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Liquid chromatography–tandem mass spectrometric assay for the PARP-1 inhibitor olaparib in combination with the nitrogen mustard melphalan in human plasma

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ABSTRACT

A bioanalytical assay for the new poly(ADP-ribose) polymerase-1 inhibitor olaparib in combination with melphalan was developed and validated. For the quantitative assay, human plasma samples were pretreated on ice using protein precipitation with 2% (v/v) acetic acid in acetonitrile containing erlotinib and melphalan-d_8 as internal standards. The extract was diluted with water and injected into the chromatographic system. This system consisted of a sub-2 μm particle, trifunctional bonded octadecyl silica column with an isocratic elution using 0.01% (v/v) of formic acid in a mixture of water and methanol. The eluate was transferred into the electrospray interface with positive ionization and the analyte was detected in the selected reaction monitoring mode of a triple quadrupole mass spectrometer. The assay was validated in a 10–5000 ng/ml calibration range for both drugs. The lowest level of this range corresponded to the lower limit of quantification. Within day precisions were 3.0–9.3%, between day precisions 6.0–9.8% and accuracies were between 101 and 110% for the whole calibration range. After validation the assay was used to assess the pharmacokinetics of olaparib in a patient with metastatic breast carcinoma. In addition, systemic exposure of melphalan was monitored in patients subjected to isolated hepatic perfusion with this drug. Both applications show that the new assay can be applied for human pharmacokinetic studies for both drugs.

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1. Introduction

Olaparib (AZD2281, KU0059436, Fig. 1A) is a novel, potent and orally bioavailable inhibitor of poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1, the major PARP isoform, is a nuclear protein and is involved in the repair of DNA damage [1,2]. Its activation is an immediate cellular response to metabolic-, chemical-, ionizing radiation-induced DNA damage, particularly under stressful conditions, like exposure to carcinogens or anti-tumor drugs, through its ability to recognize and rapidly bind DNA single or double strand breaks.

Melphalan is a phenylalanine derivative of nitrogen mustard, known also as L-phenylalanine mustard, available for more than forty years now [3]. It is a cytotoxic drug that acts as a bifunctional alkylating agent on DNA that is active against selected human neoplastic diseases [4].

A Phase I clinical trial reported by Fong et al. [5] indicated olaparib as the first PARP-inhibitor that showed anti-tumor activity as a single agent. In this study 60 patients were treated, 22 of which carried a *BRCA1* or *BRCA2* mutation and who showed a stabilization or tumor regression. These results have later been supported in a Phase II clinical study for ovarian cancer [6]. At the moment, many Phase I and II clinical studies with olaparib are ongoing [1,7], in which the drug is combined with conventional chemotherapy. Preclinical studies indicate that PARP inhibitors could enhance the efficacy of radiation therapy [8] and chemotherapies such as alkylating agents like melphalan (4-(bis (2-chloroethyl) amino-L-phenylalanine, (Fig. 1C) by preventing cancer cells from repairing DNA damage, thereby promoting apoptosis [9]. The clinical impact

Abbreviations: BRCA, *BR*east *CA*ncer gene; HESI, heated electrospray ionization; LLOQ, lower limit of quantification; PARP, poly(ADP-ribose) polymerase; SRM, selected reaction monitoring; QC, quality control.

