Journal of Chromatography B, 893–894 (2012) 92–100

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Development and validation of LC–MS/MS assays for the quantification of bendamustine and its metabolites in human plasma and urine

A.C. Dubbelman[a,b,*], M. Tibben[b], H. Rosing[b], A. Gebretensae[b], L. Nan[b], S.H. Gorman[c], P. Robertson Jr.[c], J.H.M. Schellens[a,d], J.H. Beijnen[b,d]

[a] Department of Clinical Pharmacology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[b] Department of Pharmacy & Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands
[c] Department of Global Nonclinical DMPK, Teva Pharmaceutical Industries Ltd., 145 Brandywine Parkway, West Chester, PA 19380, United States
[d] Science Faculty, Department of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 8 December 2011
Accepted 23 February 2012
Available online 3 March 2012

Keywords:
Bendamustine
Metabolites
Alkylating agent
Stability
LC–MS/MS

ABSTRACT

A sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) assay is described for the quantification of the anti-cancer agent bendamustine and its phase I metabolites γ-hydroxy-bendamustine (M3) and *N-des*-methylbendamustine (M4) and for its product of two-fold hydrolysis, dihydroxy-bendamustine (HP2), in human plasma and urine.

Like most alkylating nitrogen mustards, bendamustine is prone to chemical hydrolysis in aqueous solution. To minimize degradation of bendamustine, urine samples were stabilized by a 100-fold dilution with human plasma and then processed identically to plasma samples. Sample aliquots of 200 μL were mixed with an internal standard solution and acidified before separation of the analytes from the biomatrix with solid phase extraction. Dried and reconstituted extracts were injected on a Synergi Hydro RP column for the analysis of bendamustine, M3 and M4 or a Synergi Polar RP column for the analysis of HP2. Gradient elution was applied using 5 mM ammonium formate with 0.1% formic acid in water and methanol as mobile phases. Analytes were ionized using an electrospray ionisation source in positive mode and detected with a triple quadrupole mass spectrometer.

The quantifiable range for bendamustine, M3 and M4 was 0.5–500 ng/mL in plasma and 0.5–50 μg/mL in urine, and that for HP2 was 1–500 ng/mL in plasma and 0.1–50 μg/mL in urine. The assays were accurate and precise, with inter-assay and intra-assay accuracies within ±20% of nominal and CV values below 20% at the lower limit of quantification and within ±15% of nominal and below 15% at the other concentration levels tested. These methods were successfully applied to evaluate the pharmacokinetic profile of bendamustine and its metabolites in cancer patients treated with bendamustine.

1. Introduction

Bendamustine is an alkylating agent, comprising a nitrogen mustard moiety, a benzimidazole ring and an alkane carboxylic acid side chain. While it was already synthesized in 1963 by Ozegowski and Krebs [1] and in use for decades in Germany against a number of malignancies, it was only in 2008 that bendamustine was approved by the United States Food and Drug Administration (US FDA) for the treatment of chronic lymphocytic leukemia (CLL) and later for indolent B-cell non-Hodgkin's lymphoma (NHL) that has progressed during or following treatment with rituximab or a rituximab-containing regimen. To date, at least 80 clinical trials with bendamustine are active and recruiting patients [2], indicating that there is a lot of new interest in this rather old drug.

To support clinical trials, we developed and validated an LC–MS/MS method for the quantification of bendamustine in plasma and urine. To our knowledge, this article is the first describing the validation of a bioanalytical assay for this compound. Apart from unchanged bendamustine, the assay also allows quantification of γ-hydroxy-bendamustine (M3) and *N-des*-methylbendamustine (M4), the two known phase I metabolites of bendamustine, which have cytotoxic activity approximately equivalent to and five to ten times less than their parent, respectively [3]. Additionally, a separate assay is described to quantify the product of two-fold hydrolysis of bendamustine, dihydroxy-bendamustine (HP2), in the same samples.

The major challenge in the bioanalysis of bendamustine, similar to that of other, chemically related nitrogen mustards like

* Corresponding author at: Department of Pharmacy & Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands. Tel.: +31 20 5124073; fax: +31 20 5124753.
E-mail address: anne-charlotte.dubbelman@slz.nl (A.C. Dubbelman).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.02.039

chlorambucil and melphalan, is its limited stability [4]. In presence of water, the 2-chloroethyl groups are prone to chemical hydrolysis, leading to the formation of monohydroxy-bendamustine (HP1), which in turn can degrade to HP2. The methods described herein for sample storage and treatment aimed to minimize undesired degradation.

2. Experimental

2.1. Chemicals and reagents

Bendamustine HCl, bendamustine metabolites γ-hydroxy-bendamustine (M3), *N-des*-methylbendamustine (M4) and dihydroxy-bendamustine (HP2) and a structural analogue of bendamustine (BM-IS), used as internal standard (IS) for the bendamustine assays, were manufactured by Carbogen Amcis AG (Bubendorf, Switzerland) and provided by Cephalon Inc. (West Chester, PA, USA). Sigma–Aldrich (St. Louis, MO, USA) supplied α-dansyl-L-arginine HCl (DLA), which served as IS for the HP2 assays. Methanol (Supra-Gradient grade) was obtained from Biosolve Ltd. (Valkenswaard, The Netherlands) and formic acid (98%), ammonium formate and water (LiChrosolv) used to prepare the mobile phases were purchased from Merck (Darmstadt, Germany). Water (distilled) used for sample preparation originated from B. Braun Medical (Melsungen, Germany). Drug-free control human K_2EDTA plasma was obtained from the Slotervaart Hospital (Amsterdam, The Netherlands) and control human urine from healthy volunteers.

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

One mg/mL stock solutions for calibration standards and QC samples were prepared separately in methanol for each analyte: bendamustine, M3, M4 and HP2. They were further diluted with methanol to obtain working solutions with bendamustine, M3 and M4 combined and with HP2 exclusively, at several concentrations. Stock solutions of the internal standards BM-IS and DLA were prepared at a concentration of 10 μg/mL in methanol. The IS working solution contained 60 ng/mL BM-IS or 100 ng/mL DLA (or both for runs with study samples).

