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QUANTITATIVE ANALYSIS OF MELPHALAN AND ITS MAJOR HYDROLYSATE IN PATIENTS AND ANIMALS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

The detection of 4-bis-(2-hydroxyethyl)amino-1-phenylalanine (L-DOH) in blood samples taken from patients after treatment with melphalan [4-bis-(2-chloroethyl)amino-1-phenylalanine, L-PAM] suggests that the quantification of this major hydrolysate of L-PAM can be of considerable importance in L-PAM chemotherapy. A reversed-phase high-performance liquid chromatographic procedure has been developed for the quantitative analysis of both L-PAM and L-DOH in biological samples, with a detection sensitivity of 0.1 ppm. This method provides a distinct separation of L-PAM (retention time 12 min) and L-DOH (retention time 6.5 min), with no interference from the biological background (retention time 1.4—3 min).

INTRODUCTION

Melphalan [4-bis-(2-chloroethyl)amino-1-phenylalanine, L-PAM] is an antineoplastic alkylating agent used in the clinical treatment of multiple myeloma, ovarian carcinoma, and breast cancer [2—6]. Recent reports [7—12] indicate that the phenomenon of L-PAM degradation has been studied in whole blood, blood plasma and serum, in bile, in distilled water, and in buffer solutions at various pH values. Because of the rapid degradation of L-PAM, noted in most of these reports, the development of a technique for the distinct separation and quantification of its major hydrolysate, 4-bis-(2-hydroxyethyl)amino-1-phenylalanine (L-DOH), assumes a corresponding importance. While the measurement of L-PAM itself in various media has been achieved by gas—liquid chromatographic (GLC) [13] and high-performance liquid chromatographic (HPLC)

*A preliminary report has been presented at the FASEB meeting, Dallas, TX, 1979 [1].

methods [8, 10, 12] in the analysis of biological fluid samples, the quantification of L-DOH has been largely neglected, in part as a consequence of difficulties in analytical procedures. The GLC procedure of Goras et al. [13] requires the time-consuming and laborious derivatization of L-PAM and its hydrolytic products, and in the HPLC analyses [8, 10, 12] the biological background (t_R = 1.4—3 min) interferes with L-DOH determination (t_R = 2 min). The role of L-DOH in the mechanism by which L-PAM exerts its antineoplastic effect is not known. In order to verify such a role a quantitative HPLC analysis of both L-PAM and L-DOH in biological samples has been developed and is described in this report.

MATERIALS AND METHODS

Reagents

Melphalan (L-PAM), pharmaceutical grade, was obtained from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.) through the courtesy of Dr. W.D. Brinkman. Dihydroxylated melphalan derivative (L-DOH) was synthesized according to the procedure of Furner et al. [12]. Acetonitrile, HPLC grade, was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.) and was used as received. High-purity glacial acetic acid (Fisher Scientific) was diluted to 0.0175 *M* with double-distilled water. Other chemicals were of the highest purity available and were obtained from various commercial sources.

Instruments

A Perkin-Elmer Model 601 high-pressure liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.), equipped with two pumps and a gradient programmer, delivered the eluting solvents at a constant rate to a high-capacity reversed-phase column (10 μm HCODS/SIL X-C_{18} (Perkin-Elmer), 25 × 0.26 cm I.D.) with a variable-wavelength ultraviolet detector (Perkin-Elmer Model LC 55) attached to its outlet. The detector was interfaced with a laboratory data processing computer (Hewlett-Packard 3352-C) for instantaneous on-line quantification of the chromatographic data. Output signals were also recorded by a strip chart recorder (Perkin-Elmer Model 023).