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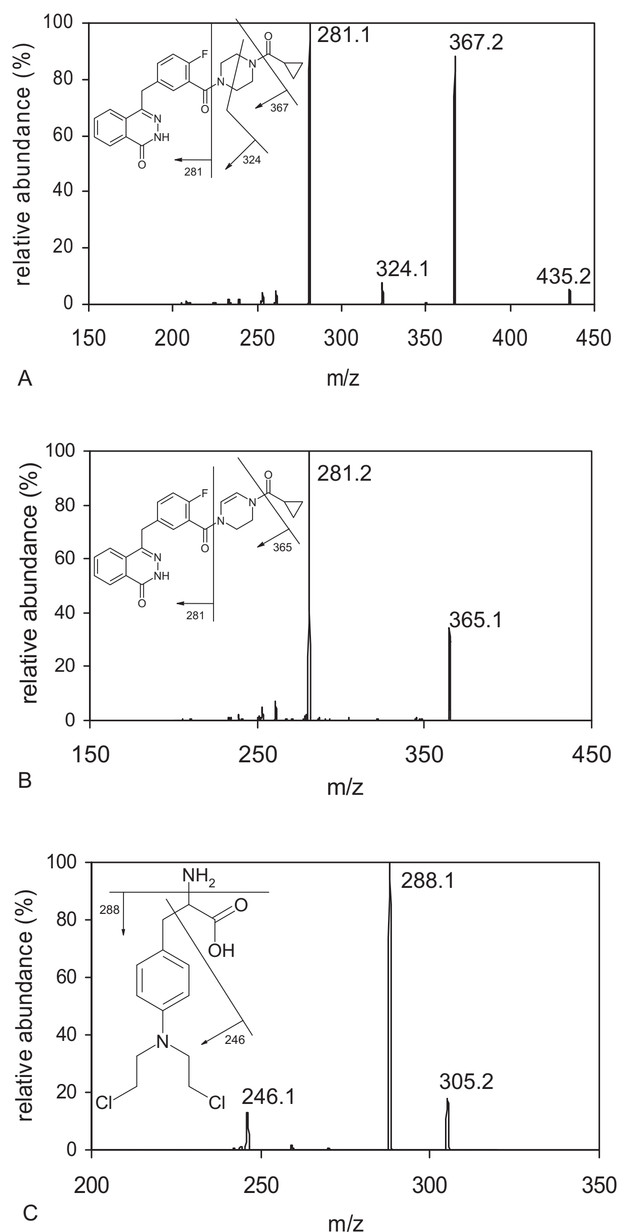

Fig. 1. Product spectra of (A) olaparib (m/z 435.2 at −25 V), (B) dehydro-olaparib (m/z 433.2 at −30 V), produced using human liver microsomes and (C) melphalan (m/z 305.1@–12 V).

of the last phenomena will be investigated in a clinical study of the combination of olaparib and melphalan in the Netherlands Cancer Institute.

For pharmacokinetic evaluation of this clinical study a bioanalytical assay will be required. While for olaparib only a bioanalytical assay was reported by Fong et al. [5] without any details, a lot of methods for melphalan are already known [3]. Typically, reverse-phase liquid chromatography with spectrophotometric detection has been applied for pharmacokinetic assessment of melphalan so far. LC–MS/MS assays for this drug, however, are still scarce and are using a relatively complicated sample treatment [10] or long chromatographic run time [11]. An LC–MS/MS assay for olaparib and melphalan in plasma was therefore developed and validated. The use of MS/MS detection in combination with an LC system using sub-2 μm particles was found to result in a high sensitivity and selectivity allowing the analysis to be executed with a simple sample pre-treatment and short analytical run times.

2. Experimental

2.1. Chemicals

Olaparib and erlotinib were obtained from Sequoia Research Products (Pangbourne, UK), melphalan (≥95%) from Sigma–Aldrich (St. Louis, MO, USA) and melphalan-d_8 from Toronto Research Chemicals (North York, ON, Canada). Water (LC–MS grade), methanol (HPLC grade) and acetonitrile (HPLC-S grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home purified by reversed osmosis on a multi-laboratory scale. Formic acid was of analytical grade and originated from Merck (Darmstadt, Germany), extra pure acetic acid (100%) from Riedel-de Haën (Sigma-Aldrich, Seelze, Germany) and analytical grade dimethyl sulfoxide (DMSO) from Acros Organics (Geel, Belgium). Pooled human lithium heparin plasma was supplied by Seralab Laboratories International (Haywards Heath, UK), plasma from individual donors by Innovative Research (Southfield, MI, USA).