Stock solutions, working solutions and QC samples were stored at −70 °C. The IS working solution and the calibration standards were freshly prepared for each analytical run.

2.2.1. CS and QC samples of the plasma assays

For the plasma assays, calibration standards were prepared by adding 10 μL working solution to 190 μL control human plasma, to give concentrations of 0.5, 1, 5, 10, 25, 125, 250, 400 and 500 ng/mL for bendamustine, M3 and M4 and of 1, 2, 5, 10, 25, 125, 250, 400 and 500 ng/mL for HP2.

The QC samples were prepared by spiking control human plasma with the appropriate QC working solution. Final concentrations at the lower limit of quantification (LLOQ), QC low, QC mid, QC high and QC above the upper limit of quantification (>ULOQ) were 0.5, 1.0, 20, 400 and 4000 ng/mL, respectively, for bendamustine, M3 and M4 and 1, 2, 20, 400 and 4000 ng/mL, respectively, for HP2.

2.2.2. CS and QC samples of the urine assays

To minimize the degradation of bendamustine in urine, samples were stabilized by 100-fold dilution in control human plasma. In this final matrix, to which we will refer as urine-plasma, a set of calibration standards with bendamustine, M3 and M4 concentrations of 5, 10, 25, 125, 250, 400 and 500 ng/mL was prepared, corresponding to concentrations in undiluted urine of 0.5–50 μg/mL. Calibration standards of HP2 in urine-plasma were prepared at concentrations of 1, 2, 5, 10, 25, 125, 250, 400 and 500 ng/mL, corresponding to undiluted urine concentrations of 0.1–50 μg/mL.

Because time and material would be saved if urine (i.e., urine-plasma) and plasma study samples could be analysed within a single analytical run with only CS curves of plasma samples, this option was also validated. To this end, calibration standards were made in plasma at the same concentrations as in urine-plasma and analysed in combination with QC samples in urine-plasma.

These QC samples contained 5, 10, 50 and 400 ng/mL bendamustine, M3 and M4 (0.5–40 μg/mL in undiluted urine) or 1, 2, 40 and 400 ng/mL HP2 (0.1–40 μg/mL in undiluted urine) for the QC LLOQ, QC low, QC mid and QC high, respectively.

2.3. Sample preparation

Immediately after collection in the clinic, urine study samples were diluted with cooled (at 2–8 °C) control human K_2EDTA plasma, at a 1:99 (urine: plasma, v/v) ratio, before storage at −70 °C. The plasma samples and urine-plasma samples were processed identically, and the final extracts were used to quantify bendamustine, M3 and M4 and HP2.

Samples were thawed and kept in an ice-water bath pending processing. Ten microlitres of IS working solution and 800 μL of 5 mM ammonium formate with 0.1% formic acid in water were added to 200-μL sample aliquots. After vortex-mixing and centrifuging for 10 min at 4 °C and 23,100 × *g*, solid phase extraction (SPE) was performed using Oasis HLB 30 mg cartridges (Waters, Etten-Leur, the Netherlands). The cartridges were conditioned and equilibrated with 1.0 mL of methanol and 1.0 mL of water, respectively. Subsequently, the acidified samples were loaded, and the cartridges were washed with 1.0 mL of 5% methanol and vacuum dried for 1 min. The analytes were eluted with 1.0 mL of methanol and the eluates were evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residue was redissolved by adding 20 μL of methanol and vortex mixing for 1 min, followed by addition of 80 μL of mobile phase A. Samples were mixed for 5 min at 1250 rpm and, after brief centrifugation, transferred to amber-coloured autosampler vials, which then were stored at 2–8 °C pending analysis. The injection volumes of the assays for bendamustine, M3 and M4 and for HP2 were 10 and 4 μL, respectively.

2.4. Liquid chromatography–tandem mass spectrometry

The HPLC system comprised a HP1100 binary pump, a degasser, a HP1100 autosampler and a switching valve (Agilent technologies, Palo Alto, CA, USA). The autosampler temperature was kept at 4 °C. Mobile phase A (5 mM ammonium formate with 0.1% formic acid in water), mobile phase B (methanol) and the flow rate (0.25 mL/min) were the same for both assays.

Bendamustine, M3 and M4 were separated on a Synergi Hydro RP column (150 mm × 2 mm i.d., particle size 4 μm; Phenomenex, Torrance, CA, USA) with the following mobile phase gradient: mobile phase B: 20% (0–0.2 min), from 20 to 50% (0.2–0.5 min), 50% (0.5–0.6 min), from 50 to 60% (0.6–6 min), 60% (6.0–8.0 min), from 60 to 20% (8.0–8.1 min) and 20% (8.0–11 min). The switching valve directed the flow eluting between 3 and 9.5 min into the mass spectrometer and the remainder to a waste container.

A Synergi Polar RP column (150 mm × 2 mm i.d., particle size 4 μm; Phenomenex) was used for the HP2 assay, with the following mobile phase gradient: mobile phase B: 15% (0–0.2 min), from 15 to 95% (0.2–3.2 min), 95% (3.2–5.2 min), from 95 to 15% (5.2–5.3 min) and 15% (5.3–8.0 min). The switching valve directed only the flow eluting between 4.5 and 6.8 min to the mass spectrometer.

Bendamustine, M3 and M4 were analysed on an API 3000 and HP2 on an API 4000 triple quadrupole mass spectrometer (MS)

Table 1
Mass spectrometric parameters for the analysis of bendamustine, its metabolites M3, M4 and HP2 with the internal standards BM-IS and α-dansyl-L-arginine (DLA).

Mass spectrometer	API 3000				API 4000	
Ion source	ESI				ESI	
Ionization mode	Positive				Positive	
Ion spray voltage (kV)	3.5				3.5	
Turbo gas temperature (°C)	350				550	
Turbo gas flow (L/min)	7				–	
Nebulizer gas (psi)	14				–	
Curtain gas (psi)	8				50	
Collision gas (psi)	5				7	
Gas 1 (nebulizer gas, psi)	–				60	
Gas 2 (turbo gas, psi)	–				70	
Analyte specific parameters	**Bendamustine**	**M3**	**M4**	**BM-IS**	**HP2**	**DLA**
Parent mass (*m*/*z*)	358	374	344	372	322	408
Product mass (*m*/*z*)	228	338	186	354	304	170
Declustering potential (V)	66	41	45	66	80	91
Focussing potential (V)	310	190	220	310	–	–
Entrance potential (V)	10	9	8.5	9	12	12
Collision energy (V)	51	35	59	37	40	43
Collision exit potential (V)	12	22	30	24	10	12
Dwell time (ms)	100	100	100	100	100	100
Typical retention time (min)	7.1	6.8	7.7	7.3	5.4	6.1

(AB Sciex, Thornhill, ON, Canada). Both instruments were equipped with a turbo ion spray interface, operating in positive mode and configured in multiple reaction monitoring (MRM).

