak at m/z 732 (Fig. 5 A) that was not present in the pre-dose plasma sample. MS^2 fragmentation of this ion was identical to the fragmentation pattern of the reference standard (not shown) of eribulin (Fig. 5 B), and therefore this peak was identified as eribulin. Major metabolites were not present in these samples, as other radioactivity containing fractions constituted only a maximum of 0.6% of the eribulin peak.

In the individual plasma extracts, the radioactivity of the samples collected at 48 and 72 h after EoI was below LLOQ for all and below LOD for most patients. Therefore, only samples collected up to 24 h after EoI were analyzed with LC-LSC-MS/MS. Eribulin represented the main peak of the radiochromatogram of all these samples (representative example Fig. 6 A). Small amounts of radioactivity eluted shortly before and after eribulin, mainly in samples drawn at EoI and 5 min after EoI. Four elution fractions contained a radioactivity level above the LLOQ in one or more patient samples and were designated as eribulin metabolites MP1 to MP4. Table 2 provides an overview of these metabolites, summarising the retention times, the number of patients in which the metabolites were detected in plasma, the time points at which the metabolites were detected and the maximum relative and absolute concentrations that were detected in a sample. The most prominent metabolites were MP2 and MP3, observed in plasma of 5 and 4 patients, respectively, and both representing maximally ≤1% of the eribulin concentration.

Urine

The radiochromatograms of the urine samples, of which Fig. 6 B is a representative example, show very little metabolism, similar to the plasma samples. The main radioactive peak eluted after 43-44 min and was identified as eribulin based on the MS and MS^2 spectra. Only a single radioactive peak other than eribulin was above the LLOQ in two patients within the 0-24 h urine pools. This peak, designated MU1, had a maximum of 3.48% of the eribulin peak and accounted for a maximum of 0.42% of the administered dose (see Fig. 7).

Faeces

Sample preparation of faecal homogenates with an evaporation and reconstitution step, similar to plasma samples, resulted in <80% recovery. It was not feasible to resolve the analytes from the dried residue using a solution compatible with the LC system. Therefore, faecal homogenates were extracted using a minimal extraction volume and the supernatant was further diluted with water until the final composition was compatible with the LC system. This approach increased recovery to 87.5 ± 6.0%.

Only small amounts of radioactivity corresponding to metabolites were present in faecal homogenates (representative example Fig. 6 C), with the largest peak in all samples identified as eribulin. The metabolite peaks were not resolved, complicating their designation. At least four metabolites appeared to be present. These were designated MF1 to MF4 and accounted for a maximum of 0.61, 0.85, 2.79 and 0.61% of the administered dose (Fig. 7).

Metabolite identification

To identify the *m/z* value of the metabolites, LC-MS spectra of pre-dose and post-dose samples were compared at the retention time of each radioactive peak. Post-dose samples were also scanned for the specific isotope pattern (Fig. 5 A) of two peaks of equal intensity with a difference of 2 in *m/z* value (due to the ^{14}C-isotope).

Plasma samples were concentrated by a factor 3 for plasma profiling, which resulted in insufficient concentration to obtain MS signals for metabolite identification. Therefore, the identity of the metabolites MP1 to MP4, each with a maximum concentration ≤1% of the eribulin concentration, was unresolved.

Since a large volume of urine was available for each collection interval, a considerable concentration step (25 times) was required for sufficient MS response. The urine sample with the highest MU1 concentration was concentrated 25 times and analyzed using LC-LTQ Orbitrap MS. At the retention time of MU1, the MS spectrum showed the specific $^{12}C/^{14}C$-eribulin related pattern, with a parent *m/z* value of 748.4244. The MS^2 spectrum of MU1 showed the same fragmentation pattern as eribulin, with subsequent losses of water and methanol, however with an 18 Da shift to the right. Based on the high-resolution mass determinations, this metabolite was identified as eribulin+H_2O. Further elucidation of the molecular structure was not possible due to the unspecific fragmentation.

The faecal homogenate containing the highest radioactivity level was concentrated twice. The concentration of MF1 was still insufficient for MS detection, but MF2, MF3 and MF4 were detected. Based on the high-resolution mass determinations, they were identified as eribulin+O, eribulin+O and eribulin+H_2O, respectively. Similar to MU1, the MS^2 spectra of MF2, MF3 and MF4 showed the same pattern as eribulin (Fig. 5 B), with a mass shift of 16, 16 and 18 Da, respectively. Also for these metabolites the unspecific fragmentation in the MS^2 spectrum impeded further structural elucidation. Table 3 summarises the main characteristics of the metabolites detected in urine and faeces: retention times, number of patients in which the metabolites were detected, time periods in which they were detected, maximum percentage of administered dose recovered as this metabolite (compared between the 6 patients), *m/z* value, proposed identity, theoretical *m/z*-value of the proposed identity and the difference between the theoretical and measured *m/z*-values.

Safety

The ^{14}C-eribulin dose was generally well tolerated. A total of 4/6 patients experienced at least one treatment-related adverse event (AE) during the study phase. The most common AE reported as treatment-related was fatigue (n=3). The majority of AEs were of grade 1 or 2; grade 3 AEs were reported for one patient. There were no deaths or serious adverse events, no dose reductions or delays due to AEs and no patients were withdrawn from study treatment due to an AE. No significant abnormalities in laboratory parameters or obvious changes in vital signs were observed. Five patients continued treatment with eribulin mesylate after the study phase at a dose of 1.4 mg/m^2 on day 1 and 8 of 21-day cycles; one patient discontinued after completion of the assessments of the study phase and prior to entering cycle 2.

Discussion

This study investigated the metabolism and excretion of eribulin in humans. It was found that eribulin undergoes limited metabolism and is primarily excreted unchanged via faeces (61.3% within 168 h). Four minor metabolites were detected in plasma, one in urine and four in faeces. Those that were identified had mass differences of +16 (+O) and +18 (+H_2O) relative to unchanged eribulin.

