Cancer Chemother Pharmacol (1989) 23: 76–80

Cancer Chemotherapy and Pharmacology

Relevance of the hydrolysis and protein binding of melphalan to the treatment of multiple myeloma*

S. Gera, E. Musch, H. K. O. Osterheld, and U. Loos

Department of Internal Medicine, University of Bonn, D-5300 Bonn, Federal Republic of Germany

Summary. Experiments to determine the hydrolysis and protein binding of melphalan (L-phenylalanine mustard, L-PAM) were carried out in vitro for therapeutic concentrations of the drug: the decrease in L-PAM concentration in plasma and whole blood during 24 h incubation at 37° C was only 5% due to hydrolysis. Serum protein binding was about 90%, whereby 60% and 20% of this binding was due to interactions with albumin and acid α_1-glycoprotein, respectively. Immunoglobulins did not participate in the binding of L-PAM. The covalently bound part of L-PAM in serum was 30% in the concentration range of 1–30 μg/ml. The binding of dihydroxymelphalan (DOH) in serum did not exceed 20%. Glucocorticoids used in combination with L-PAM for treating multiple myeloma did not influence its protein binding. Our study with 35 sera from 15 patients with multiple myeloma shows that high levels of paraproteins do not increase but may decrease the binding of L-PAM, resulting in an elevated concentration of free drug.

Introduction

For more than 20 years melphalan (L-phenylalanine mustard, L-PAM) has been the drug of choice against multiple myeloma [1]. Its functional group is a di-(2-chlorethyl)-nitrogen configuration that binds covalently to nucleic acids of tumor cells, thus inhibiting further mitotic divisions [4]. Previous publications have indicated that hydrolysis to mono- and dihydroxymelphalan (MOH, DOH) is the most important factor for the inactivation of the drug in vivo [1–3, 16]. With a recently described HPLC method [14], it is now possible to detect unlabelled L-PAM in serum simultaneously with its hydrolysis products. Therefore, our first intention was to determine the extent of its hydrolysis.

The total binding of L-PAM in serum as well as in solutions of albumin, acid α_1-glycoprotein, and immunoglobulins was measured to identify the major binding proteins of the drug. Analogous studies were carried out with DOH.

Apart from the reversible binding of L-PAM to serum proteins (which is described by the law of mass action), there is irreversible binding due to alkylation [5, 6]. Knowledge as to the extent of this binding is of practical interest for the clinician, as covalently bound L-PAM does not exert antitumor activity. In previous studies the L-PAM serum concentration used was 20 times higher than the therapeutic levels [5, 6]. In the current study, a modified ultrafiltration procedure was used to measure the covalently bound fraction of L-PAM in the therapeutic range of serum concentrations (≤ 1.5 μg/ml).

In the treatment of multiple myeloma L-PAM is combined with prednisone, since this combination has proved to be more effective than single-drug therapy. As one explanation for this augmentation of L-PAM activity, Alexanian et al. [3] have proposed the inhibition of tumor cell proliferation by prednisone. The results of our binding study with L-PAM raised the question as to whether glucocorticoids diminish the protein binding of L-PAM, since this would be an alternative explanation for the superiority of combination therapy.

In the course of multiple myeloma, serum concentrations of proteins, that are potential binding sites for L-PAM fluctuate considerably. The question as to whether this fluctuation results in changes in the protein binding of the drug was examined using 35 sera from 15 patients with multiple myeloma. The data obtained were interpreted by means of associated serum electrophoresis.

Materials and methods

Chemicals

Melphalan: Crystalline L-PAM was donated by the Deutsche Wellcome GmbH, Duisburg-Wedel. Mass spectrometry confirmed its identity.

Dihydroxymelphalan: DOH, obtained via the complete hydrolysis of L-PAM, was HPLC chromatographically pure; its structure was confirmed by mass spectrometry of the total hydrolysis product and its trimethylsilane derivative.

