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A sensitive high-performance liquid chromatographic assay for melphalan and its hydrolysis products in blood and plasma

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Summary. A sensitive high-performance liquid chromatographic assay has been developed for the measurement of the alkylating cytostatic drug melphalan (4-[*bis*(2-chloroethyl)amino]-L-phenyl-alanine, or L-phenylalanine-mustard, L-PAM) and its two hydrolysis products, monohydroxy melphalan (MOH) and dihydroxy melphalan (DOH). A reversed-phase phenyl column and a mobile phase consisting of acetonitrile/citrate buffer made possible an isocratic separation and quantification. *N,N*-[*bis* (2-hydroxy-ethyl)]toluidine has been synthesized as an internal standard structurally related to DOH. A new, accurate "kinetic" calibration procedure enabled us to determine even the concentration of the unstable MOH. The lower limit of quantification was 30 ng/ml for L-PAM and 20 ng/ml for both DOH and MOH with fluorescence detection. The use of this method is illustrated by some pharmacokinetic data in systemic and locoregional melphalan therapy.

Introduction

Melphalan (Alkeran, 4-[*bis* (2-chloroethyl)amino]-L-phenylalanine, L-phenylalanine mustard, L-PAM) is still the drug of choice in the therapy of multiple myeloma [16]. In spite of its use in clinical therapy for three decades, its pharmacokinetics has been investigated only in recent years [3, 4, 7, 26]. Few data are available about the reaction products of L-PAM in the human body. Because L-PAM is rapidly hydrolyzed in water to mono- and dihydroxy melphalan (MOH, DOH) [9], both hydrolysis products have been proposed as the major reaction products in the organism [2, 3, 9, 16, 23]. The previous assays have not been sensitive enough to measure the hydrolysis products of L-PAM in blood or plasma after therapeutic doses.

The HPLC assay described here allows the measurement of the kinetics of L-PAM as well as of MOH and DOH in the same run. The results obtained from patients under treatment present the basis for a reasonable discussion of the physiological and toxicological significance of L-PAM hydrolysis products.

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Materials and methods

Chemicals. Crystalline L-phenylalanine-mustard was donated by Deutsche Wellcome (Burgwedel, FRG). A mass spectrometer confirmed its identity.

Dihydroxy melphalan was obtained via a complete hydrolysis of L-PAM in distilled water at 60° C for 2 h. DOH was HPLC chromatographically pure; its structure was confirmed by mass spectrometry of the total hydrolysis product and of its trimethylsilyl derivative.

Monohydroxy melphalan could not be obtained as a pure crystalline compound, but only as one of the three components of the mixture after partial hydrolysis of L-PAM, and was separated in the HPLC system. Its structure was confirmed by mass spectrometry of its trimethylsilyl derivative.

N,N-[*bis* (2-hydroxy-ethyl)]toluidine, not commercially available, was synthesized from toluidine and ethylene oxide, as follows: 0.01 mol *p*-toluidine in 1 ml H_2O and 2 ml glacial acetic acid were cooled to −10° C. To the stirred mixture, 10 ml cooled ethylene oxide were added. The mixture was allowed to come to room temperature very slowly; stirring continued for 72 h. Excess reagent was removed at room temperature in a rotary evaporator, and the solution was neutralized with ammonia. The oily product was washed with water, dried, and crystallized from ethanol/diethyl ether. Its purity and structure were proved by HPLC, NMR, and mass spectrometry.

All solvents were of HPLC quality; the other chemicals were analytical grade reagents.