The plasma pharmacokinetic properties of eribulin are comparable to those reported in previous Phase I studies (Goel et al., 2009; Tan et al., 2009). The plasma concentration-time curve shows that both total radioactivity and unchanged eribulin are rapidly distributed and then slowly eliminated. This profile, as well as the extensive volume of distribution, was demonstrated before (Goel et al., 2009). Also the average $t_{½}$ of 45.6 h for eribulin was within the range of previously reported values for terminal half-life of 36 to 48 h (Cigler and Vahdat, 2010). Additionally, the recovery of unchanged eribulin in urine (8.1% after 168 h, Figure 7) is consistent with other Phase I studies, wherein 5 to 6% (Goel et al., 2009) and 7% (Tan et al., 2009) of the administered eribulin was recovered unchanged within 72 h after a single dose.

The plasma to whole blood ratio of both TRA and unchanged eribulin approximates unity, suggesting no preferential distribution of eribulin or eribulin-derived compounds to either red blood cells or plasma compartments. The comparable pharmacokinetics for eribulin and total radioactivity is indicative of limited metabolism. This was confirmed by the metabolic profile of eribulin in plasma: metabolites constituted ≤0.6% of the parent compound concentration in pooled plasma samples following 60 min after EoI and individual plasma samples revealed only four minor metabolites, MP1 to MP4, of which the most abundant comprised maximally 1% of the eribulin concentration. The low concentrations of the plasma metabolites impeded detection with LC-MS/MS; consequently, definite identification was not possible. Based on retention time only, MP1 could be identical to the urine metabolite MU1, and MP3 to the faecal metabolite MF4. Since the plasma metabolites were only detected in a few samples collected up to 15 min after the end of infusion and had a concentration of maximally 1% of the eribulin concentration in one patient, their overall systemic exposure does not exceed 10% of the parent compound, neither 10% of the total drug related exposure. This means that further tests to evaluate the safety of the metabolites may not be required according to the MIST guidelines (FDA, 2008; ICH, 2009).

The total recovery of the administered radioactivity, 86.5% at day 8 (168 h) slowly increasing to 90.4% at day 14 (312 h), is generally acceptable for a mass balance study (Beumer et al., 2006). Expired air was not analysed for the presence of $^{14}CO_2$, but considering the stability of eribulin, it is not expected that the production of $^{14}CO_2$ has contributed significantly to the loss of radioactivity, even though the ^{14}C-label was not incorporated in the ring structure of eribulin.

The quantitation of unchanged eribulin in faeces samples of one patient was not possible due to interference with large amounts of polyethylene glycol (PEG) in the samples. The PEG caused a dramatic ion suppression of eribulin in the quantitative LC-MS/MS assay, hampering reliable quantitation. The source of the PEG was probably macrogol, which was used daily by this patient to prevent constipation. Because of the interference, the LC-MS/MS results for eribulin in faeces of this patient were not included to calculate mean recoveries. PEG did not interfere with the quantitation of eribulin in plasma and urine of this patient since it appeared to be unabsorbed.

The total radioactivity that was recovered in faeces after 168 h (77.6% of the administered dose) is not completely accounted by the presence of unchanged eribulin (61.3% of the dose) and the identified metabolites MF1 to MF4 (<5% of the dose) (Figure 7). This difference can be explained by at least four factors: (i) unattributed radioactivity in the radiochromatograms, (ii) difference in LLOQ between bioanalysis and TRA analysis, (iii) incomplete recovery of eribulin during bioanalysis and (iv) presence of non-extractable metabolites. We will shortly discuss these factors and their quantitative impact.

First, not all radioactivity in the radiochromatograms of faecal samples was attributed to eribulin or one of the four identified metabolites. As the peaks were not completely resolved, a part of the unattributed radioactivity could be eribulin, MF1, MF2, MF3 and/or MF4. The other part comprises cumulative amounts of radioactivity below the LLOQ and could contain additional metabolites. On average, 5% of the administered dose was recovered in faeces, but unattributed.

Second, the LLOQ for the (LC-MS/MS) quantitation of unchanged eribulin (0.1 µg/g) was higher than the LLOQ for total radioactivity (LSC, 1.52 ng eq/g) in faeces. Consequently, samples with a concentration just below the LLOQ for quantitative bioanalysis were included to calculate TRA, but excluded when calculating the total amount of unchanged eribulin. Due to this difference in LLOQ, the underestimation of unchanged eribulin recovery in faeces would on average be 2% of the

administered dose, assumed that the ratio of unchanged eribulin to TRA was equal for all samples of the same patient.

Third, the recovery of unchanged eribulin during the bioanalysis of faeces samples may have been incomplete, which may also have resulted in an underestimation of the unchanged eribulin recovery. This would be the case if faeces samples spiked with eribulin during assay validation have a higher extraction recovery than real-life patient samples or if the extraction recovery was dependent on the substance of the original non-homogenized faecal samples. At least the latter was suspected, as it was observed that for metabolite profiling, the highest extraction recoveries were obtained from watery faeces samples, while originally dry and hard faeces samples resulted in lower extraction recoveries. To obtain an indication of the underestimation of unchanged eribulin recovery in faeces due to incomplete eribulin recovery, the highest extraction recovery across patients and the extraction recoveries of individual patients were used. This resulted in an average potential underestimation of 6% of the administered dose.

Finally, the faeces samples could have contained unknown non-extractable metabolites. However, if these were present, their quantitative contribution was limited, as the three above mentioned factors already explained the main difference between TRA in faeces and unchanged eribulin plus metabolites MF1 to MF4.

The metabolites detected in urine and faeces differed by 16 and 18 mass units from eribulin. High-resolution mass analysis indicated incorporation of O and H_2O, respectively, in eribulin. Metabolites MF2 and MF3, with molecular masses of eribulin+16, may be products of hydroxylation. Eribulin metabolites with the same molecular masses were previously reported by Zhang *et al.* in an *in vitro* study with human liver microsomes (Zhang et al., 2008). That study showed that eribulin was primarily metabolized by CYP3A4, resulting in the formation of at least four mono-oxygenated metabolites. The described product ion spectra were similar to those we found for MF2 and MF3 in this study and, likewise, only comprised fragments formed by loss of water and/or loss of methanol. Due to the limited information provided by the MS^2 spectra, further structural elucidation was impossible. The metabolites MU1 and MF4, with a mass of eribulin+18, may be products of hydration of an alkene group or products of hydroxylation with reduction of the ketone at the C-1 position. Alternatively, they

may have been formed by hydrolysis of the ketal at the C-14 position. The exact structure of these metabolites remains undetermined.