Internal standard: For hydrolysis studies, *N,N-[bis-*(2-hydroxy-ethyl)] toluidine was used as previously described by Osterheld et al. [14].

Whole blood: From a healthy proband.

Plasma: From a healthy proband.

Serum: Pooled serum was obtained from the Institut für experimentelle Hämatologie und Bluttransfusionswesen, University of Bonn.

Albumin: Stabilizer-free human albumin RHA-988 at a concentration of 5 g/dl isotonic saline, was donated for research purposes by Biotest-Pharma.

* Supported by the Ministry of Science and Research, Land Nordrhein-Westfalen, FRG

Offprint requests to: E. Musch, Medizinische Universitätsklinik Bonn, Sigmund-Freud-Str. 25, D-5300 Bonn 1, FRG

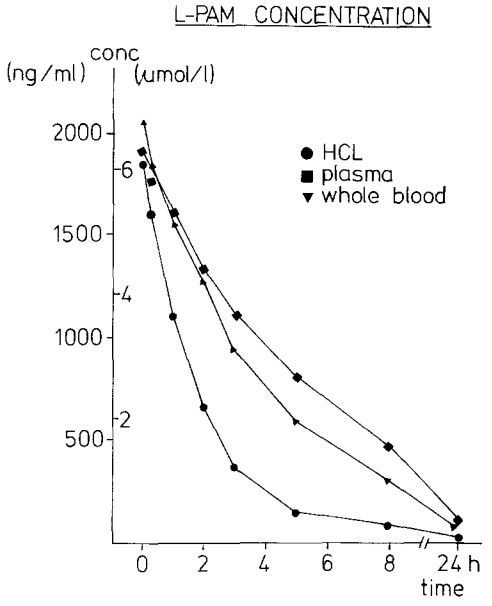

Fig. 1. L-PAM hydrolysis in 10 mmol HCl, plasma and whole blood (37° C; L-PAM concentration, 6.56 µmol/l); concentration-time plot for L-PAM

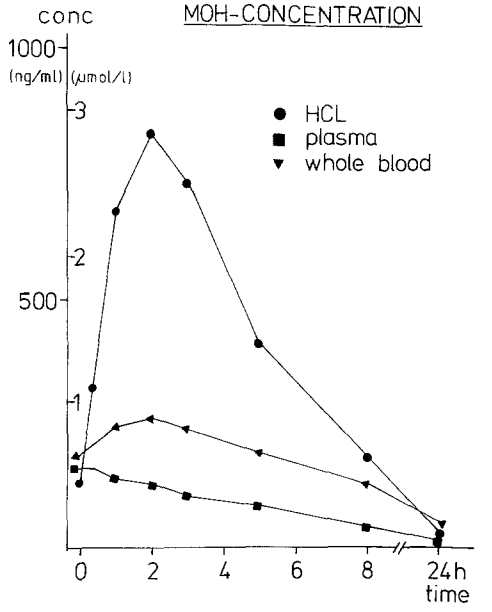

Fig. 2. L-PAM hydrolysis in 10 mmol HCl, plasma and whole blood (37° C; L-PAM concentration, 6.56 µmol/l); concentration-time plot for MOH

Acid α_1-glycoprotein: No. G-9885 (Sigma), concentration 0.75 mg/dl isotonic saline.

Immunoglobulins: Intraglobin F (Biotest-Pharma), >90% IgG antibodies; concentration 2 g/dl isotonic saline.

Prednisone: No. P 277639 (Merck)

Prednisolone: No. P 177144 (Merck)

Patients' sera: 35 sera from 15 patients with multiple myeloma who were treated repeatedly at the University of Bonn with a melphalan-prednisone combination.

HPLC conditions: as previously described by Osterheld et al. [14].