HPLC conditions. The separation was achieved on a 250 × 4.6 mm i.d. column packed with 5-µm reversed-phase phenyl Spherisorb S 5 P at room temperature. The 25-mm-long precolumn contained the same packing material, but with 10-µm particles. At an initial pressure of 180 bar, the mobile phase (acetonitrile:citrate buffer = 15:85, vol/vol) was eluted with 1.5 ml/min. The compounds were measured with a fluorescent detector at an excitation wavelength of 270 nm and an emission wavelength of 350 nm. The citrate buffer was made from 0.53 g citric acid monohydrate, 0.22 g sodium chloride, 1.3 ml 1 *M* NaOH, and deionized water to 5000 ml. The buffer was titrated with 40% citric acid in the pH range 3.00–3.25 until sufficient chromatographic separation was obtained.

Workup. Blood samples of patients were drawn into heparinized tubes, and blood was centrifuged at 0° C. Blood

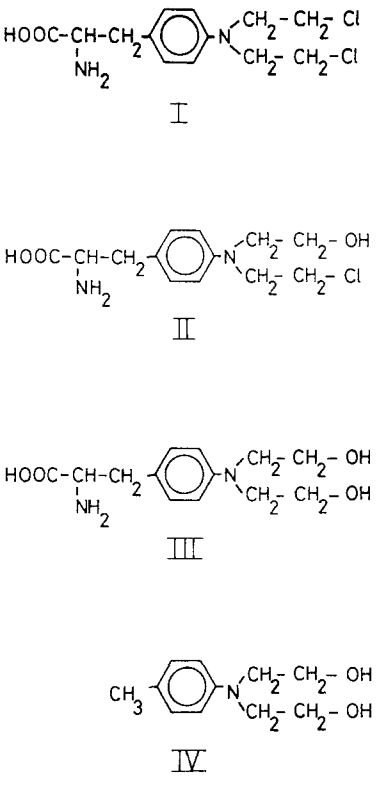

Fig. 1. Structure formulae. *I*, melphalan = L-PAM (L-phenylalanine mustard); *II*, monohydroxy melphalan = MOH; *III*, dihydroxy melphalan = DOH; *IV*, *N,N* [*bis* (2-hydroxyethyl)] toluidine = IS (internal standard)

and plasma samples were stored at −80° C until analysis. To 1 ml sample, 10 µl internal standard solution (100 µg IS in 1 ml distilled water) was added. The sample was homogenized (vortex-type mixer) and sucked through an activated Sep-Pak-C18-Cartridge (Waters) with aspirator vacuum. The cartridges were washed with 5 ml ice-cold deionized water and sucked dry. Thereafter, the compounds of interest were eluted with 1.5 ml methanol into tubes with pointed tips. The solvent was evaporated under a stream of nitrogen at room temperature; the dry samples were stored at −20° C. For analysis, the dry residue was dissolved in 0.1 ml ice-cold buffer within the next 15 days. The samples were homogenized with a vortex mixer and centrifuged. Of the buffer solution, 25 µl was injected into the HPLC system.

Calibration. A direct calibration is possible for DOH, but for L-PAM this is possible only with limited accuracy, because it hydrolyzes in aqueous solution. A freshly prepared calibration solution contains up to 5% hydrolysis products, and hydrolysis continues. For MOH, a direct calibration is impossible, because due to its instability it cannot be obtained as a pure compound.

However, we found a method of calibration via the hydrolysis characteristics of L-PAM in aqueous solution, where MOH and DOH were being formed. If the hydrolysis is interrupted (by cooling) at different intervals, mixtures are obtained with different concentrations of L-PAM, MOH, and DOH. These concentrations are unknown at the time of measurement. An adequate number of such mixtures was chromatographed. From the peak ratios obtained and the solution of the set of linear equations, we could calculate retrospectively the actual concentrations in each of these mixtures.[1]

These hydrolysis mixtures served as calibration stock solutions for spiking blank samples of plasma and whole blood. It was guaranteed by cooling on ice that the hydrolysis of melphalan did not continue in these calibration samples. The validity of this calibration procedure was confirmed by a conventional calibration of L-PAM and DOH (correlation coefficient ≥0.996, see below). Known amounts of L-PAM and DOH were spiked to plasma and whole blood.

