Rapid HPLC analysis of melphalan applied to hyperthermic isolation limb perfusion*

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Hyperthermic isolated limb perfusion (HILP) with melphalan (MH) as a standard cytotoxic drug has been performed in 28 patients suffering from malignant melanoma. MH has been administered by HILP via extracorporeal circulation system. The drug given locoregionally reduces subsequent toxicity of organs. For all that residues can leak into the systemic circulation during HILP. Because of known carcinogenic potential and secondary cancer formation, the main interest of this work is to determine MH concentration profile in the patient plasma during and after HILP and evaluation of its potential toxicity in patients. Reversed-phase HPLC assay, which uses isocratic elution and fluorimetric detection has been shown to be sensitive, reliable and suitable for routine analyses. The assay was validated for the concentration range of 50–2500 ng.ml^{-1} with the limit of detection (LOD) 6.881 ng.ml^{-1}. The samples were treated by methanol precipitation with the recovery more than 80%. The stability of standard solutions and methanolic extracts of MH were also followed. The concentration profile of MH in patient samples has been pursued in three time points during and after chemoperfusion (45 min after application of MH in extracorporeal circulation, 10 min after the joining the extremity to systemic circulation and one hour after the great vessels reconstruction). The concentrations of MH ranged 100–1500 ng.ml^{-1} and varied from patient-to-patient. Some complications were observed after HILP in 11 patients and are correlated with the higher concentrations of MH (over 150 ng.ml^{-1}) found in plasma.

Key words: Melphalan, hyperthermic isolation limb perfusion, malignant melanoma, isocratic HPLC, fluorescence detection.

Melphalan or phenylalanine mustard, MH, a bifunctional alkylating agent, is the standard cytotoxic drug in hyperthermic isolated limb perfusion HILP for melanoma, mainly in recurrent malignant melanoma (MM) of extremities, satellitosis and in-transit metastasis. It exerts a cytotoxic effect through the formation of interstrand or intrastrand DNA cross-links or DNA-protein cross-links via the two chlorethyl groups of the molecule [8, 13, 16]. It is also extensively used in the treatment of ovarian cancer, breast cancer, neuroblastoma, multiple myeloma, advanced malignant melanoma and localized soft tissue sarcoma [1].

The main advantage of HILP is that by isolating a limb from the rest of the body, a high dose of cytotoxic drug can be given locoregionally, which minimizes systemic exposure to the drug and subsequent toxicity to vital organs [12, 17]. HILP has been performed at the National Cancer Institute in Bratislava since 1995.

Although the MH is administrated by HILP via extracorporeal circulation system, the persistance of the drug residues in blood and its potential depositing in the organism after chemoperfusion can affect tissue toxicity and/or its damage. From that reasons attention is being focused on the determination of MH residues in peripheral circulation system during and after HILP.

Some analytical methods have been developed to quantify MH in biological samples. High-performance thin-layer

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chromatography (HPTLC) has been developed for estimation of MH in rabbits [15]. Mass spectrometry (MS) [9], gas chromatography-chemical ionisation-mass spectrometry (GC-CI-MS) [10] provide good results but they involve the use of expensive equipment and time consuming sample pre-treatment and are not currently available in laboratories. Several high performance liquid chromatographic HPLC methods with ultraviolet, fluorescence and electrochemical detection using isocratic or gradient elutions have been developed [3, 4, 7, 11, 14, 18]. Newly, rapid and automated 96 well solid phase extraction (SPE)/LC-MS-MS method has been succesfully validated and used to support clinical pharmacokinetic studies of MH [5].

The introduced validated HPLC method based on reversed-phase chromatography, isocratic elution, fluorescence detection has been used for the determination of MH concentration in plasma samples. The obtained concentration levels attend for leakage monitoring of cytostatic.

Material and methods

Patients eligibility. The patients with histologically proved MM have undergone HILP on the base of recommendation of melanoma expert group. We registered simultaneous diseases in some of patients as follows: hypertension (Patients No. 2, 4, 11, 14, 21, 22, 24, 25), bradycardia (Patients No. 2, 7), ischemia (Patients No. 1, 2, 14, 22), epilepsy (Patient No. 15), cardiac-pulmonary complications (Patients No. 4, 18, 19), allergy (Patients No. 12, 17), chronic gastritis (Patient No. 22), hepatopathy (Patients No. 20, 21), diabetes (Patients No. 14, 24). Biochemical and hematological parameters of patients before and after HILP, the registration of HILP course, futher therapies were also available.