The results of the present study may help to explain findings in other clinical studies. For example, the limited contribution of metabolism to the elimination of eribulin can explain that although eribulin is predominantly metabolized by CYP3A4 *in vitro* (Zhang et al., 2008), co-administration of the CYP3A4 inhibitor ketoconazole with eribulin to patients has no effect on eribulin exposure (Devriese et al., 2011a), and neither do CYP3A4 inducers or inhibitors on eribulin systemic clearance (Reyderman et al., 2011). Furthermore, the small contribution of urinary excretion to total excretion of eribulin may help to explain that eribulin is well tolerated at full dose in patients with moderate and severe renal dysfunction (Synold et al., 2010). Similarly, the important role of biliary excretion may explain that in patients with moderate hepatic impairment, dose reductions of eribulin are supported by a population pharmacokinetic / adverse event model based on data from Phase 1 and 2 studies (Devriese et al., 2011b). Hepatic impairment prolonged the elimination half-life of eribulin and the dose-normalised exposure ($AUC_{0-\infty}$) of patients with mild and moderate hepatic impairment was higher by 1.75-fold and 2.79-fold, respectively, compared with normal hepatic function (Devriese et al., 2011b).

In conclusion, eribulin was rapidly distributed and slowly eliminated following a 2-5 min IV bolus injection of ~2 mg (~80 µCi) ^{14}C-eribulin. Eribulin was primarily excreted unchanged in faeces and metabolism played only a minor role in the elimination. Renal clearance represented a minor component (<10%) in eribulin clearance. No major metabolites were found in plasma; each metabolite represented ≤0.6% of eribulin in pooled plasma samples.

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Authorship contributions

Participated in research design: Dubbelman, Rosing, Huitema, Koetz, Lymboura, Reyderman, Lopez-Anaya, Schellens, Beijnen

Conducted experiments: Dubbelman, Jansen

Contributed new reagents or analytic tools: Beijnen

Performed data analysis: Dubbelman, Jansen, Mergui-Roelvink, Reyderman, Lopez-Anaya

Wrote or contributed to the writing of the manuscript: Dubbelman, Rosing, Jansen, Mergui-Roelvink, Huitema, Koetz, Lymboura, Reyderman, Lopez-Anaya, Schellens, Beijnen

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Footnotes

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Figure legends

Figure 1: Chemical structure of ^{14}C-eribulin. The asterisk indicates the position of the ^{14}C-label.

Figure 2: Mean (± SD) log-linear plasma concentration-time curves of total radioactivity (TRA, by LSC) and eribulin (by LC-MS/MS) in plasma up to 144 h after the end of a 2-5 min IV infusion of ~2 mg (~80 µCi) ^{14}C-eribulin in cancer patients. Inset: first 6 hours.

Figure 3: Mean (± SD, n=6) whole blood to plasma concentration ratio of total radioactivity (TRA, by LSC) and eribulin (by LC-MS/MS), after a single IV dose of ~2 mg (~80 µCi) ^{14}C-eribulin to cancer patients.

Figure 4: Mean (± SD) cumulative urinary and faecal excretion of eribulin (by LC-MS/MS) and ^{14}C-eribulin derived radioactivity (by LSC) of cancer patients after a single IV dose of ~2 mg (~80 µCi) ^{14}C-eribulin. n=6, only for eribulin in faeces n=5, as the results of one patient were excluded due to LC-MS/MS interference with co-medication (macrogol)

Figure 5: Mass spectrum of radiolabeled eribulin, showing the typical pattern of the ^{12}C- and ^{14}C-eribulin peaks and their Na-adduct peaks (A) and MS^2 spectrum of eribulin (m/z 730) (B).

Figure 6: Representative radiochromatograms of a 5 min after EoI plasma sample (A), a 0-24 h urine sample (B) and a 48-72 h faecal sample (C), collected after administration of ~2 mg ^{14}C-eribulin (~80 µCi) to a cancer patient. The left vertical axes display the activity of the fraction and the right axes the corresponding concentration in ng eribulin equivalents /mL (for plasma) or the total amount excreted in that sample pool in µg eribulin equivalents (for urine and faeces). The insets show an enlargement of the region of metabolite elution, the lower limit of quantitation (LLOQ) is marked with a dashed line.

Figure 7: Overview of the average mass balance of ^{14}C-eribulin in 5 or 6 patients with advanced solid tumours during 168 h excreta collection after an IV-bolus injection of ~2 mg (~80 µCi) ^{14}C-eribulin. TRA: total radioactivity; BA: quantitative bioanalysis using LC-MS/MS, MP: metabolite profiling using LC-LSC. Max % of dose: maximum percentage of the administered dose that was excreted for a patient as this metabolite (assuming that excretion recovery was proportional for all radioactive material).

Table 1: Administered doses and pharmacokinetic parameters (mean ± SD) of eribulin and total radioactivity in cancer patients (n=6) after a 2-5-min bolus injection of ~2 mg (~80 µCi) of ^{14}C-eribulin.

Actual administered doses				
Dose	2.14 ± 0.44	mg		
Activity	85.5 ± 17.9	µCi		
Pharmacokinetic parameters				
	Eribulin		Total radioactivity	
C_{max}	444 ± 144	ng/mL	449 ± 137	ng eq/mL
$t_{½}$	45.6 ± 8.68	h	42.3 ± 17.2[a]	h
AUC_{0-t}	627 ± 386	ng.h/mL	568 ± 392	ng eq.h/mL
$AUC_{0-\infty}$	681 ± 425	ng.h/mL	753 ± 403[a]	ng eq.h/mL
CL	3.93 ± 2.10	L/h	-	
CL_r	0.30 ± 0.13	L/h	-	
V_z	247 ± 123	L	-	
Dose-normalized parameters				
	Eribulin		Total radioactivity	
C_{max}	222 ± 76.4	ng/mL/mg	224 ± 74.1	ng eq/mL/mg
AUC_{0-t}	301 ± 165	ng.h/mL/mg	269 ± 153	ng eq.h/mL/mg
$AUC_{0-\infty}$	328 ± 189	ng.h/mL/mg	357 ± 148	ng eq.h/mL/mg

Data are presented as mean ± SD.

eq: equivalents; C_{max}: maximum observed plasma concentration; $t_{1/2}$: terminal half-life; AUC_{0-t}: area under the plasma concentration-time curve; $AUC_{0-\infty}$: area under the plasma concentration-time curve from zero to infinity; CL: clearance; CL_r: renal clearance; V_z: apparent volume of distribution in the terminal phase.