The exact calibration procedure was performed as follows: 3 ml 100 µg/ml L-PAM solution and 3 ml 100 µg/ml internal standard solution in 10 mmol HCl, respectively, were mixed. Hydrolysis was carried out at 50° C. After 5, 15, 30, and 60 min 1-ml samples were withdrawn and cooled on ice. From these samples, 20-µl aliquots were chromatographed directly and used to spike blank plasma and blood calibration samples immediately. Thus, these samples contained (at a beginning concentration of 50 µg/ml) 1 µg IS and 1 µg (3.28 nmol) partly hydrolyzed melphalan (L-PAM + MOH + DOH).

Quality control. At the beginning of each measuring day, the following chromatograms were evaluated: the mixture of the three compounds and internal standard in aqueous solution, spiked pool plasma as described above, the calibration samples, and the blanks (samples obtained before the drug was given).

Plasma kinetics in systemic and regional melphalan therapy. As an application of our HPLC method, we show some kinetic data in the i.v. administration and hyperthermic limb perfusion of melphalan.

In the systemic treatment of eight patients with multiple myeloma (mean age ±SD, 61±7 years), 0.5 mg/kg melphalan was injected as a bolus into an antecubital vein.

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[1] The quantification of HPLC peaks requires the following equation:

$$C_{subst} = f_{subst} \cdot PR_{subst} \quad (1)$$

c_{subst}: concentration of a substance
f_{subst}: calibration factor of a substance
PR_{subst}: peak ratio ($area_{substance}/area_{internal\ standard}$).

The sum of the molarities of all three substances is constant and equal to the initial molarity of L-PAM, i.e.:

$$\text{starting concentration} = C_{L\text{-}PAM} + C_{MOH} + C_{DOH} = \text{constant}, \quad (2)$$

or

$$\text{starting concentration} = f_{L\text{-}PAM} \cdot PR_{L\text{-}PAM} + f_{MOH} \cdot PR_{MOH} + f_{DOH} \cdot PR_{DOH}. \quad (3)$$

First, the three calibration factors (f) (in the medium 10 mmol HCl) are unknown. The starting concentration of L-PAM and the peak ratios (PR) (calculated from the chromatogramms of the hydrolysis mixtures) are known quantities. If they are inserted in Eq. (3), as many different, independent equations are obtained as chromatogramms at different hydrolysis intervals have been recorded. At least three chromatograms are required for calculation. We chromatographed four hydrolysis mixtures and obtained four equations. By known mathematical procedures, we calculated the unknown calibration factors in HCl. This enabled us to calculate the concentrations in each of these hydrolysis mixtures that were used to spike plasma and blood samples. With these known concentrations, exact calibration could be performed.