Perfusion technique. The technique of HILP has been standardized by specialists at oncosurgical department in the National Cancer Institute and is well described by other investigators [4, 5, 11]. Briefly, isolation of the blood circulation of the limb was achieved by clamping the major artery and vein (v. iliaca externa) after heparinization 5000–9000 units i.v.. The extremities were warmed from initial temperature of venous blood of 38.5 °C to 41 °C of venous blood. Alkeran (Glaxo Wellcome, UK) was administrated in the total dose 1.5 $mg.kg^{-1}$ of patient weight for 60 minutes. After HILP, the extremities have been rinsed four times, the cannulaes were removed. Arteries and veins were reconstructed and deaerated. Finally, the patients were admitted to intensive care unit.

Reagents and standards. Melphalan hydrochlorid, MH, was donated by Dr. Finlay (Glaxo Wellcome, UK). Stock solutions containing 1 $mg.ml^{-1}$ were freshly prepared in methanol and further diluted with methanol to give a series of working standards. Methanol and acetic acid were of HPLC grade and were supplied by Merck (Darmstadt, Germany).

Apparatus and chromatographic conditions. The HPLC system consisted of Rheodyne 7010-091 injector (20 μl loop), 2150 HPLC pump coupled with a 2152 HPLC controller (LKB Bromma, Sweden), FP 920 fluorescence intelligent detector (Jasco, Tokyo, Japan). MH was measured at an excitation wavelength of 265 nm and emission wavelength of 360 nm. Data were evaluated by software CSW (Data Appex, Prague, Czech Republic). The chromatographic column was a Separon SGX C18, 150x3 mm i.d., particle size 7 μm (Tessek, Prague, Czech Republic). The mobile phase, consisting of methanol:water (60:40, v/v), was run at a flow-rate of 0.8 $ml.min^{-1}$ at ambient temperature. The pH of mobile phase was adjusted by acetic acid to 3.2–3.4.

Plasma samples and sample preparation. Blood samples were collected from a peripheral vein in heparinized tubes before HILP, in the middle of perfusion (45 min after application of MH in extracorporeal circulation), 10 min after the joining the extremity to systemic circulation and one hour after the great vessels reconstruction. In some patients, the plasma samples were collected from day 1 until day 4 after chemoperfusion. The blood was centrifuged at 8000 g for 5 min at 4 °C. Resulting plasma was collected, frozen and kept at –70 °C until analysis. 500 μl of plasma was transferred into Eppendorf tube and 800 μl of methanol was added. The tube was shaken in a vortex mixer for 60 s and centrifuged at 2200 g for 15 min at 4 °C. The supernatant (1200 μl) was collected into glass tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 250 μl of methanol and chromatographically analyzed.

Results

Patients characteristics. The HILP has been performed in 28 patients (26 of them with histologically proved MM, 1 patient with malignant fibrous histiocytoma, 1 patient with pleomorphous liposarcoma) at the National Cancer Institute in Bratislava since 1998. The introduced series of patients included 21 females (median age 52 years, range 33–73) and 7 males (median age 48 years, range 18–76). The localization, type of MM and classification criteria are shown in Table 1.

HPLC assay validation. The sample pre-treatment procedure involving deproteinization with methanol is simple, rapid, giving sufficient recovery and non-degradating of the drug. The recoveries of MH in plasma were 91.0, 80.0, 73.7, 99.1, 89.7% for concentrations 50, 100, 250, 500 and 2500 $ng.ml^{-1}$ (n=6).

Calibration samples were prepared by spiking 500 μl drug-free human plasma with appropriate volumes of stan-

Table 1. Localization and classification of MM in patients

Patient No./Sex/Age	Localization	Tumor type	Criteria
1/F/54	Right LL, planta	SSM	Breslow 2.2 mm, Clark IV
2/F/60	Left LL	SSM	Breslow 4.5 mm, Clark III
3/F/52	Right LL, thigh	non-specified	non-classified
4/M/48	Left LL, thigh	pleomorphous	non-classified
5/F/52	Right LL, thigh	LMM	non-classified
6/F/52	Left LL, calf		Breslow 4.0 mm, Clark IV
7/M/18	Right LL, knee	non-specified, probably NM	Breslow 4.2 mm, Clark III
8/F/52	Right LL, thigh	NM	Breslow 2.6 mm, Clark IV
9/F/41	Left LL	SSM	Breslow 2.9 mm, Clark III
10/F/45	Left LL, knee	SSM	Breslow 4.5 mm, Clark IV
11/F/47	Right LL, above knee	non-specified	Breslow 3.0 mm, Clark IV
12/F/53	Left LL, thigh	SSM	Breslow 1.1 mm, Clark IV
13/F/33	Left LL, knee+thigh	LMM	non-possible to classify by Clark and Breslow
14/F/58	Left LL, above knee	non specified	Breslow 3.0 mm, Clark IV
15/M/37	Right LL, thigh	SSM	Breslow 3.2 mm, Clark IV
16/F/65	Right LL, above knee	NM	non-classified
17/F/59	Right LL, above knee	SSM	Breslow 10.2 mm, Clark V
18/F/42	Right LL, thigh	SSM	Breslow 5.0 mm, Clark IV
19/F/52	Left LL, above knee	NM	Breslow 1.4 mm, Clark III
20/M/50	Left LL, above knee	SSM	Breslow 5.0 mm, Clark III
21/M/46	Right LL, thigh	NM	Breslow 6.7 mm, Clark V
22/F/73	Left LL, above knee	SSM	Breslow 2.0 mm, Clark III
23/F/34	Left LL, thigh	NM	Breslow 5.5 mm, Clark IV
24/F/69	Right LL, thigh	SSM	Breslow 4.1mm, Clark III
25/M/53	Right LL, thigh	SSM	Breslow 1.7mm, Clark IV
26/M/76	Right LL, planta	ALM	Breslow 1.0 mm, Clark III
27/F/67	Right LL, dorsal	SSM	Breslow 2.0 mm, Clark IV
28/F/44	Right LL, above knee	Pleomorphous liposarcomal	non-classified