LC–MS/MS data were acquired and processed using the software application Analyst™ (AB Sciex). Table 1 summarizes the MS operating parameters for both assays.

2.5. Validation procedures

The plasma assays were fully validated for calibration model, accuracy and precision, specificity and selectivity, recovery and matrix effect, carry-over and stability according to the FDA guidance of bioanalytical method validation [5,6]. A partial validation was performed for the assays in urine and included assessment of the calibration model, accuracy and precision, carry-over and stability.

3. Results and discussion

3.1. Method development

3.1.1. Sample collection and storage

Collection of urine samples for clinical pharmacokinetic studies often occurs over 24-h periods, whereby individual portions are pooled in a refrigerated container. Knowing, however, that compounds containing nitrogen mustard moieties may be unstable in aqueous solutions, it was questionable whether this approach would be suitable for urine collection for bendamustine analysis.

Stability tests in control human urine ($n = 6$, pH range 6.5–7.6) confirmed that bendamustine is very unstable in aqueous solution. After 18 h of storage at 2–8 °C, 47 ± 20% (mean ± SD) of the bendamustine was lost. The degradation of bendamustine in urine appeared pH dependent: at pH 7.6 only 27% of the initial concentration was found while 75% was detected at pH 6.5.

Based on these and similar results, other possibilities for storage of urine samples containing bendamustine were investigated. Literature on the stability of nitrogen mustard-containing compounds like melphalan and chlorambucil describes that these compounds are more stable at a pH <3 [4], when treated on ice-water instead of at room temperature [7,8] and in the presence of 5% human serum albumin or human plasma as compared to aqueous buffers [9]. This led to the idea to stabilize bendamustine by a 100-fold dilution with human plasma. The additional advantage of this method is that the final biomatrix will be virtually identical to plasma, meaning that the urine-plasma samples can be processed and analysed in the same manner as plasma samples.

3.1.2. Sample preparation

To prevent analyte degradation, a low pH (obtained by addition of a low pH buffer or an acid solution), treatment on ice-water and amber-coloured vials were used as standard conditions during sample preparation. A method previously developed and validated at BASi (West Lafayette, IN, USA) served as a point of departure for the development and validation of the method for analysis of bendamustine, M3, and M4 in plasma.

During the development of the sample preparation procedure, five standard methods were systematically tested and the most sensitive method (high signal-to-noise ratio combined with a high recovery) was selected. Four plasma aliquots spiked with bendamustine, M3, M4 and HP2 and one blank plasma aliquot were all treated with the following methods: (i) protein precipitation (PPT) with perchloric acid, (ii) liquid–liquid extraction (LLE) with tert-butyl methyl ether (TBME), (iii) LLE with diethyl ether, (iv) SPE using Oasis Max cartridges and (v) SPE using Oasis HLB cartridges. A matrix-free dilution of a working solution was used as a reference to calculate the recovery. Signal-to-noise ratio was calculated as the peak height of an analyte in the spiked plasma sample and the signal height at the same retention time in the blank plasma sample.

The results of these tests are visualised in the plots presented in Fig. 1. The LLE methods appeared unsuitable to extract the polar compound HP2 from the plasma samples. Although the recovery and sensitivity of the simple PPT method was higher than of the LLE methods, the SPE methods resulted in the highest overall recovery. The signal-to-noise ratio of each compound was highest in the SPE method using the Oasis HLB cartridges; therefore, this method was selected.

Attempts to further decrease the conversion of bendamustine to HP2 during sample processing included evaporation at a lower temperature (25 °C instead of 35 °C), acidification with 1% perchloric acid instead of 5 mM ammonium formate with 0.1% formic acid in water, and addition of an excess of sodium chloride at the start of the sample preparation. None of these methods resulted in significant reduction in the formation of HP2. The SPE method using Oasis HLB cartridges (see Section 2.3) was validated.