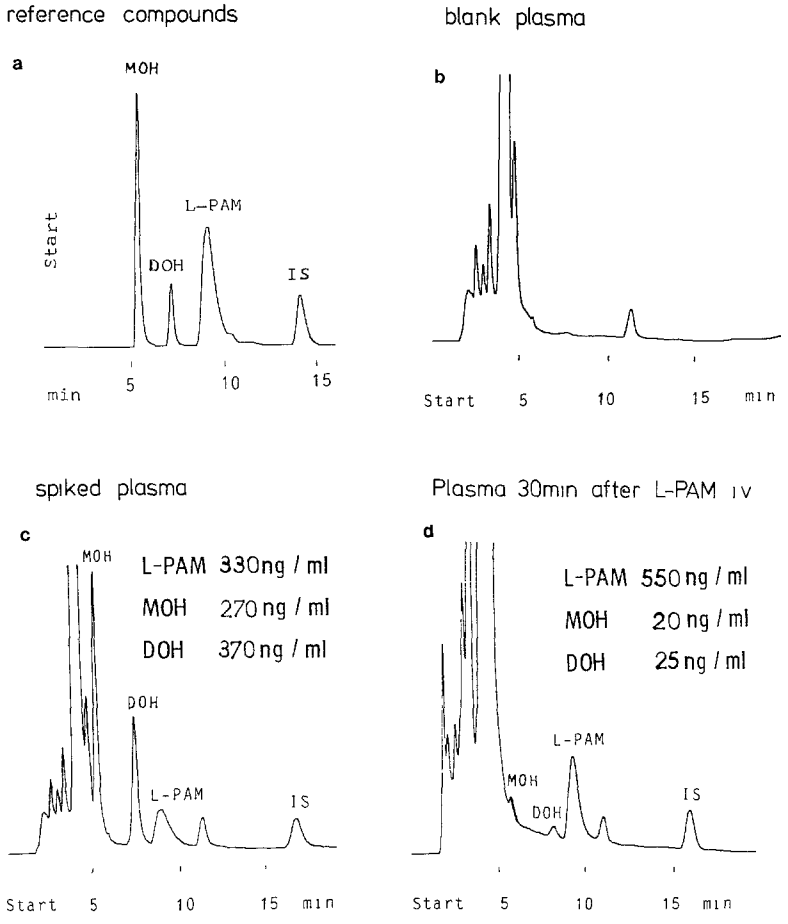

Fig. 2a–d. HPLC chromatograms of **a**, the reference compounds, L-PAM (10 µg/ml) and IS (10 µg/ml), 30 min at 37° C in H_2O, 20 µl injection; **b**, plasma of a patient before treatment; **c**, spiked plasma; **d**, plasma of a patient, sample obtained 30 min after a 0.5 mg/kg i.v. injection of L-PAM

Venous blood samples were taken from another cannula into heparinized tubes just prior to the injection and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, and 5 h following melphalan administration.

Hyperthermic regional perfusion therapy was carried out in eight patients with locoregional metastases of malignant melanoma (mean age ±SD, 53±8 years, melphalan dosage 10 mg per liter extremity volume, mean achieved perfusion temperature 41° C over about 1 h, method described by Wieberdink et al. [24]). Blood samples were drawn at 10-min intervals from the beginning of the procedure.

All specimens were put on ice in order to prevent spontaneous hydrolysis of melphalan. Blood samples were centrifuged and plasma was stored at −80° C until analysis.

Results

The HPLC peaks containing L-PAM, DOH, and internal standard were collected; solvent and buffer salts were removed; the identities of these compounds were confirmed by mass spectrometry. The absence of interfering peaks was demonstrated by chromatograms of the blood samples of each patient, obtained at time zero. Thus, the assay was specific without other interferences.

For recovery evaluation of the method, three blank plasma samples and three blank whole-blood samples were processed as usual, except that no internal standard was added. The hydrolysis mixture, containing internal standard, was added to the extracts prior to chromatography. The mean values were used as the 100% recovery values. Ten spiked, blank plasma samples and ten spiked, blank whole-blood samples were processed as usual. The mean recoveries (±SD) calculated were:

	from plasma	from whole blood
L-PAM	66±3%	69±4%
MOH	77±3%	80±3%
DOH	56±5%	64±5%
internal standard	88±2%	85±2%

With this method, the lower limit of quantification was found to be 30, 20, and 20 ng/ml for L-PAM, MOH, and DOH, respectively. The lower limit of detection (signal-to-noise ratio = 2:1) was about 5 ng/ml or 15 nmol/l.

For studying the reproducibility of the assay, one sample of pool plasma, spiked with 2.88 µmol/l L-PAM, 1.22 µmol/l MOH, and 2.46 µmol/l DOH, was measured ten times. The coefficients of variation were 2.06%, 2.10%, and 2.76% for L-PAM, MOH, and DOH, respectively. Data are given in Table 1.