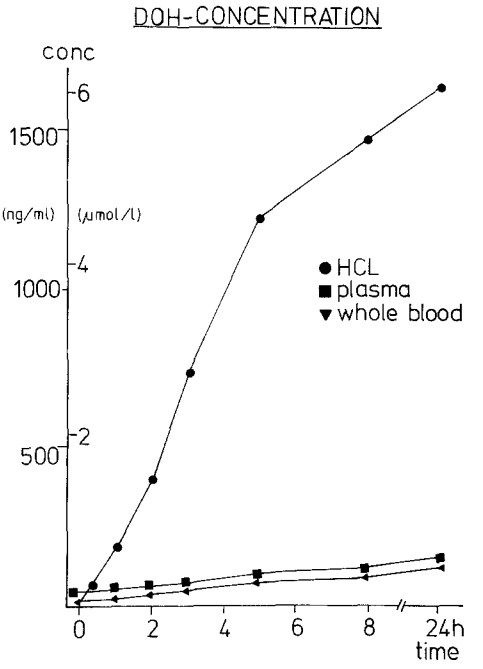

Fig. 3. L-PAM hydrolysis in 10 mmol HCl, plasma and whole blood (37° C; L-PAM concentration, 6.56 µmol/l); concentration-time plot for DOH

Hydrolysis experiments in vitro. Equal starting concentrations of L-PAM (2 µg/ml) and internal standard (1 µg/ml) were produced in 10 mmol HCl, in plasma, and in whole blood. The samples were exposed to identical treatment-shaking in a 37° C water bath – and aliquots were taken for analysis at 0, 0.25, 1, 2, 3, 5, 8, and 24 h. The concentration-time curves of L-PAM and its hydrolysis products are shown in Figs. 1–3.

Binding studies. For all binding studies ultrafiltration was carried out with Centrifree micropartition chambers (Amicon). A prerequisite for use of this method was the absence of absorption of L-PAM and DOH to the YMT membranes. This was shown by centrifugation and subsequent analysis of aqueous solutions of the substances. All protein containing solutions were spiked with various concentrations of L-PAM and DOH. After an incubation period of 10 min at 37° C, each solution was transferred into an ultrafiltration chamber and centrifuged for 10 min at 2000 *g*. In the L-PAM binding studies, the ultrafiltrate was analyzed directly by HPLC. However, the detection of DOH required desalination of the ultrafiltrate prior to HPLC analysis by means of Sep-Pak cartridges (Millipore), as previously described by Osterheld et al. [14]. No internal standard was used to avoid any influence on the protein binding of L-PAM and DOH.

Measurement of the L-PAM covalently bound in serum was carried out with a modified ultrafiltration procedure, as it was impossible to reproduce the L-PAM extraction by methanol described by Chang et al. in 1978 [5]. In principle, this method consists of repetitive ultrafiltration until L-PAM can no longer be detected in the ultrafiltrate. After each centrifugation, the volume lost above the membrane is replaced by isotonic saline. After the last centrifugation, the sum of all L-PAM masses removed from the sample di-