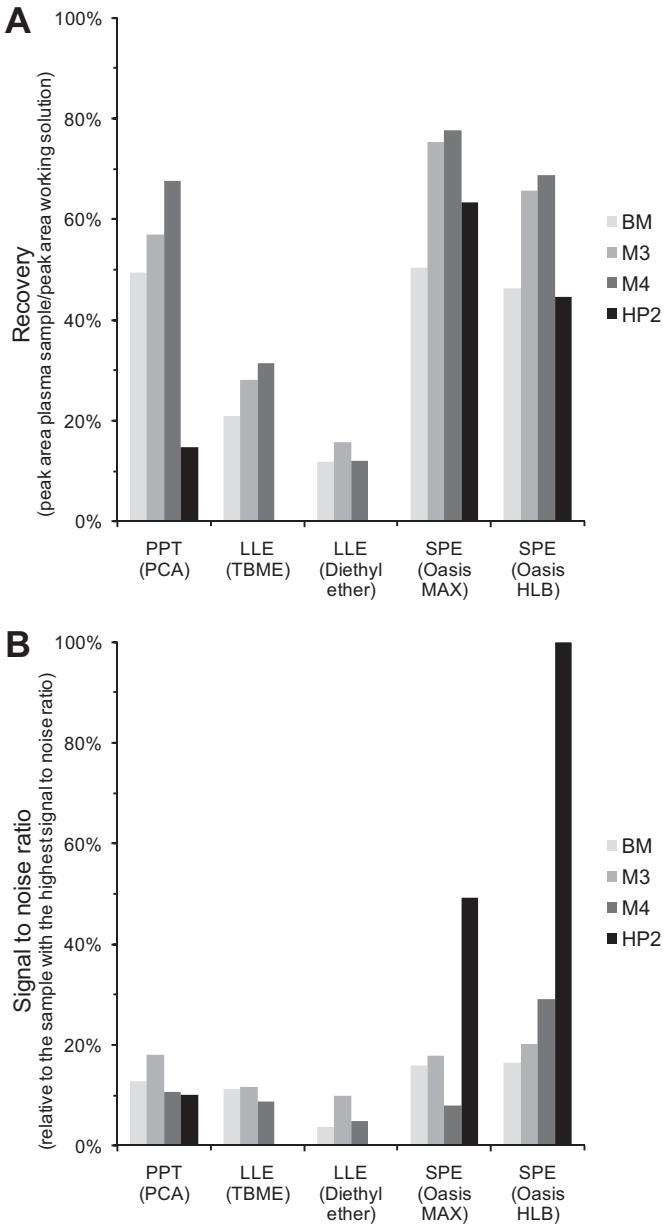


Fig. 1. Sample preparation recovery of each analyte with different preparation procedures (A) and signal-to-noise ratio of each analyte compared to the sample with the highest signal-to-noise ratio (B). BM: bendamustine; PPT: protein precipitation; PCA: perchloric acid; LLE: liquid–liquid extraction; TBME: tert-butyl methyl ether; SPE: solid phase extraction.

3.1.3. Liquid chromatography–tandem mass spectrometry

Simultaneous quantification of bendamustine, M3, M4 and HP2 was complicated by the high polarity of HP2 and by the absence of stable isotope-labelled internal standards. Initially, all analytes were combined in one chromatographic system; however, matrix effect caused a biased quantification of HP2. Attempts to compensate for this effect by use of alternative internal standards were not successful. Therefore, a separate chromatographic method for HP2 was developed. The Synergi Polar RP column was chosen for its ability to retain highly polar compounds. Using this column, HP2 quantification was less influenced by matrix effects; however, sensitivity was reduced. To accurately and precisely quantify HP2 at the target LLOQ of 1 ng/mL, the method was transferred to a more sensitive mass spectrometer (API 4000).

To optimize the mass spectrometric parameters and to find suitable product ions for multiple reaction monitoring, each analyte was infused directly into an electrospray ionization source. Settings for gasses and ion spray voltage were optimized by flow injection analysis. Table 2 shows the final transitions that were selected and their proposed fragmentation patterns. Figs. 2 and 3 show representative chromatograms of bendamustine, M3, M4 and HP2 in a plasma QC LLOQ sample.

3.2. Validation procedures

3.2.1. Calibration model

For the bendamustine, M3 and M4 assay and the HP2 assay, 9 non-zero calibration standards were prepared and analysed in duplicate in three analytical runs. The simplest model that adequately described the concentration–response relationships for all analytes was a quadratic curve weighted with $1/x$, whereby x is the concentration.

The calibration ranges of bendamustine, M3 and M4 were 0.5–500 ng/mL in plasma and 5–500 ng/mL in urine-plasma, corresponding to a 0.5–50 μg/mL range in undiluted urine. The HP2 calibration curve in both plasma and urine-plasma ranged from 1.0 to 500 ng/mL, corresponding to a 0.1–50 μg/mL range in undiluted urine.

Calibration curves were accepted if two-thirds of the non-zero calibration standards, including a LLOQ and an ULOQ, had a deviation within ±15% of nominal (±20% at the LLOQ) [5,6]. All calibration curves in plasma and urine-plasma met these acceptance criteria and had correlation coefficients (r^2) of 0.997 or better.

3.2.2. Accuracy and precision

To assess the accuracy and precision of the plasma assays, five replicates of the QC LLOQ, QC low, QC mid and QC high in plasma were analysed in 3 analytical runs with bendamustine, M3 and M4 and with HP2. The QC samples prepared in urine-plasma were analysed in one analytical run together with plasma and urine-plasma calibration standards.

Table 3 summarizes the intra-assay accuracies (the percentage difference of the measured from the nominal concentration) and precisions (CV values) of the analytes in plasma. For the assays in urine-plasma, the QC samples were quantified using either calibration standards in urine-plasma or in plasma and for both cases the accuracy and precision data are tabulated in Table 4. Because the difference between the calculated concentrations of the two quantification methods was very small (2% on average), it was concluded that study samples in urine-plasma can be quantified using calibration standards prepared in plasma.

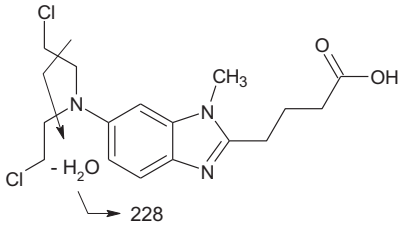
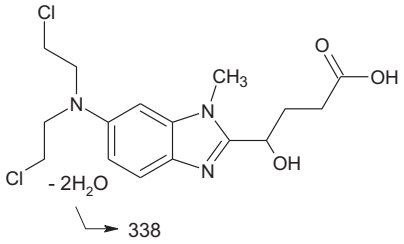
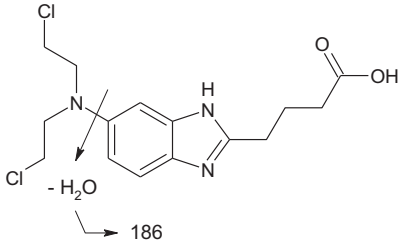
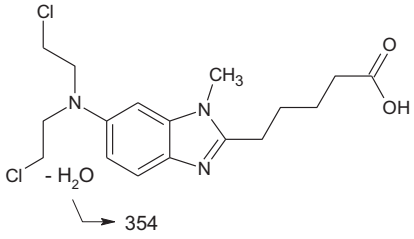
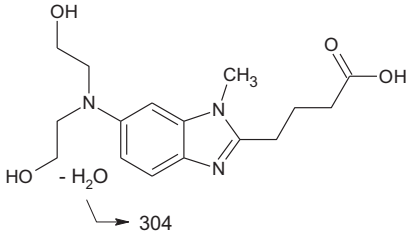
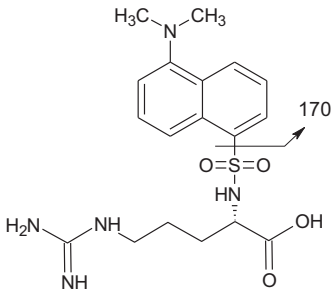
The biases and precisions of all analytes were within the acceptance criteria (within ±20% and ≤20%, respectively, at the LLOQ level and within ±15% and ≤15% at the other QC levels) and the signal-to-noise ratios of the analytes at the LLOQ level were above 5 [5,6].