We determined the day-to-day precision by the following procedure: Aliquots of a plasma pool (spiked with internal standard solution and partially hydrolyzed melphalan, i.e., L-PAM, MOH, and DOH) were stored at −80° C. One aliquot was always processed with patient samples and measured as the first sample. Over a period of 3 months, measurements of ten series yielded coefficients of variation of 10.2%, 5.9%, and 9.4% for L-PAM, MOH, and DOH, respectively. Data are given in Table 1.

Table 1. Reproducibility and day-to-day precision: means, standard deviations, and coefficients of variation (spiked plasma measured ten times on the same day, or over a period of 3 months, respectively)

	Reproducibility			Day-to-day precision		
	L-PAM	MOH	DOH	L-PAM	MOH	DOH
Mean (µmol/l)	2.88	1.22	2.46	1.24	0.96	1.08
SD (µmol/l)	0.059	0.025	0.068	0.126	0.056	0.101
CV (%)	2.06	2.10	2.76	10.16	5.89	9.35

Table 2. Accuracy: ten measurements of plasma and whole blood spiked with both L-PAM and DOH. Amounts added, and percentage deviations of the amounts found, and coefficients of correlation are shown

Accuracy					
L-PAM			DOH		
Amounts added (ng/ml)	% deviation of the amounts found		Amounts added (ng/ml)	% deviation of the amounts found	
	in plasma	in blood		in plasma	in blood
100	− 7.6	− 4.6	50	− 8.2	+ 7.6
200	+ 6.1	− 5.7	50	+ 6.4	−10.8
250	− 0.4	+ 2.1	100	+ 7.1	+ 5.4
250	−11.4	− 6.2	100	+ 2.2	− 6.3
250	+ 5.2	+ 6.2	200	− 2.2	+12.1
400	− 2.1	+ 7.1	250	+12.1	+ 7.7
500	+ 6.7	− 0.6	250	− 8.2	− 2.3
500	− 2.3	+ 5.2	500	+ 9.0	+ 7.6
1,000	− 2.2	+ 5.8	500	+ 4.8	− 6.6
10,000	− 8.7	− 1.1	5,000	− 3.5	− 2.0
	Coefficient of correlation				
	in plasma	in blood		in plasma	in blood
	0.996	0.997		0.996	0.996

Table 3. Calibration: Calculated calibration factors, equations for the linear regression lines, and their coefficients of correlation in plasma and whole blood

Calibration			
Substance	f = calibration factor (µmol/l)	Calibration line y = ax + b	R = coefficient of correlation
L-PAM			
in plasma	1.307	y = 0.765 x + 0.009	0.999
in blood	1.196	y = 0.836 x + 0.01	1.000
DOH			
in plasma	1.193	y = 0.858 x + 0.087	0.997
in blood	0.981	y = 1.019 x + 0.024	0.999
MOH			
in plasma	0.721	y = 1.387 x + 0.054	0.998
in blood	0.651	y = 1.537 x + 0.028	0.999

In order to evaluate the accuracy of the method, ten 1-ml aliquots of pooled plasma and ten 1-ml aliquots of pooled whole blood were spiked with both L-PAM and DOH. Amounts added and deviations of the amounts found are shown in Table 2. The coefficients of correlation were ≥0.996.

Calibration

The linearity was controlled in the range from 0–100 µg/ml for L-PAM and DOH, and from 0–5 µg/ml for MOH, respectively (coefficient of correlation, ≥0.997). The calibration factors in plasma and whole blood, the equations

for the calibration lines, and their correlation coefficients are given in Table 3.

By this calibration procedure there is no need of repeated pipetting or diluting, hence errors can be avoided. The different concentration steps are the "internal" result of L-PAM hydrolysis. The internal standard compensates other possible error sources. The accuracy control confirmed the calibration results via known added amounts of L-PAM and DOH (i.e., as in the conventional calibration procedure).