[a]n=5 (for one patient too few data points were above the limit of quantitation to estimate an terminal disposition rate constant for the total radioactivity, therefore $t_{1/2}$ and $AUC_{0-\infty}$ could not be calculated for this patient)

Table 2: Eribulin metabolites detected in plasma samples of individual patients

Peak ID	**Retention time** min	**n**	**Timepoints**	**Max. relative concentration** % of eribulin peak	**Max. absolute concentration** ng eq/mL (% of eribulin)
MP1	35-36	1	EoI	0.31%	2.12 (0.31 %)
MP2	37-38	5	Upto 15 min after EoI	1.04%	3.64 (0.48 %)
MP3	42-43	4	Upto 15 min after EoI	0.99%	4.28 (0.56 %)
MP4	45-46	1	Upto 15 min after EoI	0.70%	2.15 (0.70 %)

n: number of patients for which this metabolite was observed in plasma; Max. relative concentration: maximum peak area of the metabolite relative to the eribulin peak area in the same radiochromatogram; Max. absolute concentration: maximum absolute concentration of the metabolite in a plasma sample, with corresponding relative concentration between parentheses

Table 3: Eribulin metabolites detected in urine and faeces

Peak ID	Retention time min	n	Time periods	Max. contribution % of administered dose	Accurate mass *m/z* $[M+H]^+$	Proposed ID	Theoretical mass *m/z* $[M+H]^+$	Δ ppm
MU1	35-36	2	0-24 h	0.42%	748.4244	Eribulin+H_2O	748.4267	-3.07
MF1	31-32	1	48-72 h	0.61%	unk	-	-	-
MF2	33-34	1	48-72 h	0.85%	746.4116	Eribulin+O	746.4110	0.80
MF3	35-37	2	24-72 h	2.79%	746.4111	Eribulin+O	746.4110	0.13
MF4	41-42	1	48-72 h	0.61%	748.4272	Eribulin+H_2O	748.4267	0.67

n: number of patients for which this metabolite was observed in urine / faeces; Max. contribution: maximum percentage of administered dose recovered as this metabolite (compared among the 6 patients); Δ ppm: deviation of accurate mass from theoretical mass in parts-per-million; unk: unknown