LL – lower limb, SSM – superficial spreading melanoma, NM – nodular melanoma, LMM – lentigo maligna melanoma, ALM – acral lentiginous melanoma.

dard solutions in order to obtain concentrations 50, 100, 250, 500, 1000 and 2500 ng.ml^{-1}. The samples were taken through the deproteinization procedure described above and the peak heights were plotted against the corresponding concentration. Regression analysis was performed for obtaining the calibration curve. The equation for calibration line was found to be y = 0.232x – 2.575, correlation coefficient 0.9998. The unknown MH concentrations in patient plasma samples were determined from the equation generated by the least-squares regression analysis.

The retention times of MH showed intra- and inter-day variation less than 1% (RSD) and the peak heights determined varied less than 5% (RSD). The relative retention time of MH was 4.3 min, analysis time was 10 min. No interferences were observed within the time in which the MH was detected. The precision of the method (% RSD) in human plasma was determined by assessing the agreement between measurements of spiked samples prepared independently from the calibration standards. Spiked samples at five concentrations were analyzed in six replicates for the evaluation of intra-day variability and in five replicates on three days for evaluation of inter-day variability. The data were examined by analysis of variance (ANOVA). The results are summarized in Table 2 and show that at all concentrations, the precision (% RSD) does not exceed the acceptance criteria of 15%. The accuracy expressed as a bias (%) exceeds in two cases of $\pm$15% (50 and 100 ng.ml^{-1}).

The limit of detection was taken as the amount of MH giving a signal to noise ratio greater than 3. The limit of detection (LOD) determined for MH was 6.881 ng.ml^{-1}. Furthermore, the limit of quantification (LOQ) was taken as the minimum quantifiable concentration presenting a CV value $\leq$ 2.5% (n=15) and was 28.945 ng.ml^{-1}.

Stock solutions of MH (1 mg.ml^{-1}) in methanol were stable at least 3 months. The stability of working solutions was determined by comparing peak height data obtained from a dilution of a freshly prepared solution against the equivalent solutions (100 and 2500 ng.ml^{-1}, respectively) that had been stored at 4 ˚C. These solutions were stable over the week, recoveries averaged 98% after 24 h, 90% after 5 days. In plasma samples spiked with 100 and 2500 ng.ml^{-1}, MH concentration decreased after 1 h at 4 ˚C with losses averaging 60 and 70%, respectively. Methanolic extracts were stable during 30 min whatever the studied concentrations. Their recoveries averaged after 1 h at 4 ˚C for 100 ng.ml^{-1} 85% and for 2500 ng.ml^{-1} 90% .

Table 2. Intra- and inter-assay reproducibilities of HPLC analysis. Precision and accuracy of HPLC assay

Spiked concentration added (ng.ml^{-1})	Mean calculated concentration (ng.ml^{-1})	RSD (%)	Deviation from theoretical value (%)
Intra-day *(n6)*			
50	41.926 ± 5.579	13.3	16.1
100	93.834 ± 9.371	9.9	6.2
250	238.288 ± 15.350	6.4	4.7
500	515.269 ± 19.905	3.9	3.1
2500	2711.590 ± 111.748	4.1	8.5
Inter-day *(n15)*			
50	46.225 ± 5.378	11.6	7.5
100	112.445 ± 2.907	2.6	12.4
250	233.842 ± 17.200	7.4	6.5
500	491.434 ± 37.168	7.6	1.7
2500	2374.841 ± 183.213	7.7	5.0