Five replicate samples of bendamustine, M3, M4 and HP2 at QC >ULOQ level that were diluted 10- and 100-fold with control plasma had a maximum deviation of −14.3% and a maximum CV of 6.06%, indicating that study samples with concentrations above the ULOQ can be diluted 10- and 100-fold with acceptable accuracy and precision values [5,6].

3.2.3. Specificity and selectivity

Six different batches of control plasma were spiked at the LLOQ level with either bendamustine, M3 and M4 or HP2 to investigate the selectivity. The mean deviations from the nominal concentrations were 0.4%, −3.6%, −13.3% and 7.5% with CV values of 3.2%, 11.3%, 12.1% and 5.0%, respectively. Peaks appearing in double-blank samples of these batches, caused by endogenous disturbances and co-eluting with an analyte, were maximally 14.6%,

Table 2
Analytes with their selected mass transitions and proposed fragmentation pathways.

Compound	Transition	Proposed fragmentation
Bendamustine	358 → 228	
M3	374 → 338	
M4	344 → 186	
BM-IS	372 → 354	
HP2	322 → 304	
α-Dansyl-L-arginine	407 → 170	

13.8%, 13.2% and 10.1% of the LLOQ for bendamustine, M3, M4 and HP2, respectively, and no interferences were detected at the retention time of the internal standards BM-IS and DLA. Selectivity was therefore considered sufficient [5,6].

Cross-analyte interference was tested by spiking plasma with one of the analytes at the ULOQ level and analysing it with both the assay for bendamustine, M3 and M4 and the assay for HP2. In the assay of bendamustine, M3 and M4, the maximum cross-analyte

Table 3
Assay performance data for the analysis of bendamustine, M3 and M4 and for the analysis of HP2 in human plasma.

Analyte	Internal standard	QC	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Inter-assay accuracy (%)	Inter-assay precision (%)	*n*
Bendamustine	BM-IS	LLOQ	0.502	0.487	−3.07	16.2	14
		Low	1.00	0.952	−4.76	4.42	15
		Mid	20.1	17.9	−10.8	3.16	15
		High	402	379	−5.77	4.88	15
M3	BM-IS	LLOQ	0.503	0.517	2.85	15.6	14
		Low	1.01	0.974	−3.52	6.16	14
		Mid	20.1	17.5	−12.8	2.82	15
		High	402	373	−7.16	3.29	15
M4	BM-IS	LLOQ	0.497	0.490	−1.49	14.2	14
		Low	0.994	0.895	−10.0	10.0	15
		Mid	19.9	18.2	−8.54	3.69	15
		High	398	367	−7.74	3.44	15
HP2	DLA	LLOQ	1.04	1.01	−3.26	7.40	15
		Low	2.09	2.01	−3.64	5.71	15
		Mid	209	21.4	2.36	9.59	15
		High	417	420	0.719	5.23	15

n: number of replicates.

interference that was observed originated from bendamustine, generating a peak of 2.66% of the LLOQ of M3 in the transition window of M3.

BM-IS produced a peak in the window of M3 (as shown in Fig. 2), probably resulting from the loss of $H^{35}Cl$ of a ^{37}Cl-isotope of the internal standard. However, the peak, which is baseline-separated from the M3 peak, will be essentially invariant because the BM-IS concentration is the same in all samples; this peak is not expected to impact the quantification of M3.

In the HP2 assay, the interferences of M3, M4, BM-IS and DLA on HP2 were less than 10% of the LLOQ of HP2, and no interference was observed in the transition window of DLA. The only significant interference that was observed was of bendamustine on HP2 quantification. The plasma sample spiked with bendamustine at 500 ng/mL gave a peak in the transition window of HP2 at the retention time of HP2 and with an area of 48% of the LLOQ of HP2, which was 1 ng/mL. As HP2 was not observed as a significant impurity in the stock solution of bendamustine, it was likely formed by chemical degradation during sample preparation and analysis. Because the sample preparation procedures were already optimized to minimize degradation of bendamustine, we calculated in which cases this interference would adversely affect the results of study samples.

To this end, a maximum interference of 20% of the LLOQ level was considered acceptable and it was assumed that 0.1% (≈48% of 1 ng/mL per 500 ng/mL bendamustine) of the original bendamustine concentration is added to the HP2 concentration due to degradation during sample processing. In that case, a sample with original concentrations of 1 ng/mL HP2 and 200 ng/mL bendamustine would be quantified at 1.2 ng/mL HP2, which is the maximum accepted limit. The HP2 quantification results of study samples with a measured bendamustine: HP2 concentration ratio above 200:1.2 (i.e., 167:1) will be affected by the degradation of bendamustine during sample preparation and should be interpreted with caution. Considering the short half-life time of bendamustine and the plausibility of bendamustine to be converted into HP2 in the body,

Table 4
Assay performance data for the analysis of bendamustine, M3 and M4 and for the analysis of HP2 in human urine: plasma (1:99, v/v) (referred to as urine-plasma), with respect to CS samples in urine-plasma and plasma.