Figure 1

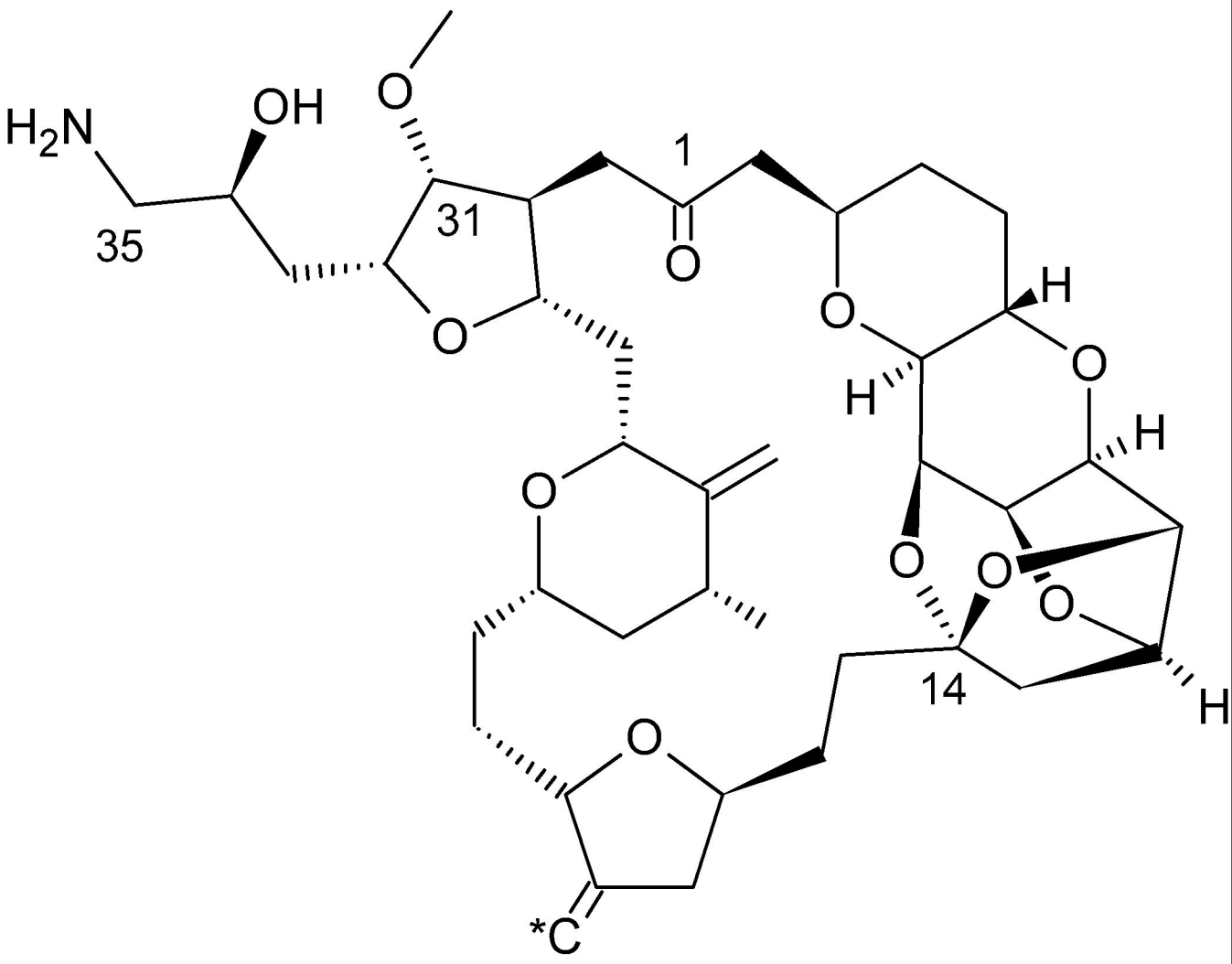


Figure 2

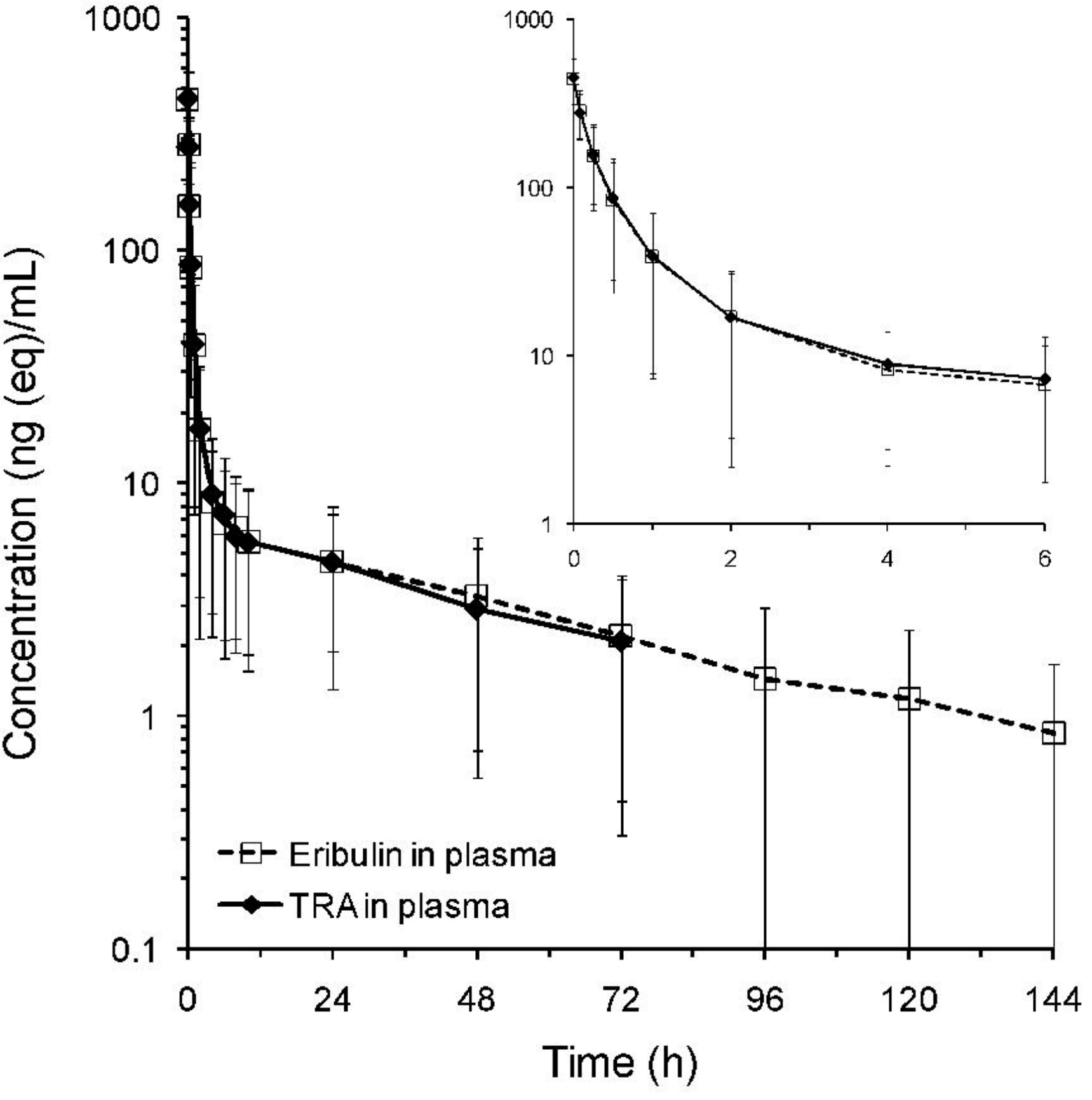

1000
100
10
1
0.1
Concentration (ng (eq)/mL)
0
24
48
72
96
120
144
Time (h)
Eribulin in plasma
TRA in plasma
1000
100
10
1
0
2
4
6