Table 1. Binding of L-PAM to sera of patients with multiple myeloma

Patient	Type of myeloma	Week	Total fraction [g/dl]	Albumin [g/dl]	α_1-Globulin [g/dl]	α_2-Globulin [g/dl]	β-Globulin [g/dl]	γ-Globulin [g/dl]	L-PAM binding [%]
ST	IgG kappa	1	10.1**	4.07	0.22	0.53	0.61	4.70**	90
		8	9.3*	3.02	0.23	0.54	0.63	4.84**	86
		14	9.3*	3.58	0.33	0.62	0.81	3.96**	88
		20	9.3*	4.0	0.31	0.57	0.63	3.79**	87
		51	9.7*	*3.26	0.26	0.55	0.60	5.03**	78
KR	IgG kappa	1	9.2*	5.23*	0.18	*0.33	0.57	2.90*	95
		10	7.6	3.73	0.28	*0.39	0.66	2.54*	86
GR	IgG kappa	1	8.1	3.69	0.28	0.61	0.69	2.80*	86
		7	6.4	4.0	0.28	0.53	0.73	0.86	89
BR	IgG kappa	1	7.6	4.32	0.34	0.80*	0.84	1.28	86
			7.3	4.48	0.28	0.70	0.75	1.08	87
MÜ	IgG kappa	1	11.7**	*3.41	0.27	0.53	0.63	6.84**	83
		7	11.1**	**3.15	0.20	0.42	0.62	6.67**	70
		13	11.0**	*3.43	0.28	0.44	0.69	6.16**	67
SA	IgG kappa	1	8.6	3.80	0.29	0.51	3.54**	*0.46	87
KÖ	IgG kappa	1	9.9*	3.55	0.22	0.70	0.57	4.85**	91
HE	IgG kappa	1	8.2	4.47*	0.21	0.53	0.80	2.19*	90
		20	8.3	3.71	0.29	0.63	0.78	2.89*	90
		27	8.9	3.89	0.24	0.66	0.85	3.26*	90
		31	9.1*	3.80	0.22	0.56	0.84	3.65*	88
DI	IgG kappa	1	7.6	3.60	0.34	0.73	0.65	2.28*	88
		15	7.4	*3.47	0.25	0.53	0.75	2.42*	87
SC	IgG kappa	1	6.7	*3.28	0.19	0.40	2.26*	*0.56	87
SP	IgG kappa	1	7.9	3.85	0.35	0.76	2.37**	*0.55	87
MI	IgA kappa	1	8.3	*3.22	0.28	0.54	3.83**	*0.43	88
		6	8.8	*3.50	0.31	0.49	4.31**	**0.19	83
LI	kappa chains	1	6.3	3.56	0.27	1.04*	0.92	*0.51	91
		6	6.3	3.56	0.31	1.06*	0.92	*0.45	85
		19	6.9	4.05	0.30	0.84*	0.93	*0.78	83
		28	*5.2	*3.28	0.22	0.58	0.62	*0.50	88
SO	lambda chains	1	7.3	*2.98	0.28	1.34*	**0.00	2.69*	82
		4	7.1	*2.72	0.31	1.29*	0.82	1.97*	86
		10	*5.8	*2.98	0.29	1.08*	1.10*	*0.35	82
		19	*6.0	*3.05	0.29	1.02*	1.13*	*0.51	83
HA	lambda chains	1	6.5	4.60	0.20	0.50	0.77	*0.44	91

* Moderately elevated or decreased concentrations
** Grossly elevated or decreased concentrations

vided by the applied L-PAM mass represents the recovery rate, *R*. The percentage of the irreversibly bound fraction of L-PAM, *IRR%*, is given by $IRR\% = (1-R) \cdot 100\%$. The L-PAM concentrations used were 1, 5, 10 and 30 µg/ml serum. The initial incubation period of L-PAM-containing serum was 30 min at room temperature. Centrifugations were carried out at 10° C.

L-PAM binding in the presence of glucocorticoids was measured in sera that had previously been spiked with 20 µg/ml (and 50 µg/ml) prednisone or prednisolone and incubated at room temperature for 15 min. In each case the L-PAM concentration used was 5 µg/ml. A control test without glucocorticoids completed the experiment.

Measurement of L-PAM binding in sera of patients with multiple myeloma was carried out in vitro analogous to the above described first binding study. The L-PAM concentration in each case was 1 µg/ml.