Analyte	QC	Nominal conc.[a] (ng/mL)	CS samples in urine-plasma			CS samples in plasma		
			Measured conc.[a,b] (ng/mL)	Intra-assay accuracy (%)	Intra-assay precision (%)	Measured conc.[b] (ng/mL)	Intra-assay accuracy (%)	Intra-assay precision (%)
Bendamustine	LLOQ	4.70	3.93	−16.3	4.77	4.07	−13.3	4.61
	Low	9.40	8.75	−6.87	4.64	8.90	−5.28	4.57
	Mid	47.0	45.1	−3.96	5.54	45.3	−3.57	5.55
	High	376	372	−1.12	4.18	373	−0.798	4.17
M3	LLOQ	5.47	4.50	−17.7	3.31	4.55	−16.8	3.21
	Low	10.9	10.7	−2.20	3.96	10.7	−2.20	3.96
	Mid	54.7	57.1	4.31	5.76	56.8	3.77	5.79
	High	437	434	−0.778	3.63	448	2.43	3.79
M4	LLOQ	4.63	4.48	−3.20	9.14	4.86	4.88	8.53
	Low	9.25	9.65	4.41	6.36	10.1	8.69	6.16
	Mid	46.3	51.9	12.1	3.80	52.6	13.6	3.75
	High	370	411	11.0	2.48	425	14.8	2.64
HP2	LLOQ	1.04	0.895	−13.9	1.77	0.904	−13.1	1.75
	Low	2.09	2.07	−1.05	1.30	2.06	−1.53	1.30
	Mid	20.9	23.4	12.2	2.08	23.1	10.7	2.11
	High	417	409	−1.92	4.51	414	−0.82	4.67

Conc.: concentration.
[a] Reported concentrations are in urine-plasma instead of urine.
[b] Samples were analysed in five replicates

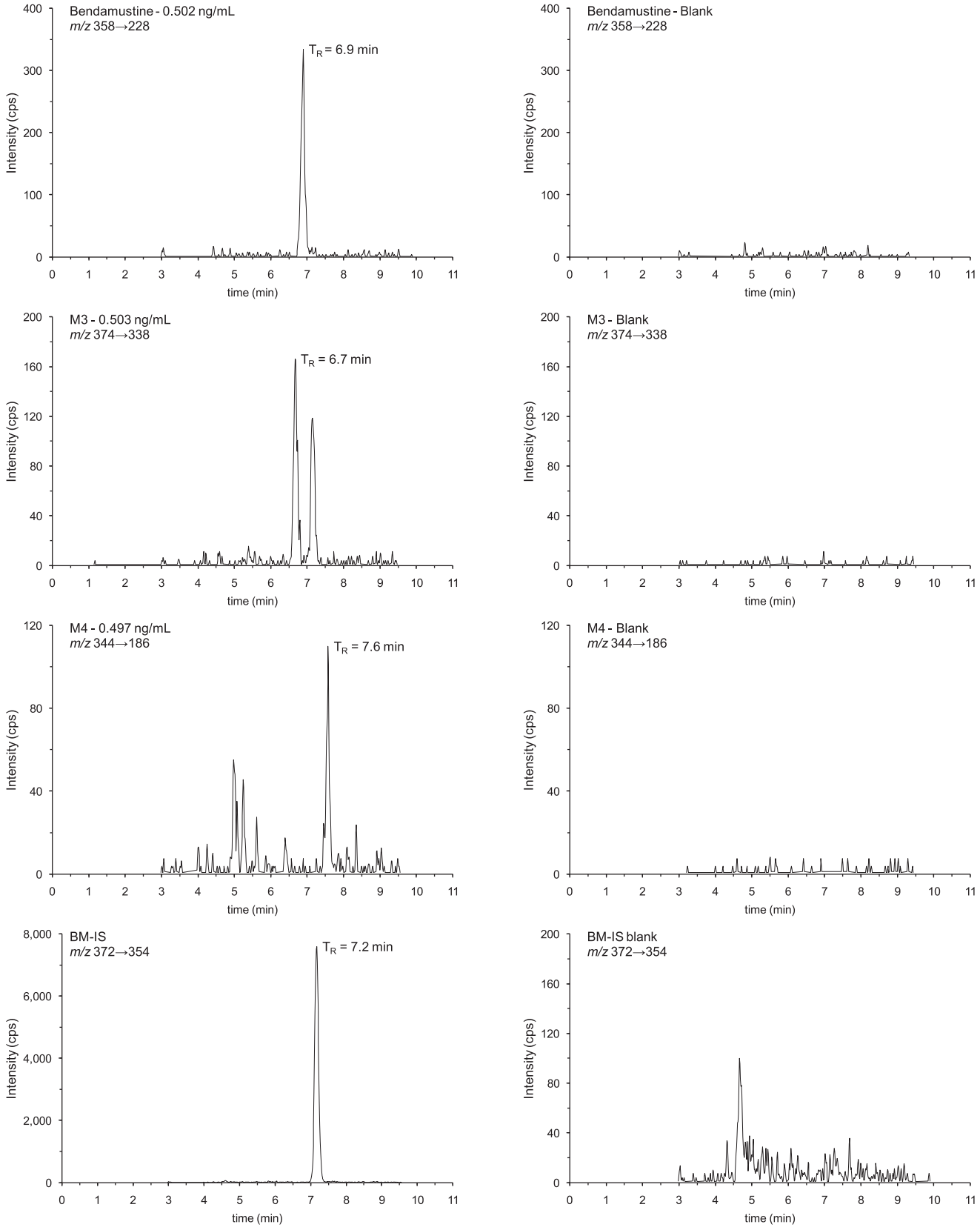


Fig. 2. MRM chromatograms of bendamustine, M3, M4 and the internal standard BM-IS of a QC LLOQ (left) and a blank (right) plasma sample.