2.2. Equipment

The LC–MS/MS equipment consisted of an Accela pump and autoinjector and a TSQ Quantum Ultra triple quadrupole mass spectrometer with heated electrospray ionization (HESI) (Thermo Fisher Scientific, San Jose, CA, USA). Data were recorded and the system was controlled using the Thermo Fisher Xcalibur software (version 2.07)

2.3. LC–MS/MS conditions

Partial-loop injections (1 μl) were made on an Acquity UPLC® BEH C18 column (30 mm × 2.1 mm, d_p = 1.7 μm, Waters, Milford, USA), protected by the corresponding VanGuard pre-column (Waters, 5 mm × 2.1 mm). The column temperature was maintained at 40 °C and the sample rack compartment of the autosampler at 4 °C. The eluent comprised a mixture of 0.1% (v/v) formic acid in water (10%, v/v), water (47%, v/v) and methanol (43%, v/v), that was pumped at 0.6 ml/min. The whole eluate was transferred into the electrospray probe, starting at 0.3 min after injection by switching the MS divert valve. The HESI was tuned in the positive ionization mode by introducing 0.6 ml/min of a mixture of 0.1% formic acid in water (10%, v/v), methanol (45%, v/v), water (45%, v/v) and 5 μl/min of 10,000 ng/ml of olaparib and melphalan, respectively. The highest response for olaparib was obtained with a 4000 V spray voltage, a 400 °C capillary temperature, a 360 °C vaporizer temperature, and the nitrogen sheath, ion sweep and auxiliary gasses set at 50, 2 and 30 arbitrary units, respectively; the skimmer voltage was set off. The SRM mode was used with argon as the collision gas at 1.7 mTorr. The tube lens offset was 106 V for erlotinib, 118 V for olaparib and 112 V for melphalan and melphalan-d_8. Olaparib was monitored at m/z 435.2 → 281.1; 367.2 at −30 V and −20 V collision energies, respectively; erlotinib at m/z 394.1 → 278.1 at −30 V; melphalan at m/z 305.1 → 246.1; 288.2 at −22 V and −12 V collision energies, and melphalan-d_8 at m/z 313.2 → 254.1; 296.1 at −20 V and −15 V. All transitions were monitored using 0.1 s dwell times and the mass resolutions were set at 0.7 full width at half height (unit resolution) for both separating quadrupoles.

2.4. Sample pre-treatment

All samples were kept on ice during the sample preparation. To a volume of 100 μl of human plasma, pipetted into a 1.5 ml polypropylene tube, 150 μl of 8 ng/ml erlotinib and 24 ng/ml melphalan-d_8 in 2% (v/v) acetic acid in acetonitrile were added.

The tube was then closed and shaken vigorously for *ca.* 15 s using vortex-mixing. After centrifugation of the sample at $13 \times 10^3 \times g$ at 4 °C for 10 min, 125 μl of the supernatant was pipetted into a 250 μl glass insert placed in an autoinjector vial. Before closing the vial, 125 μl of water was added and finally, 1 μl of the mixture was injected into the column.

2.5. Validation

A laboratory scheme based on international guidelines was used for the validation procedures [12–14].

2.5.1. Calibration

Stock solutions of olaparib and melphalan at 1 and 0.5 mg/ml and melphalan-d_8 (IS) at 1 mg/ml were prepared in methanol, erlotinib (IS for olaparib) was prepared in dimethyl sulfoxide at 4 mg/ml. Melphalan stock solutions were stored at −80 °C and all others at −30 °C. The 1 mg/ml stock solutions of olaparib and melphalan were combined and diluted to a 10,000 ng/ml calibration sample in pooled plasma, stored in a polypropylene tube at −80 °C. Additional calibration samples were prepared daily at 5000, 2500, 500, 250, 125, 25 and 10 ng/ml by dilution with the blank pooled plasma. The 10, 25 and 5000 ng/ml calibration samples were processed in duplicate for each daily calibration, whereas the levels in between were processed only once. Least-square linear regression was employed to define the calibration curves using the ratios of the peaks of olaparib and melphalan and the IS erlotinib and melphalan-d_8.