Results

The changes in concentrations of L-PAM, MOH, and DOH in plasma and whole blood during 24-h incubation at 37° C are displayed in Figs. 1–3. The protein binding of L-PAM in serum and in solutions of albumin (pure and with added acid α_1-glycoprotein) and immunoglobulins is shown in Fig. 4. The covalently bound fraction of L-PAM

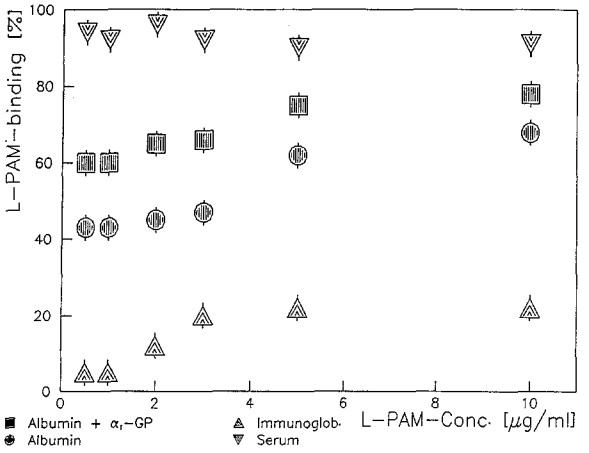

Fig. 4. Binding of L-PAM to serum proteins

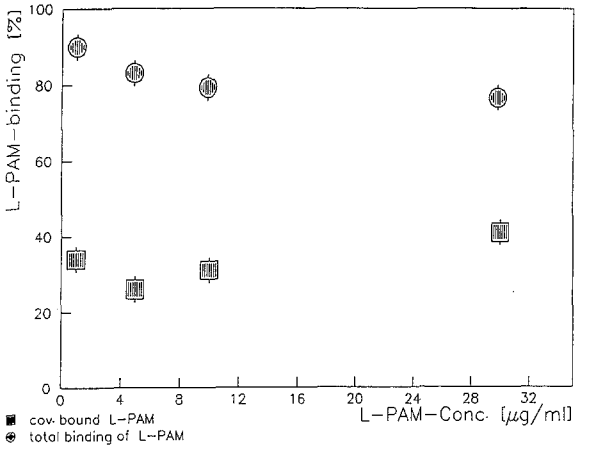

Fig. 5. Total and irreversible binding of L-PAM in serum

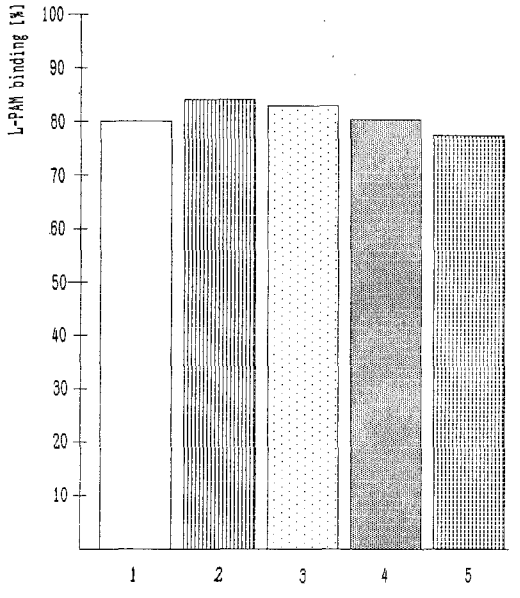

Fig. 6. Binding of L-PAM to serum proteins in the presence of glucocorticoids (L-PAM, 5 µg/ml): 1, without glucocorticoids; 2, +20 µg/ml prednisone; 3, +50 µg/ml prednisone; 4, +20 µg/ml prednisolone; 5, +50 µg/ml prednisolone

in the concentration range of 1–30 µg/ml serum was about 30% (Fig. 5). The binding of DOH in pooled serum as well as in sera of 4 patients with multiple myeloma did not exceed 20%. The histogram (Fig. 6) displays the binding of L-PAM in the presence of various concentrations of prednisone and prednisolone. L-PAM binding in 35 sera of 15 patients with multiple myeloma including the associated serum electrophoresis, is shown in Table 1.