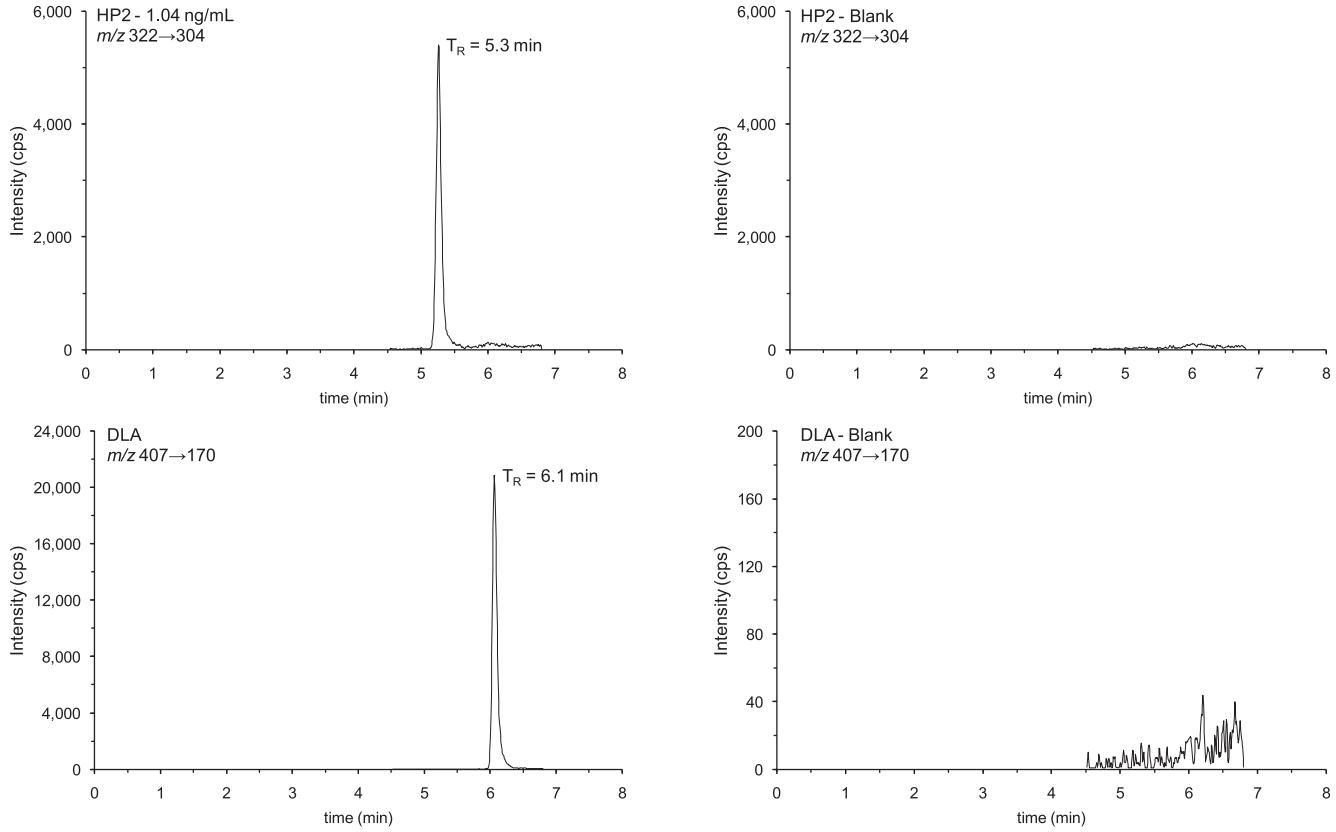


Fig. 3. MRM chromatograms of HP2 and the internal standard α-dansyl-L-arginine (DLA) of a QC LLOQ (left) and a blank (right) plasma sample.

concentration ratios above 167 may occur during the infusion of bendamustine, but are not expected to persist.

3.2.4. Sample preparation recovery and matrix effect

The sample preparation recoveries of bendamustine, M3 and M4, determined in triplicate at three concentrations in plasma by comparing processed spiked samples with processed blank samples that had been spiked with working solutions, were consistent across the concentration range for the three analytes: 76.4–82.8%, 79.3–86.5% and 74.9–82.7%, respectively. The matrix effect was determined by comparing the latter samples to matrix-free working solutions and ranged from −32.2 to −6.4%, −18.7 to −1.2% and −23.3 to −2.4%, for bendamustine, M3 and M4, respectively. The sample preparation recovery of BM-IS was 79.6% and the matrix effect was −30.6%.

For HP2, the sample preparation recovery ranged from 81.4 to 83.9% and the matrix effect ranged from −31.3 to −18.5%. The sample preparation recovery of DLA was 89.2% and the matrix effect −19.1%.

3.2.5. Carry-over

Carry-over of bendamustine, M3 and M4 caused peaks in the first blank plasma sample after an ULOQ sample with an area of 49–89% of the LLOQs in plasma. In the second blank plasma sample, the carry-over was reduced to less than 20% of the LLOQ. As a consequence, a blank sample needs to be injected after ULOQ and QC high samples and between study samples with high expected concentrations of one or more of the analytes. No carry-over effect was observed for BM-IS with this assay. In urine-plasma, carry-over of bendamustine, M3 and M4 in the first blank was limited to ≤6.3% of the LLOQ with 0.95% carry-over of BM-IS.

The carry-over of HP2 was limited: 7.1% in the first blank plasma sample after an ULOQ sample and 10.1% in urine-plasma. No carry-over was observed for DLA.

Table 5
Long-term and short-term stability of stock solutions of bendamustine, its metabolites and the internal standards in methanol.

Condition	Analyte	Nominal conc. (mg/mL)	Measured conc.[a] (mg/mL)	CV (%)	Dev. (%)
−70 °C, 12 Mon	Bendamustine	1.02	1.06	1.10	4.57
−70 °C, 9 Mon	M3	0.986	0.954	1.07	−3.23
−70 °C, 12 Mon	M4	1.00	0.960	0.856	−4.03
−70 °C, 7 Mon	HP2	1.04	1.07	1.39	3.03
−70 °C, 12 Mon	BM-IS	0.106	0.117	1.26	10.4
−70 °C, 9 Mon	DLA	0.105	0.0928	1.06	−9.09
Icewater, 3 h	Bendamustine	1.02	0.994	0.837	−2.56
Icewater, 3 h	M3	0.986	0.944	2.16	−4.29
Icewater, 4 h	M4	1.00	0.984	0.00	−1.58
Icewater, 6 h	HP2	1.04	1.03	1.37	−1.17

Conc.: concentration; CV: coefficient of variation; dev.: deviation; Mon: months; h: hours.
[a] Samples were analyzed in three replicates

3.2.6. Stability

Stability experiments were executed in triplicate at the concentration levels QC low and QC high. Biases were calculated against the initial concentrations and analytes were considered stable in a matrix if the difference was within ±15%. For diluted stock solutions and working solutions of the analytes other than the internal standards, the maximum allowed deviation was ±5%.

Stock solutions of bendamustine, M3, M4 and HP2 in methanol were stable for at least 12, 9, 12 and 7 months at −70 °C, respectively. The internal standards BM-IS and DLA were stable for at least 12 and 9 months, respectively, under the same storage conditions. The stability of the analyte stock solutions on ice-water (during sample preparation) was at least 3 h. Table 5 provides an overview of the stability of the analytes in methanol.