Figure 3

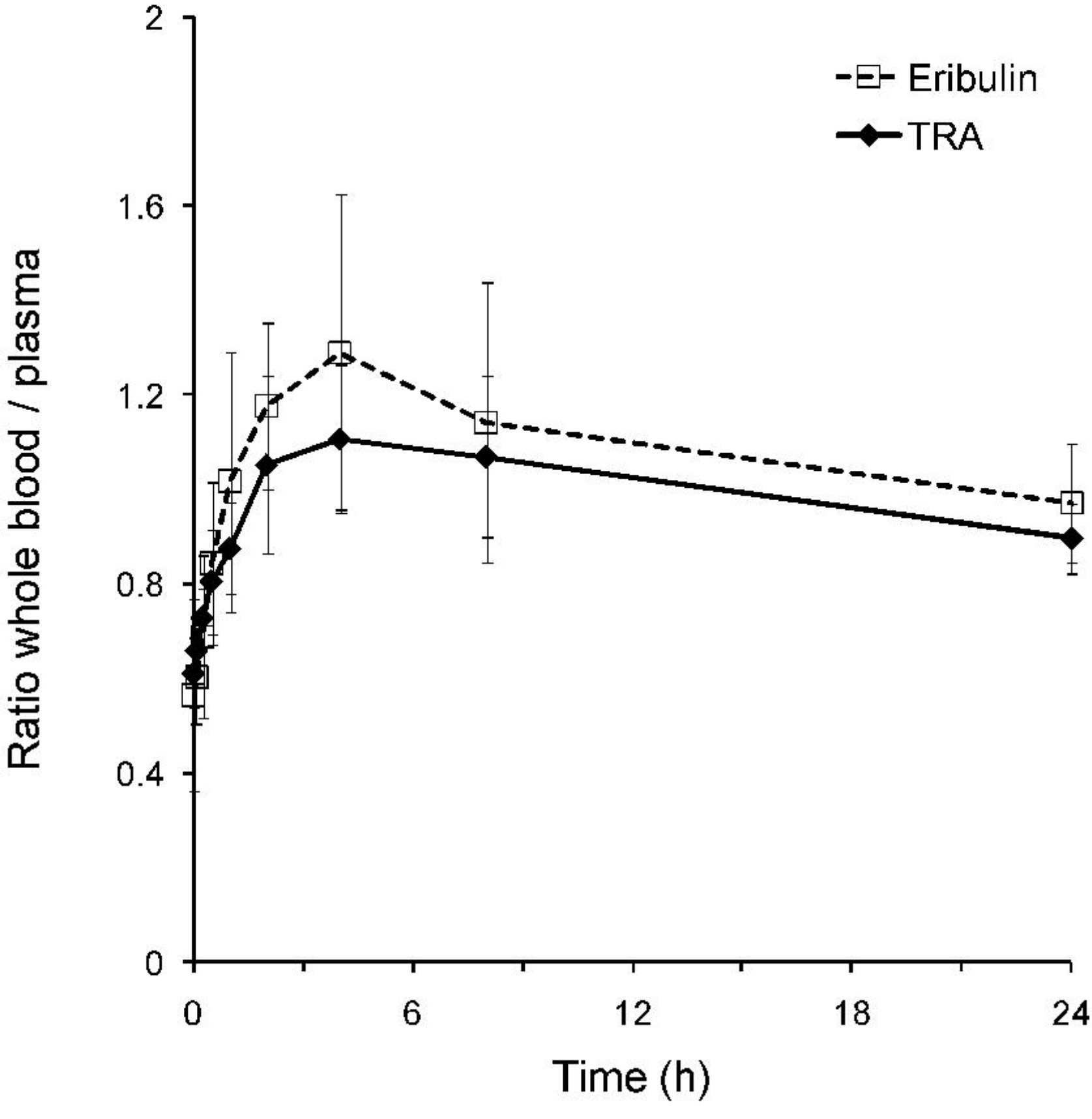

Eribulin
TRA
Ratio whole blood / plasma
2
1.6
1.2
0.8
0.4
0
0
6
12
18
24
Time (h)

Figure 4

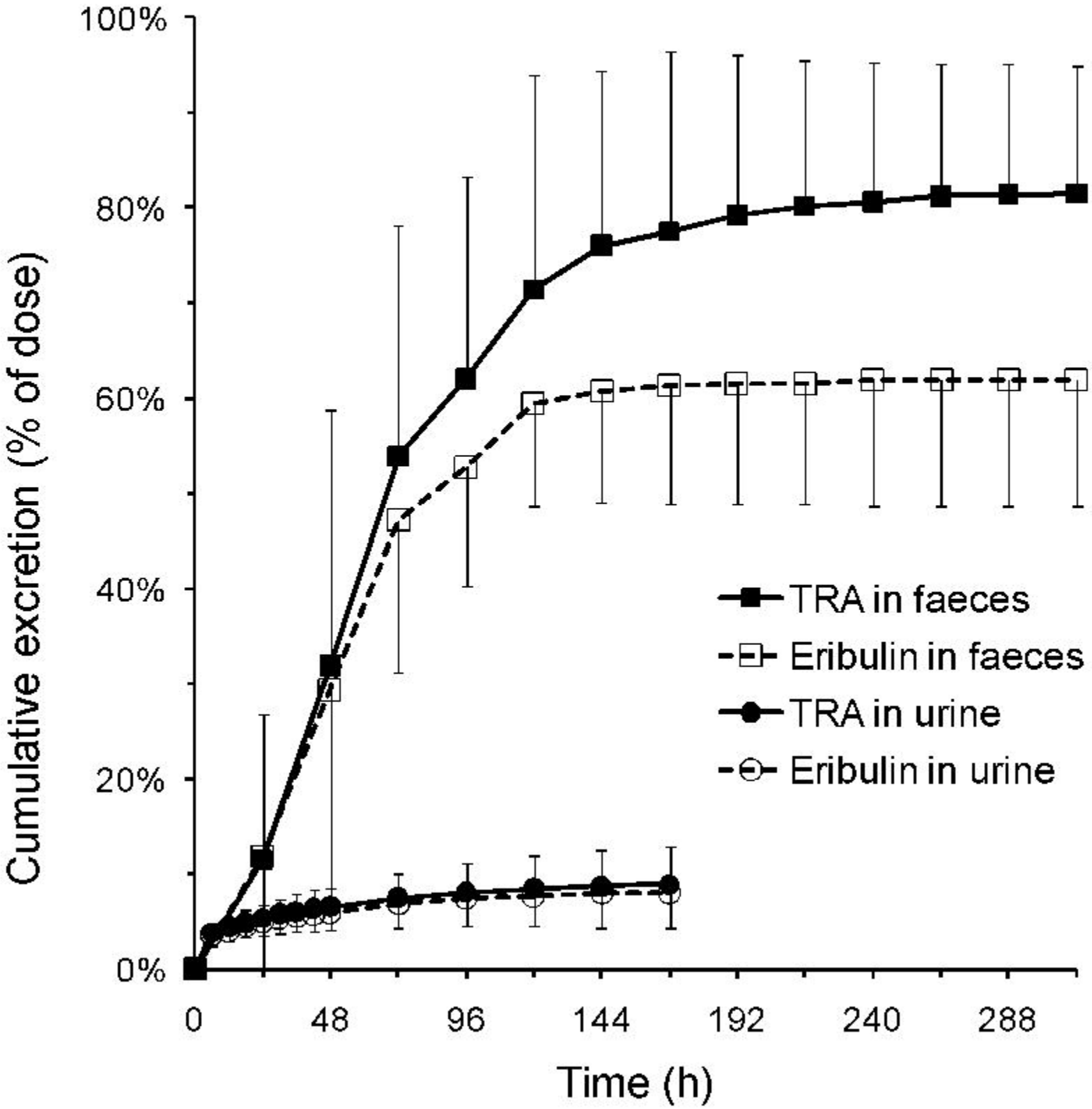

100%
80%
60%
40%
20%
0%
Cumulative excretion (% of dose)
0
48
96
144
192
240
288
Time (h)
TRA in faeces
Eribulin in faeces
TRA in urine
Eribulin in urine