2.5.2. Precision and accuracy

The 0.5 mg/ml stock solutions of olaparib and melphalan were used to obtain validation (quality control; QC) samples in pooled plasma at 4000 (QC-high), 400 (QC-med), 30 (QC-low) and 10 ng/ml (QC-LLOQ). The QC samples were stored in polypropylene tubes at −80 °C. Precisions and accuracies were determined by sextuple analysis of each QC in three analytical runs on three separate days for all QCs (total: $n = 18$). Relative standard deviations were calculated for both the within day precisions (repeatability) and the between day precisions (reproducibility).

2.5.3. Selectivity

Six individual human plasma samples were processed to test the selectivity of the assay. The samples were processed without analytes and ISs and with olaparib and melphalan at the LLOQ level (10 ng/ml), supplemented with the ISs.

2.5.4. Recovery

The total recovery was determined in quadruplicate by comparing processed samples (QC-high, -med, -low) with reference solutions in water–acetonitrile (1:1; v/v) at the three validation levels (QC-high, -med, -low). The total recoveries of the ISs were assessed using identical procedures at the erlotinib and melphalan-d_8 concentrations used in the assay. The matrix effect was determined by an infusion experiment with six individual blank plasma samples. A solution of 5 μg/ml of all analytes in acetonitrile–water (50:50 v/v) was infused continuously into the detector after post-column mixing with the eluent after injecting blank pre-treated samples. No divert valve was used during the runs to show the matrix effect during the whole run time.

2.5.5. Stability

The stability of olaparib and melphalan was investigated in QC-high and -low plasma samples stored in polypropylene tubes. Quadruplicate analysis of these samples was performed after storage at 4 °C for 24 h, three additional freeze–thaw cycles (thawing at 4 °C during *ca.* 2 h and freezing again at −80 °C for at least one day), and storage at −80 °C for 2 months, respectively. Furthermore, validation runs were reinjected after additional storage of the extracts at 4 °C for three days. Finally, the responses of olaparib and melphalan from the stock solutions in methanol after 24 h at 4 °C and after 2 months of storage in the freezer (−30 °C for olaparib and −80° for melphalan) were compared to fresh stock solutions.

2.6. Patient samples

2.6.1. Olaparib

A female patient, 46-year-old, was diagnosed with a breast carcinoma with metastatic cells in the lungs, liver and skeleton. The patient was treated with 50 mg olaparib twice daily without co-medication. Blood samples were obtained at *ca.* 0, 0.5, 1, 2, 3, 4, 6 and 8 h in lithium heparin tubes after oral administration of the drug. After centrifugation at ambient temperature and at $2 \times 10^3 \times g$ for 10 min, plasma was separated and stored at −80 °C until assaying by the presented method. The samples were additionally screened for the dehydro-metabolite of olaparib using m/z 433.2 → 281.1 and 365.2 at −30 and −20 V collision energies, respectively.

2.6.2. Melphalan

Eleven patients with metastasis of colorectal cancer in the liver were treated by isolated hepatic perfusion with melphalan. Livers were perfused with 10–20 μg/ml melphalan for 30 min (Valkenburg-van Iersel et al., in preparation). Blood samples were collected before, during and after treatment in polypropylene tubes without anti-coagulant to monitor systemic melphalan exposure. Samples were stored at −80 °C and melphalan was initially assayed using an existing LC–UV method (LLOQ = 100 ng/ml) based on Sparidans et al. [3] at the Department of Clinical Pharmacy and Toxicology at the Leiden University Medical Center. These whole blood samples were re-analysed using the new LC–MS/MS method with plasma calibration and whole blood QC samples. For all available samples exceeding a melphalan concentration of 150 ng/ml ($n = 20$) results of both assays were compared.