Table 3. MH concentrations in patient samples

Patient No./Sex/Age	Dose of Alkeran in mg	The concentration of MH in ng.ml^{-1}		
		1	2	3
1/F/54	100	538.262	144.912	n.d.
2/F/60	75	95.753	45.601	n.d.
3/F/52	80	120.701	1515.687	39.760
4/M/48	100	151.634	70.300	19.053
5/F/52	100	603.312	533.276	157.284
6/F/52	80	515.339	353.563	n.d.
7/M/18	50	350.162	319.344	n.d.
8/F/52	75	219.967	n.d.	n.d.
9/F/41	80	421.888	217.334	n.d.
10/F/45	100	n.d.	n.d.	n.d.
11/F/47	100	n.d.	n.d.	n.d.
12/F/53	80	163.804	177.276	n.d.
13/F/33	80	181.063	127.022	n.d.
14/F/58	100	321.741	344.804	n.d.
15/M/37	100	n.d.	n.d.	n.d.
16/F/65	100	n.d.	n.d.	n.d.
17/F/59	100	204.429	30.927	n.d.
18/F/42	70	n.d.	n.d.	n.d.
19/F/52	80	435.747	158.098	n.d.
20/M/50	100	43.163	38.256	n.d.
21/M/46	100	462.526	241.954	n.d.
22/F/73	100	157.514	46.239	n.d.
23/F/34	80	122.041	77.364	n.d.
24/F/69	90	47.184	111.344	n.d.
25/M/53	100	n.d.	n.d.	n.d.
26/M/76	100	397.295	208.975	89.447
27/F/67	100	1057.905	2943.095	n.d.
28/F/44	80	n.d.	n.d.	n.d.

1 – sample collected 45 min from the beginning of chemoperfusion, 2 – 10 min after the joining the limb to normal (systemic) circulation, 3 – 1 h after great vessels reconstruction, n.d. – not detected.

Results of HPLC analysis. The validated HPLC method was applied for analysis of patient plasma samples. The obtained concentrations of MH in patient samples are included in Table 3. The concentrations of MH in the sample collected 45 min after application of MH were in the range 100–1100 ng.ml^{-1} (50% of patients in the range 150–500 ng.ml^{-1}, 18% less but up to 150 ng.ml^{-1}, 7% in the range 500–1100 ng.ml^{-1}, 25% of patients MH was not detected). The lower concentrations were measured in the plasma samples collected after 10 min, when the extremity was joined to systemic circulation. The concentration was in the range 100–350 ng.ml^{-1} in 39% of patients, under 100 ng.ml^{-1} in 21%, under LOQ was in 29% of patients and over 500 ng.ml^{-1} in 11% of patients. The concentration of MH in the samples collected from the day 1 until day 4 after the operation was found to be very low, in the range between LOD and LOQ. The quantification of these results is charged of inaccuracy.

Discussion

The methods determining MH include HPTLC, GC and LC with various detection modes (ultraviolet, fluorescence, electrochemical and MS) and various sensitivity to measure the levels of MH in biological fluids. MH shows to be unstable in water solutions. Its stability is strongly affected by the pH and chloride ion concentration. These reasons should be taken into account in the purification of the analyte from biological matrix and also in proper analysis. Extraction methods as protein precipitation and liquid-liquid extraction are employed in the literature [11, 14, 18]. SPE [6], in some cases, full-automated [3] or rapid automated 96-well SPE [5] were applied as clean-up procedures too. The reversed-phase or cyanopropyl cartridges were used and recoveries were higher than 80% for both [2]. The recoveries obtained using methanol protein precipitation reported here were reproducible and with sufficient values. The recoveries were not improved when acidified methanol was used for the precipitation. The sample pre-treatment procedure is rapid, not expensive and avoiding degradation of the drug.

The method validated for the concentration range 50–2500 ng.ml^{-1} has good reproducibility and accuracy is not exceeding 20% and the limit of quantitation (28 ng.ml^{-1}) at the detection used was comparable with the earlier published LC methods [4, 6, 18]. The run time of the sample was 10 min what is sufficient, mainly for routine analysis. The results of chromatographic analysis may be available to medical doctors in 30 min after chemoperfusion.

Regional perfusion permits regional drug delivery in which the same high concentrations can be maintained for a desired time in the perfused organ, while the rest of the body is spared. For all that, the MH concentrations found in

patient plasma indicate its leakage into blood circulation. This leakage can be reliably monitored by validated HPLC method. The higher plasma concentrations (over 150 $ng.ml^{-1}$) of MH found in 11 of patients are probably in correlation with occurring complications, as anemia, thrombocytopenia and leucopenia, subcutaneous necrosis and edema formation, allergic cutaneous reactions. The chemoperfusion has not been effective in three cases. After HILP, in three cases relaps was confirmed three months after surgery.

The complete evaluation of potential risk of MH residues to regional and systemic toxicities with regard to treatment response is futher required to study.

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