Figure 5

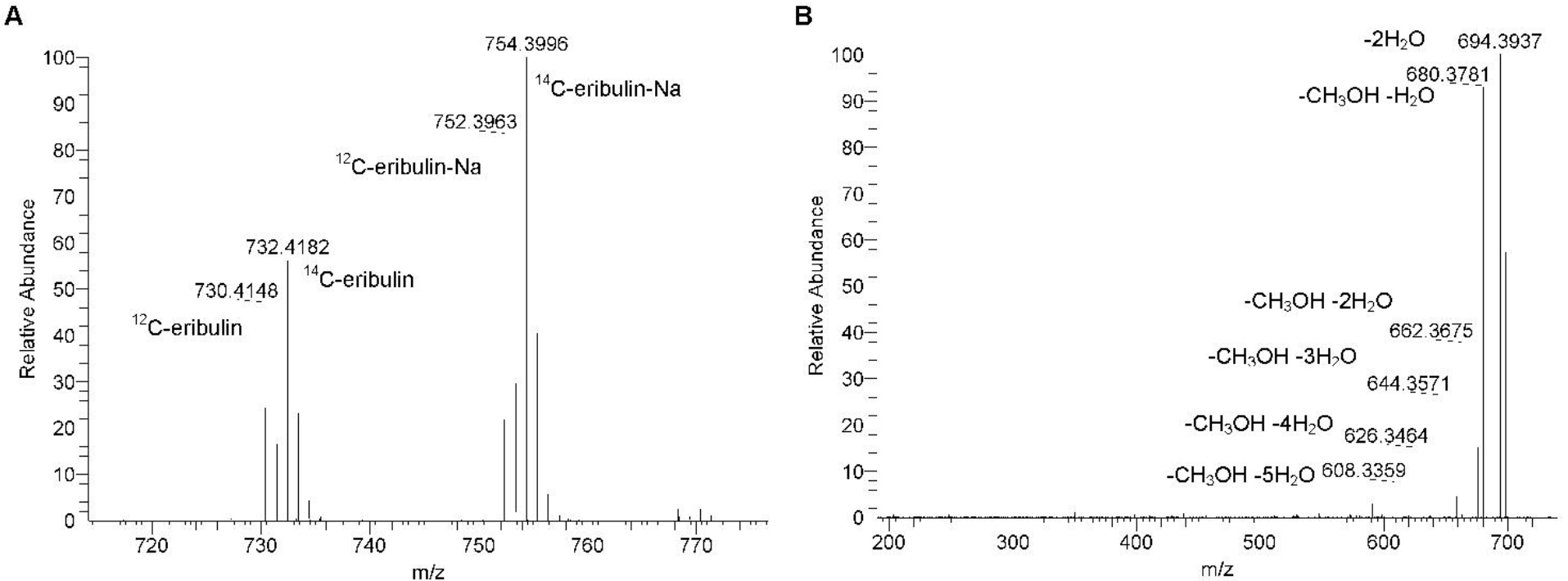

A
754.3996
14C-eribulin-Na
752.3963
12C-eribulin-Na
732.4182
14C-eribulin
730.4148
12C-eribulin
Relative Abundance
m/z
B
-2H2O
694.3937
680.3781
-CH3OH -H2O
-CH3OH -2H2O
662.3675
-CH3OH -3H2O
644.3571
-CH3OH -4H2O
626.3464
-CH3OH -5H2O
608.3359
Relative Abundance
m/z