3. Results and discussion

3.1. Method development

A simple pre-treatment procedure like protein precipitation was investigated as the first option because we expected a high selectivity and sensitivity of the MS/MS detection in combination with an LC system using 2 μm particles. Product spectra of both drugs are shown in Fig. 1. Protein precipitation with acetonitrile showed high extraction recoveries for both compounds. Water was added to the extract to obtain sufficiently sharp, symmetric peaks (Fig. 2) because the eluent contained less organic modifier than the extract.

The method is a modification of a previous method that used erlotinib as IS for the bioanalysis of axitinib in human plasma [15]. Both compounds show similar retention times compared to olaparib and melphalan. A few modifications were used: (1) due to the instability of melphalan, samples were pre-treated on ice and acetic acid was added to the precipitating agent; (2) because relative melphalan responses showed a high variability as reported in Table 1, especially at lower levels, using erlotinib as a IS, stable isotopically labeled melphalan was required to meet acceptable validation results; (3) ion source parameters and the amount of formic acid in the eluent were optimized for the most efficient ionization of olaparib, being the main drug of this investigation; for melphalan also sufficient sensitivity was observed under the

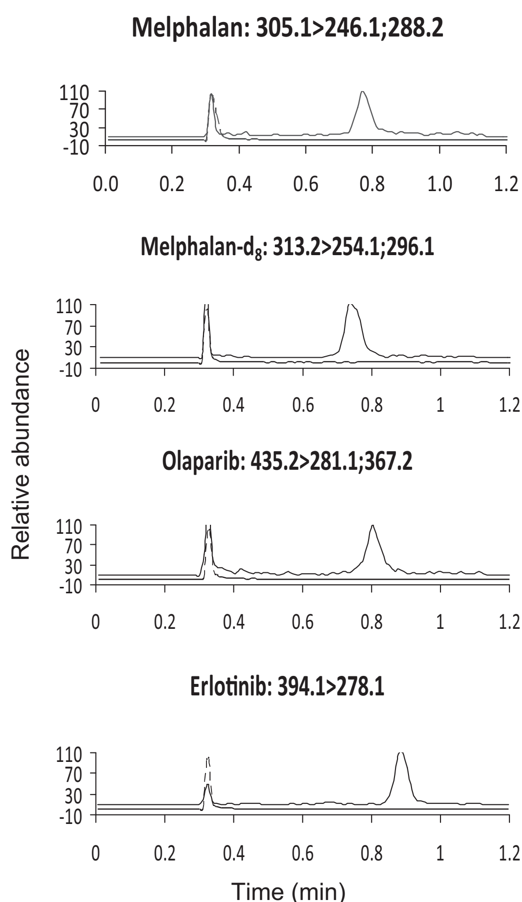

Fig. 2. SRM chromatograms of olaparib, melphalan and their ISs in plasma extracts: blank plasma and LLOQ spiked plasma with ISs are shown for each compound. An artificial offset was given the chromatogram of the spiked samples. The responses shortly after 0.3 min are caused by swiching the divert valve.

for this compound near to optimal conditions; (4) the amount of methanol in the eluent was adjusted to obtain retention times in the range 0.7–1.0 min and (5) light protection was not required, and therefore not used, for both drugs.

Table 1
Assay performance data of olaparib and melphalan from 18 validations (QC) samples in 3 analytical runs. Results for melphalan with the use of erlotinib as IS have also been added for comparison.