Discussion

Figures 1–3 show that the hydrolysis of L-PAM does not play an important role in plasma and whole blood during 24-h incubation at 37° C. The decrease in L-PAM concentration due to hydrolysis is only 3.5% and 4.6% in plasma and whole blood, respectively. L-PAM probably binds covalently to proteins or to nucleophils such as bicarbonate and phosphate. However, in 10 mmol HCl, DOH finally amounts to 96.5% of the starting L-PAM concentration. The absence of protein and other potential binding sites allows hydrolysis to take place extensively.

Our findings are consistent with the in vitro studies of Chang et al. [5] who could also demonstrate that bovine serum albumin or human plasma proteins retarded the hydrolysis rate of L-PAM in vitro. The authors could even show a reverse correlation between protein concentration and hydrolysis rate of L-PAM. As the decay of L-PAM in the current study was significantly slower than the plasma elimination rate demonstrated by Alberts et al. [1, 2] and Tattersall et al. [16] in vivo, the importance of renal clearance in plasma elimination of L-PAM has to be considered: urinary excretion of L-PAM dosage averaged 10.9 ± 4.9% after oral administration and 13.0 ± 5.4% after intravenous injection according to Alberts et al. [1, 2]. 20%–50% of ^{14}C labelled L-PAM was recovered in urine over 24 h (Tattersall et al. [16]).

Because of the high protein binding of L-PAM it appears advisable to carry out bolus injection of the drug to obtain short-term, high concentrations of unbound agent [10, 13]. The major binding protein is albumin. L-PAM binding to acid α_1-glycoprotein may be of clinical significance, as its concentration is often elevated in chronic inflammatory and neoplastic diseases [8, 15, 17]. However, serum electrophoresis of our patients yielded normal concentrations of this fraction in each case. The low binding of L-PAM to immunoglobulins above the concentration of 2 µg/ml is probably due to interactions with the solution stabilizer.

Discussing the clinical significance of the extent of L-PAM binding to plasma proteins one has to consider tissue binding of the drug as well. From a theoretical point of view, variations of plasma protein binding of a drug will be of therapeutic relevance only if associated with relative low affinity of the drug to the tissue compartment [9, 18]. A low degree of distribution in tissues will result in a high variation of the free portion of the drug, which may result in increased pharmacological or toxic effect.

So far very few animal studies have been performed yielding highest concentrations of labelled L-PAM in the following tissues: tumour (Walker-carcinoma), liver, gallbladder, kidneys, spleen, and intestine [7, 12]. Studies establishing exact tissue invasion constants are being performed by our group using clinical hypothermic isolation perfusion with melphalan and will be reported later [11].

The extent of covalent binding of L-PAM is independent of the concentration of the drug. Our result at 30 μg/ml is consistent with the findings of Chang et al. [5] obtained by methanol extraction. Further study is required to determine which chemical groups are alkylated by L-PAM and whether their physiological functions are impaired by this chemical reaction.

The marked aqueous solubility of DOH compared with that of L-PAM is manifest in a low serum protein binding for DOH. The high renal excretion of this substance, previously described by Alberts et al. [1] and Tattersall et al. [16] is due to either its glomerular filtration or the hydrolysis of L-PAM in the urine.

Glucocorticoids do not influence the binding of L-PAM. Thus, the superiority of the L-PAM-prednisone combination is unlikely to be due to pharmacokinetic interactions.

Table 1 shows that despite considerable fluctuations of serum protein concentrations, L-PAM binding does not vary much inter- and intraindividually during intermittent chemotherapy of multiple myeloma (M: 85.9%, SD: 5.4%). Low concentrations of albumin are not necessarily associated with the diminution of L-PAM binding (patients *DI*, *SC*, and *LI*). Paraproteins do not participate in the L-PAM binding; indeed, the presence of high concentrations of paraproteins seems to slightly diminish the binding of the drug (patients *ST*, *MI*, and *MÜ*).

Acknowledgements. This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Federal Republic of Germany. Technical assistance by Ms. H. Leufgens and Ms W. Supplie is gratefully acknowledged, and we thank Dr. G. von Unruh for his advise concerning chemical reactions and Mr. C. Hemley for reading the manuscript.