Figure 6

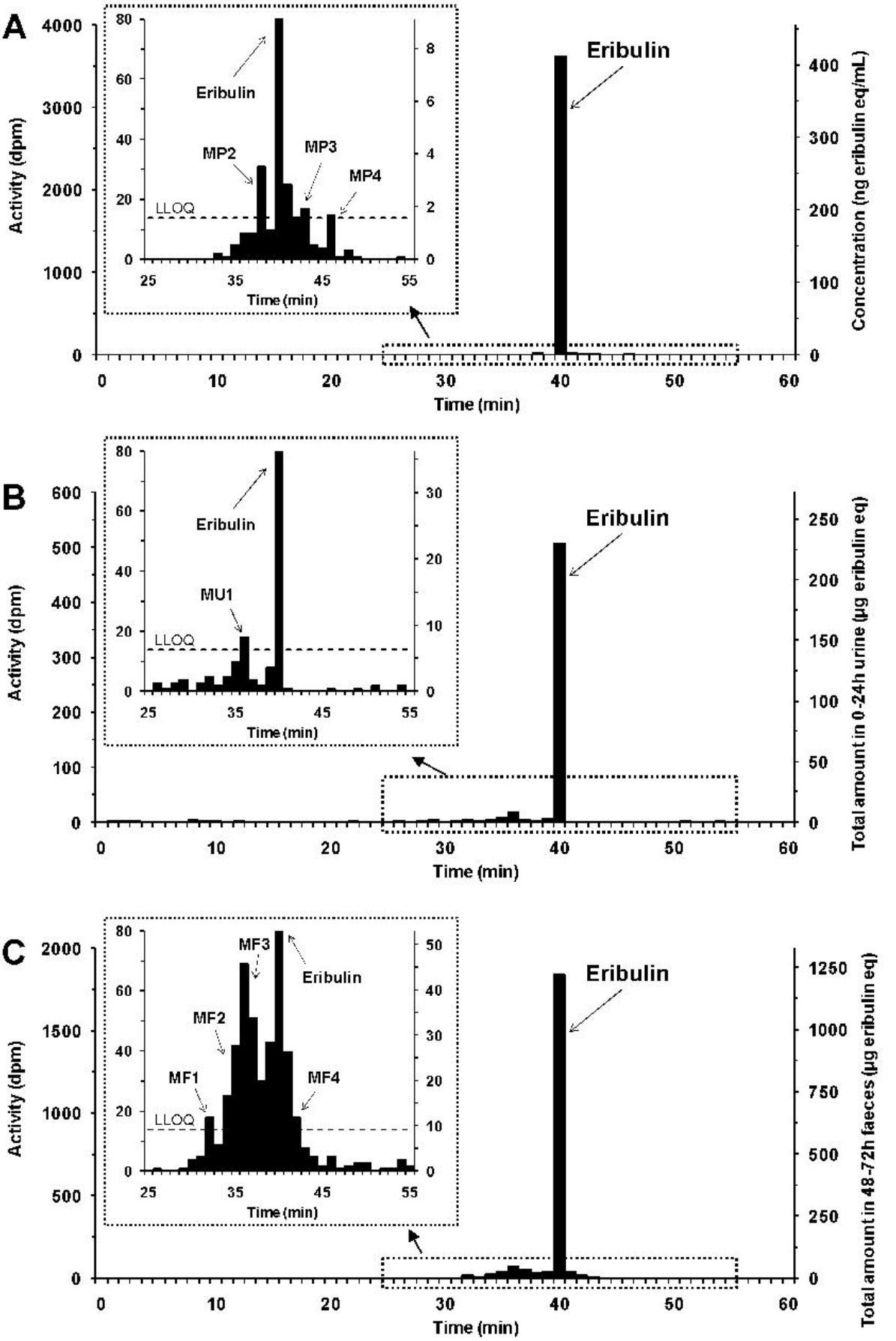
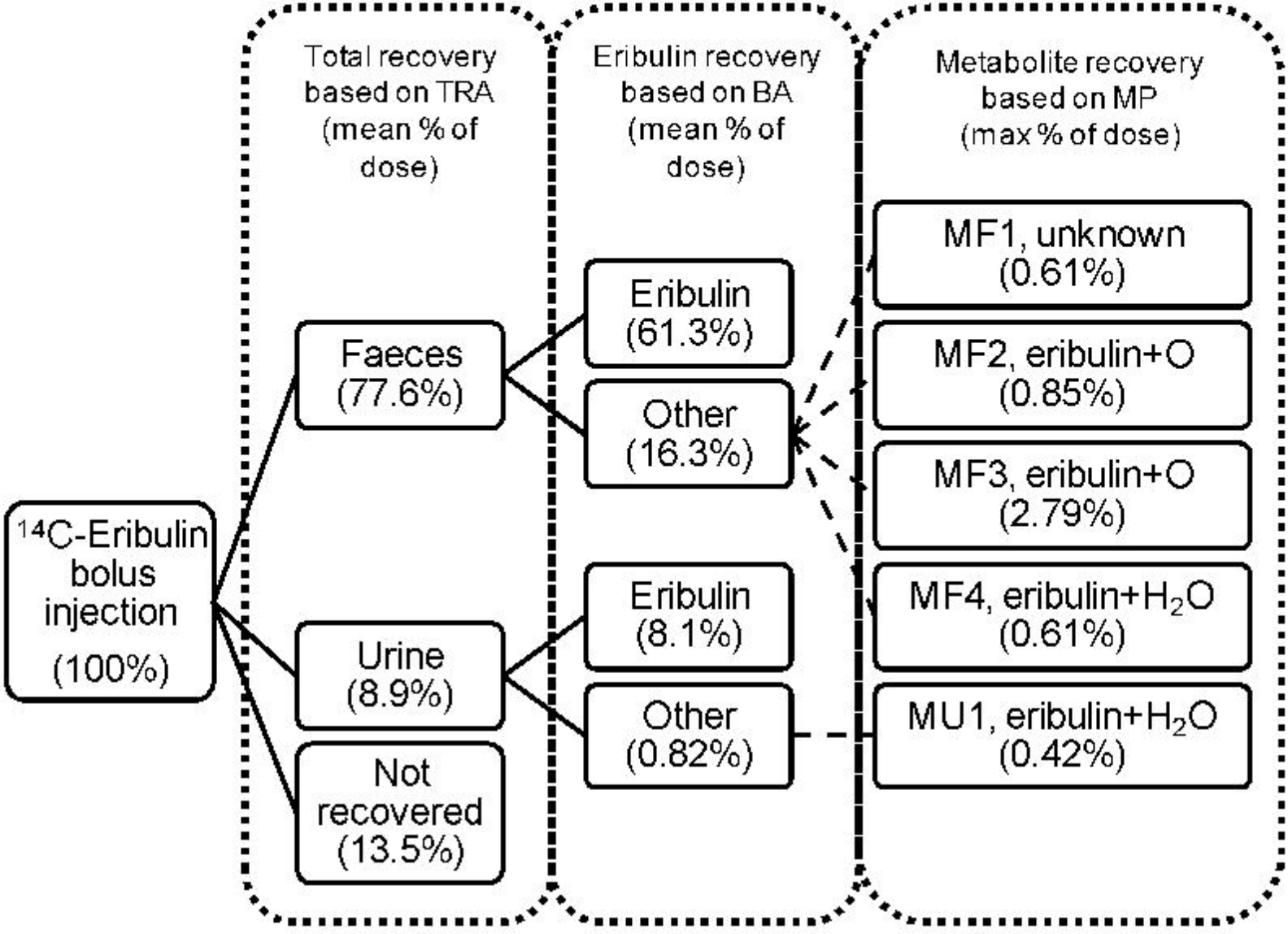

A
Activity (dpm)
Concentration (ng eribulin eq/mL)
Time (min)
Eribulin
MP2
MP3
MP4
LLOQ
B
Activity (dpm)
Total amount in 0-24h urine (µg eribulin eq)
Time (min)
Eribulin
MU1
LLOQ
C
Activity (dpm)
Total amount in 48-72h faeces (µg eribulin eq)
Time (min)
Eribulin
MF1
MF2
MF3
MF4
LLOQ

Figure 7


Total recovery based on TRA (mean % of dose)
Eribulin recovery based on BA (mean % of dose)
Metabolite recovery based on MP (max % of dose)
14C-Eribulin bolus injection (100%)
Faeces (77.6%)
Urine (8.9%)
Not recovered (13.5%)
Eribulin (61.3%)
Other (16.3%)
Eribulin (8.1%)
Other (0.82%)
MF1, unknown (0.61%)
MF2, eribulin+O (0.85%)
MF3, eribulin+O (2.79%)
MF4, eribulin+H2O (0.61%)
MU1, eribulin+H2O (0.42%)