Nominal concentration (ng/ml)	Within day precision (%)	Between day precision (%)	Accuracy (%)
Olaparib			
10	8.9	9.3	107.9
30	5.3	6.0	105.4
400	3.0	4.1	104.9
4000	5.8	7.8	102.5
Melphalan			
10	9.3	9.8	110.3
30	3.7	4.9	108.0
400	3.7	4.8	106.8
4000	4.6	7.0	103.1
Melphalan with erlotinib as IS			
10	14.0	**30.7**	102.0
30	6.0	11.3	92.3
400	3.3	9.3	96.3
4000	7.0	9.0	100.7

Bold value: exceeds 20%

Table 2
Back calculated concentrations of olaparib and melphalan from the calibration samples using a linear calibration ($n = 3$) with a quadratic weighting factor ($1/x^2$).

Nominal concentration (ng/ml)	Precision (%)	Accuracy (%)
Olaparib		
10	3.8	98.2
25	4.6	100.4
125	2.1	99.9
250	1.9	101.0
500	2.8	99.9
2500	4.5	98.6
5000	1.3	100.0
Melphalan		
10	0.8	99.8
25	3.6	101.6
125	4.3	100.8
250	2.4	101.2
500	4.4	101.1
2500	1.8	100.4
5000	3.0	98.0

3.2. Validation

SRM chromatograms of all compounds are depicted in Fig. 2, showing chromatograms of blank and QC-low spiked plasma samples.

3.2.1. Calibration

A 10–5000 ng/ml olaparib range was chosen to assess expected levels in the range 50–5000 ng/ml for a 100 mg twice daily regimen [5]. A linear function was used for both assay calibrations. For all calibration samples (30 samples in 3 calibration curves) the concentrations were back calculated from the ratio of the peaks area (of analyte and IS; olaparib–erlotinib and melphalan–melphalan-d_8) using the calibration curves of the run in which they were included. No deviations of the average of each level higher than 2.0% were observed (Table 2), indicating the suitability of the linear regression model with quadratic weighting. The average regression parameters of the linear regression functions ($n = 3$) were $y = 0.024(\pm 0.019) + 0.0294(\pm 0.0015) \times x$ for olaparib and $y = 0.003(\pm 0.027) + 0.0344(\pm 0.0021) \times x$ for melphalan with regression coefficients (r^2) 0.9989 ± 0.0009 for olaparib and 0.9989 ± 0.0006 for melphalan, respectively; the functions show reproducible calibration parameters.

3.2.2. Precision and accuracy

Assay performance data from the validation samples at four concentrations are reported in Table 1. Between day variations and deviations of the accuracy lower than 10.5% were observed for all levels. The precision and the accuracy therefore met the required ±15% (±20% for the LLOQ) [12–14].

3.2.3. Selectivity

The analysis of six batches of blank samples showed no interfering peaks in the SRM traces for both compounds in human plasma. Blank responses could not be distinguished from the detector noise (Fig. 2) and are therefore <20% of the LLOQ response [16]. The signals of the LLOQ level (10 ng/ml) were easily distinguishable from blank responses; concentrations found at the LLOQ level ($n = 6$) were 10.7 ± 0.7 ng/ml for olaparib and 8.5 ± 0.9 ng/ml for melphalan, respectively, both show the applicability of the investigated LLOQ levels [12–14].

3.2.4. Recovery

The extraction recoveries showed only small losses for both target compounds (Table 3). Total recoveries for both internal standards ($n = 8$) were $87.9 \pm 5.4\%$ for melphalan-d_8, and $93.3 \pm 5.7\%$ for erlotinib using analyses of the QC-low levels of the analytes.

Table 3
Total recoveries (%) of both analytes ($n = 8$).

Concentration (ng/ml)	Olaparib	Melphalan
30	91.2 ± 6.9	93.4 ± 5.0
400	105.5 ± 13.4	98.9 ± 11.3
4000	76.6 ± 7.3	72.9 ± 7.4

Table 4
Stability data of olaparib (A) and melphalan (B) in plasma, reporting the percentages (%) of the initial concentration.