Supplementary Tables 1 and 2 show the stability data for bendamustine and its metabolites in the biological matrices. When stored at −70 °C, bendamustine, M3 and M4 and HP2 were stable for at least 8–9 months in plasma and in urine-plasma. All analytes remained stable after three freeze–thaw cycles from −70 °C to ice-water in both plasma and urine-plasma, except for M4 in plasma, for which 2 cycles appeared to be the maximum. Stability of the analytes in plasma and urine-plasma on ice-water was demonstrated for 6 h, and the final extracts of plasma samples were stable for at least two days for bendamustine, M3 and M4 and 14 days for HP2 at 4 °C.

The stability of the analytes was also tested in undiluted urine at 4 °C, to mimic the situation of urine samples collected in the clinic and refrigerated but not instantly diluted with plasma. All analytes were stable for at least 2 h under these conditions.

3.2.7. Reinjection reproducibility

Reinjection of duplicate CS samples and triplicate QC samples of bendamustine, M3 and M4 in plasma was reproducible after 2 days storage at 4 °C, as the results met the acceptance criteria of the calibration curve, accuracy and precision. For analysis of HP2, reinjection reproducibility was demonstrated after 24 h storage at 4 °C.

4. Clinical application

The purpose of the validated assays was to support clinical pharmacokinetic studies of bendamustine. To demonstrate their applicability, we present the concentration profiles over time of bendamustine, M3, M4 and HP2 in plasma of a representative patient who had received bendamustine (Fig. 4).

A 120 mg/m^2 dose of bendamustine was administered as a 1-h intravenous infusion to a cancer patient, as part of a clinical phase I study investigating the metabolism and excretion of bendamustine in patients with relapsed or refractory malignancies. Blood samples were collected at several time points using K_2EDTA tubes and put on ice. Within 30 min after collection, samples were centrifuged for 10 min at 1200 × *g* and 4 °C; plasma was isolated and then stored at −70 °C pending analysis. Fig. 4 shows the plasma concentration–time curves for bendamustine and its metabolites in this patient. The maximum concentration of bendamustine was 8187 ng/mL, which was achieved at the end of the infusion. Concentrations then declined rapidly in a multiphasic manner. The phase I metabolites M3 and M4 showed a similar general time course as bendamustine, but reached much lower concentrations. The concentrations of HP2 early in the sampling period were much lower compared to those of bendamustine, but this metabolite had a longer elimination half-life than the others. The maximum ratio between bendamustine and HP2 was 164:1, occurring halfway through the infusion; hence, the critical ratio (i.e., 167:1) above which degradation of bendamustine during sample processing would be predicted to affect HP2 quantification was barely achieved and only transiently.

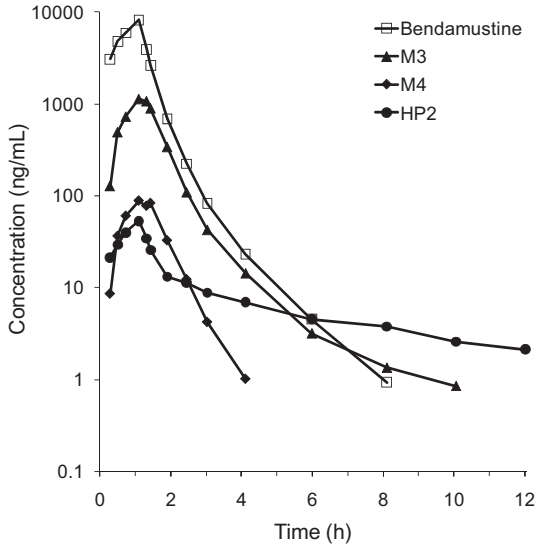


Fig. 4. Representative plasma concentration–time curves of bendamustine and its metabolites M3, M4 and HP2 following a 1-h infusion with 120 mg/m^2 bendamustine in a cancer patient.

5. Conclusion

By systematically comparing various sample preparation techniques and optimizing LC–MS/MS settings, we developed a sensitive assay for the quantification of bendamustine and its metabolites M3, M4 and HP2. The assays were validated according to the US FDA guidelines and applicable for human K_2EDTA plasma and for human urine that has been diluted with control plasma to minimize chemical hydrolysis of bendamustine. The assays were able to quantify bendamustine, M3 and M4 from 0.5 to 500 ng/mL and HP2 from 1 to 500 ng/mL in 200-μL plasma aliquots, with the possibility to dilute samples containing higher concentrations 10- or 100-fold with control plasma prior to analysis. The quantifiable range in urine was from 0.5 to 50 μg/mL for bendamustine, M3 and M4 and from 0.1 to 50 μg/mL for HP2. The assays are considered very suitable and are now in use to support clinical pharmacologic studies of bendamustine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.039.

References

[1] W. Ozegowski, D. Krebs, J. Prakt. Chem. 20 (1963) 178.
[2] United States National Institutes of Health, 2011, http://clinicaltrials.gov/ct2/results?term=bendamustine&no_unk=Y (accessed 1.11.11).
[3] J. Teichert, F. Baumann, Q. Chao, C. Franklin, B. Bailey, L. Hennig, K. Caca, K. Schoppmeyer, U. Patzak, R. Preiss, Cancer Chemother. Pharmacol. 59 (2007) 759.
[4] A.G. Bosanquet, Cancer Chemother. Pharmacol. 14 (1985) 83.
[5] H. Rosing, W.Y. Man, E. Doyle, A. Bult, J.H. Beijnen, J. Liq. Chromatogr. Relat. Technol. 23 (2000) 329.
[6] US FDA, 2001, www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf (accessed 9.1.12).
[7] C.G. Adair, J.M. Bridges, Z.R. Desai, Cancer Chemother. Pharmacol. 17 (1986) 185.
[8] A. Mirkou, B. Vignal, S. Cohen, M. Guillaumont, O. Glehen, J. Guitton, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 3089.
[9] E. Watson, P. Dea, K.K. Chan, J. Pharm. Sci. 74 (1985) 1283.