Plasma kinetics in patients

The concentration time integrals (area under the curve, AUC) of melphalan and its hydrolysis products in systemic therapy are shown in Table 4. One example of the kinetics after i.v. bolus injection of melphalan in a patient is shown in Fig. 3. It should be pointed out that the two hydrolysis products reached only very low concentrations and could hardly be detected after the first hour.

A larger portion of MOH was found in hyperthermic extremity perfusion therapy (Table 4). Figure 4 presents an example of a typical plasma concentration time curve for melphalan and its hydrolysis derivatives after locoregional administration of 10 mg melphalan per liter limb volume at a mean perfusion temperature of 41° C. In this mode of application, melphalan showed an increased decline of plasma concentration compared to the concentration time plot of systemic therapy (Fig. 3). In terms of the higher dose of melphalan and hyperthermia, the hydrolysis derivatives were markedly increased. The concentrations of MOH exceeded those of DOH. The sum of the AUC of the hydrolysis products amounted to 29%±6.4% of the AUC of melphalan during the perfusion period. Therefore, even in hyperthermia, inactivation of L-PAM by hydrolysis plays only a minor role. Most of the drug remains available as the alkylating active substance during perfusion.

Table 4. Areas under the plasma concentration time curves (AUC) of L-PAM, MOH, and DOH in locoregional therapy (10 mg melphalan/l limb volume, 10 to 50 min in hyperthermic isolation perfusion) and in systemic therapy (0.5 mg/kg melphalan i.v., extrapolated integral)

Patient	Extremity	$AUC_{L\text{-}PAM}$	AUC_{MOH} (min * ng/ml)	AUC_{DOH}
Locoregional therapy				
R. U.	arm	185360	52250	6260
S. T.	arm	127770	36335	5295
D. H.	leg	309610	55680	13305
E. E.	leg	509030	99260	10180
M. M.	leg	445690	119675	7880
R. H.	leg	444740	107415	5705
S. E.	leg	229800	78580	8035
W. A.	leg	292055	85665	4800
Systemic therapy				
A. M.		78635	760	220
H. K.		56948	–	180
M. C.		64503	450	675
S. A.		74666	–	–
S. C.		95751	620	935
S. F.		100826	575	520
S. M.		97494	–	375
Z. M.		43906	–	–

Discussion

This newly developed HPLC assay allows the simultaneous, specific, and sensitive quantification of melphalan and its hydrolysis products, MOH and DOH, in the blood and plasma of patients on melphalan therapy. Until now, MOH and DOH, the prominent products of spontaneous hydrolysis, have been regarded as the main reaction products in man [2, 3, 9, 16, 23]. Especially DOH has been discussed as a compound that is eliminated only very slowly [2]. Ahmed and Hsu [2] have shown a typical chromatogram of plasma from a patient on oral melphalan therapy (days 1–5; dose, 5 mg/m^2 per day). In this patient they found a large peak attributed by them to DOH at day 6, even after a therapy-free interval of more than 3 weeks. This finding has not yet been verified. In contrast, Alberts et al. [3] have supposed that melphalan is mainly eliminated by DOH. The situation is probably even more compli-

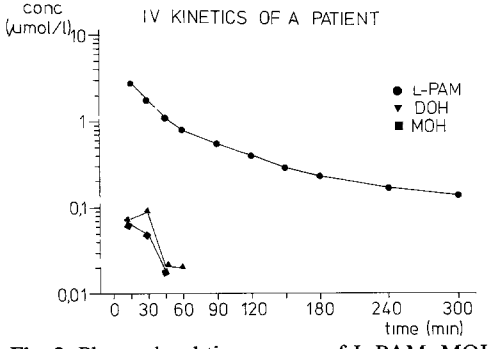

Fig. 3. Plasma level time course of L-PAM, MOH, and DOH in a patient after a 0.5 mg/kg i.v. injection of L-PAM