	QC-high	QC-low
Olaparib		
24 at 4 °C	112.5 ± 3.2	99.7 ± 0.5
3 freeze–thaw cycles	88.8 ± 2.0	102.5 ± 4.3
2 months at −80 °C	112.8 ± 1.5	112.6 ± 0.1
Melphalan		
24 at 4 °C	109.9 ± 3.3	104.1 ± 4.0
3 freeze–thaw cycles	90.7 ± 1.1	98.0 ± 4.5
2 months at −80 °C	98.3 ± 3.1	98.8 ± 7.7

The infusion experiments showed no matrix effects at the retention times of the analytes but only ion suppression starting around 0.15 min after injection and ending before 0.5 min for all compounds. This ion suppression was visible as a negative tailing peak at *ca.* 0.2 min in the infusion chromatograms, shortly after the dead time of the chromatographic system. Overall, all recoveries are acceptable for successful validation of the assay [12–14].

3.2.5. Stability

The stability of olaparib and melphalan in plasma after different storage procedures is shown in Table 4. No losses higher than 11.8% were found with low standard deviations. Re-injection of calibration and validation samples, after additional storage at 4 °C for 3 days resulted again in successful performances, additional storage before injection is therefore allowed. Recoveries of the analytes in the stock solutions were >95% for all conditions investigated: olaparib and melphalan for 2 months at −30 and −80 °C, respectively and both compounds at 4 °C for 24 h. All stability results can be considered satisfactory for the validation [12–14].

3.3. Patient samples

Because the study with combined administration of olaparib and melphalan was not yet started, separate series of samples for both drugs were analysed to demonstrate the applicability of the new assay for both compounds. Unfortunately, melphalan samples were only available in whole blood and not in the matrix of this study, lithium heparin plasma. Whole blood QC samples were successfully co-analysed in the analytical runs for melphalan.

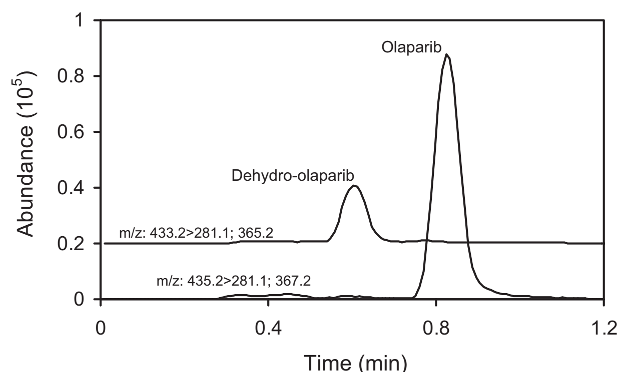

Fig. 3. SRM chromatogram of a patient sample 8 h after administration of 50 mg olaparib, an artificial offset was given to the metabolite trace.

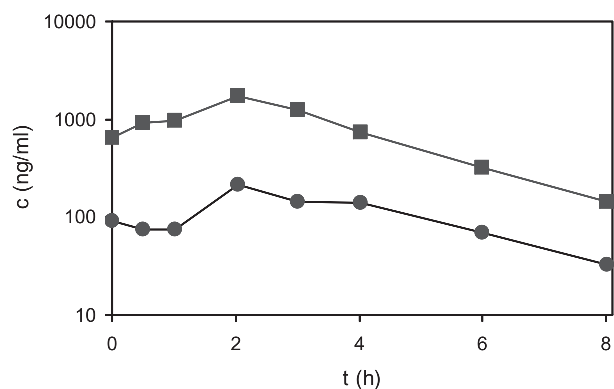

Fig. 4. Pharmacokinetic curves of olaparib and dehydro-olaparib of a patient treated with 50 mg olaparib twice daily. Metabolite concentrations were obtained by calculating the SRM response relative to the parent compound.