References

1. Alberts DS, Chang SY, Chen H-SG, Moon TE, Evans TL, Furner RL, Himmelstein K, Gross JF (1979a) Kinetics of intravenous melphalan. Clin Pharmacol Ther 26: 73–80
2. Alberts DS, Chang SY, Chen H-SG, Evans TL, Moon TE (1979b) Oral melphalan kinetics. Clin Pharmacol Ther 26: 737–745
3. Alexanian R, Haut A, Khan AU, Lane M, McKelvey EM, Migliore PJ, Stuckey WJ, Wilson HE (1969) Treatment for multiple myeloma. J Am Med Assoc 208: 1680–1685
4. Begleiter A, Lam H-YP, Grover J, Froese E, Goldenberg GJ (1979) Evidence for active transport of melphalan by two amino acid carriers in L5178Y lymphoblasts in vitro. Cancer Research 39: 353–359
5. Chang SY, Alberts DS, Farquhar D, Melnick LR, Walson PD, Salmon SE (1978b) Hydrolysis and protein binding of melphalan. J Pharm Sci 67: 682–684
6. Chirigos MA, Mead JAR (1964) Experiments on determination of melphalan by fluorescence. Interaction with protein and various solutions. Anal Biochem 7: 259–268
7. Cohn P (1957) The distribution of radioactivity in tissues of the rat following the administration of a nitrogen mustard derivative. Br J Cancer 1: 258–267
8. Israili ZH, El-Attar H (1983) Binding of some acidic drugs to α_1-acid glycoprotein. Clin Pharmacol Ther 33: 255
9. Jusko WJ, Gretch M (1976) Plasma and tissue protein binding of drugs in pharmacokinetics. Drug Metabol Rev 5 (1): 43–140
10. Kurz H (1969) Einfluß der Proteinbindung auf die Verteilung von Arzneimitteln nach schneller und langsamer Injektion. Naunyn Schmiedeberg's Arch Pharmacol 263: 233
11. Loos U, Musch E, Engel M, Hartlapp JH, Hügl E, Dengler HJ (1987) Pharmacokinetics of melphalan during intermittent therapy of multiple myeloma. Submitted for publication Eur J Clin Pharmacol
12. Milner AN, Klatt O, Young SE, Stehlin JS, Jr (1965) The biochemical mechanism of action of 1-phenylalanine mustard, I. distribution of 1-phenylalanine mustard-h^3 in tumor bearing rats. Cancer Res 25: 259–264
13. Ohnhaus EE (1979) Arzneimittelwechselwirkungen durch Beeinflussung der Plasmaeiweißbindung. Internist 20: 225–228
14. Osterheld HKO, Musch E, von Unruh GE, Loos U, Rauschecker H, Mühlenbruch BJ (1988) Sensitive high-performance liquid chromatographic assay for melphalan and its hydrolysis products in blood and plasma. Cancer Chemother Pharmacol 21: 156–162
15. Piafsky KM, Borga O (1977) Plasma protein binding of basic drugs: II. Importance of α_1-acid glycoprotein for interindividual variations. Clin Pharmacol Ther 22: 545–549
16. Tattersall MHN, Jarman M, Newlands ES, Holyhead L, Milstead RAV, Weinberg A (1978) Pharmaco-kinetics of melphalan following oral or intravenous administration in patients with malignant disease. Eur J Cancer 14: 507–513
17. Urien S, Albengres E, Zini R, Tillement JP (1982) Evidence for binding of certain acidic drugs to α_1-acid glycoprotein. Biochem Pharmacol 31: 3687–3689
18. Wilkinson GR (1983) Plasma and tissue binding considerations in drug disposition. Drug Metabol Rev 14 (3): 427–465

Received August 12, 1987/Accepted May 15, 1988