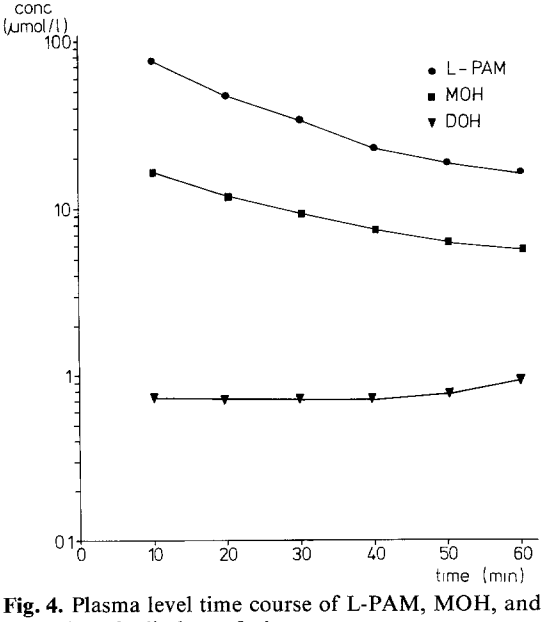

Fig. 4. Plasma level time course of L-PAM, MOH, and DOH in a hyperthermic limb perfusion at a mean temperature of 41° C (10 mg L-PAM/liter volume of the limb)

cated, for it is still unknown if the DOH found in the urine was formed in the blood or in the bladder.

Several assays available for the determination of L-PAM [10, 18–20, 23] were not suitable, for routine use on patients. Only HPLC assays [1, 2, 5, 8, 11–13, 15, 17, 21, 25] allowed pharmacokinetic studies of melphalan [3, 4, 7, 26], but not of its hydrolysis products. In these HPLC assays, reversed-phase C_{18} columns were used. Whereas L-PAM had a reasonable retention time, the very polar hydrolysis products were eluted with the front and, in biological samples, were buried in interferences. Methods using radioactively labelled L-PAM [6, 9, 13] have yielded some results concerning DOH and MOH, but radioactivity should be avoided in patients. Ahmed and Hsu [2] and Taha et al. [22] have been able to detect L-PAM and DOH, but not MOH, in plasma. The sensitivity of their methods is not sufficient for clinical samples.

To overcome these problems, we developed a new and sensitive HPLC method. With a phenyl column and an isocratic system, L-PAM, MOH, and DOH peaks could be specifically separated from serum interferences. Compared to the UV detection used by most authors, fluorescence detection increased sensitivity and specificity. We synthesized an internal standard, structurally related to DOH, which guaranteed a high reproducibility of the measurement (coefficient of variation $<3\%$). The cartridge solid-phase extraction resulted in a sufficient extraction rate for all substances ($>60\%$). The loss may be partly due to irreversible protein binding of melphalan [8].

A further advantage of our assay was the kinetic calibration method that permitted even the determination of the MOH concentration. It was based on a known initial melphalan concentration and its hydrolysis characteristics, resulting in calibration points for all three substances. The evaluation of the accuracy of the method described confirmed the validity of their calibration procedure (coefficient of correlation >0.996). The lower limit of quantitative measurement amounted to 30 ng/ml for L-PAM and to 20 ng/ml for the other two compounds. Even 5 ng/ml was detectable at a signal-to-noise ratio of 2:1.

In the patients with systemic melphalan therapy, we could only detect minor concentrations of the hydrolysis derivatives. In locoregional hyperthermic perfusion therapy, there was a higher percentage of detectable MOH, indicating a different pharmacokinetic behavior for melphalan. This probably depended on the missing renal excretion or the higher limb temperature in this therapeutic form. In both applications, the hydrolysis of melphalan was not the superior degradation pathway, contrary to previous discussions in the literature [2, 3, 9, 16, 23]. The alkylation reaction of the bifunctionally cytotoxic drug seems to be more important. However, more clinical data are needed.

With the specific and sensitive HPLC method described, it is now possible to evaluate the relevance of MOH and DOH in the pharmacokinetics of melphalan.

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