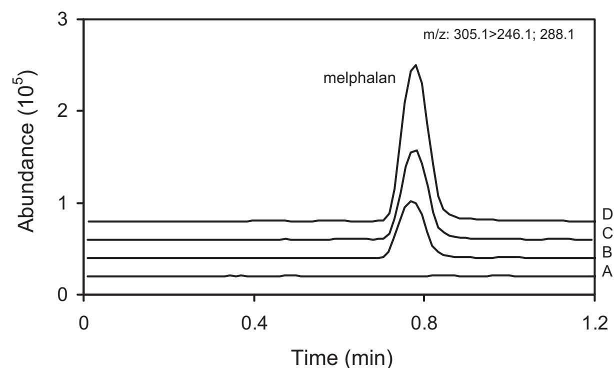

Fig. 5. Chromatograms of melphalan in whole blood taken previous to isolated hepatic perfusion with melphalan (A; blank sample) and 2 (B; 84 ng/ml melphalan), 5 (C; 140 ng/ml melphalan) and 10 (D; 177 ng/ml melphalan) min, respectively, after termination of this treatment. An artificial offset was given to all chromatograms.

3.3.1. Olaparib

A chromatogram of an olaparib patient sample is shown in Fig. 3 and the pharmacokinetic curves of this patient in Fig. 4. In addition to olaparib, its dehydro-metabolite was clearly present in all samples of this patient (Figs. 3 and 4). The structure of the metabolite was previously elucidated (dehydrogenation could be localized in the piperazine ring) based on its product spectrum (Fig. 1B), showing prominent m/z 281.1 and 365.2 products and no product with a broken (dehydro-)piperazine ring,

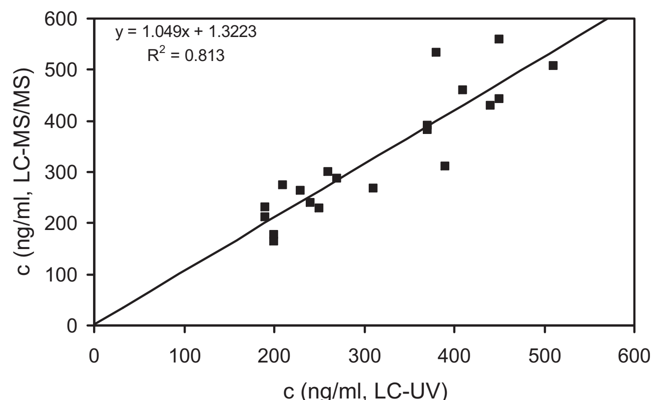

Fig. 6. Linear relation between melphalan concentrations measured by an existing LC–UV and the new LC–MS/MS assay.

after comparison with the spectrum of the parent compound (Fig. 1A). Metabolites of olaparib have not been reported previously, clinical activity and toxicity of this metabolite need further investigation.

3.3.2. Melphalan

Chromatograms of whole blood samples of a patient are shown in Fig. 5. The correlation between the results of the clinical samples (150–600 ng/ml melphalan), using the existing LC–UV and the new LC–MS/MS method, is shown in Fig. 6. The figure shows an acceptable correlation, absence of a deviation at the origin and a slope resulting in drug levels being only *ca.* 5% higher in the new assay. Therefore, the new LC–MS/MS method can be used as a faster and more sensitive alternative for the existing LC–UV melphalan assay in the future.

4. Conclusions

The first validated assay for olaparib has now been fully reported for human plasma samples, melphalan can be simultaneously determined. The LC–MS/MS assay uses a simple pre-treatment method, and thereby proves the redundancy of solid-phase extraction for olaparib [5]. It also shows values of accuracy, precision, recovery and stability for both analytes allowed by international guidelines [12–14]. For melphalan the new assay combines two advantages of both existing LC–MS/MS assays for this drug: short run times [10], it is the first chromatographic bioanalytical melphalan assay using sub-2 μm particles, and simple sample pretreatment [11]. The sensitivity for melphalan is in the lower range compared to levels reported for existing LC methods with other, less selective, detection methods [3]. The new assay can be applied to clinical olaparib studies, with or without co-administration of melphalan, in the future. Finally, the dehydro-metabolite of olaparib has been reported for the first time.

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