Analytical procedures

Standard L-PAM solution. The solution of L-PAM (5 μg/ml) was prepared in 5% trichloroacetic acid to simulate the solution in which the drug was extracted from biological samples. To minimize L-PAM hydrolysis, the solution was prepared immediately before use. A 10-μl aliquot of the solution was injected on to the column maintained at 50°C and solvent programming was begun at once with a flow-rate of 1.5 ml/min and chart speed of 0.5 cm/min. Within 14 min the composition of the solvent, initially 12% acetonitrile and 88% 0.0175 *M* acetic acid, was converted by a concave gradient programmer to 80% acetonitrile and 20% 0.0175 *M* acetic acid. Detector sensitivity was varied from 0.002 to 0.2 absorbance units full scale. L-PAM and its hydrolysate L-DOH were detected at 263 nm. This procedure allowed reproducible measurement of L-PAM levels as low as 0.1 ppm.

Standard curve of L-PAM and L-DOH in human blood. Aliquots of L-PAM

or L-DOH solution (1 mg/ml) were added to human blood, obtained from the blood blank, to produce concentrations ranging from 0.1 to 1.0 μg/ml of blood. Each blood sample was centrifuged and the plasma collected. A 1-ml aliquot of plasma was treated with 0.5 ml of 5% trichloroacetic acid solution, vortexed and centrifuged. The supernatant was filtered through a Pillicon molecular filter (0.45 μm, Millipore). A 10-μl aliquot of the clear filtrate from each concentration was injected on to the column and standard curves obtained by plotting absorbance against concentration (μg/ml) as shown in Fig. 2 (see under Results).

Clinical application of the analytical technique. Blood samples from patients receiving L-PAM chemotherapy were obtained under the supervision of John Costanzi, Director of the Oncology Division, University of Texas Medical Branch at Galveston. In L-PAM chemotherapy [Southwest Oncology Group (SWOG) protocols], patients receive the drug orally for five days (5 mg/m^2 per day), then rest for three weeks before medication is administered again. Day 0 is the last day of the resting period before a new cycle of medication; day 6 is 24 h after the last dose of the drug. The blood of patients at days 0 and 6 of a treatment cycle was analyzed.

All blood samples were prepared for chromatographic analysis by the same procedures used to prepare samples for determination of the L-PAM standard curve as previously described.

Time course of L-PAM and L-DOH in rats. Male Sprague-Dawley rats (Charles River, Wilmington, MA, U.S.A.), ranging in weight from 190 to 200 g, were used. The animals were fed standard lab chow and tap water ad libitum. A fresh stock solution of L-PAM (4 mg/ml) was prepared in 0.12 *N* HCl. The dose of L-PAM (20 mg/kg) was administered orally through an esophageal tube. Groups of three rats were killed by decapitation to collect blood samples at intervals of 0, 0.5, 1, 1.5, 2, 4, 8, 12, 24, 48, and 72 h. The samples were prepared for analysis by the procedures previously described.

RESULTS

Fig. 1 illustrates the HPLC analysis of L-PAM (5 μg/ml) in 5% trichloroacetic acid (A) and of L-PAM plus L-DOH "spiked" in human blood samples (B). The separation of L-PAM from L-DOH is distinct, allowing individual quantification. The peak corresponding to L-DOH (t_R = 6.5 min) is separated both from the L-PAM peak (t_R = 12 min) and from background interference caused by the biological molecules (t_R = 1.4—3 min).

The standard curves for L-PAM and L-DOH in human blood (Fig. 2) demonstrate the sensitivity and reproducibility of the new method. Each point on the graph represents the mean of at least three separate analyses, and linearity is evident for both compounds from 0.1 to 1.0 μg/ml (10 μl injection volume); linearity actually persisted to levels as high as 20 μg/ml (not shown in the graphs). None of the amino acids such as phenylalanine, tyrosine, or dihydroxyphenylalanine (DOPA) was found to interfere with the L-PAM or L-DOH assay. This technique allows quantification with a high degree of accuracy: in ten separate analyses of a standard concentration of L-PAM or L-DOH (1 μg/ml), for instance, the range of difference in area under the L-PAM or L-DOH peak is only ± 10% of the mean.

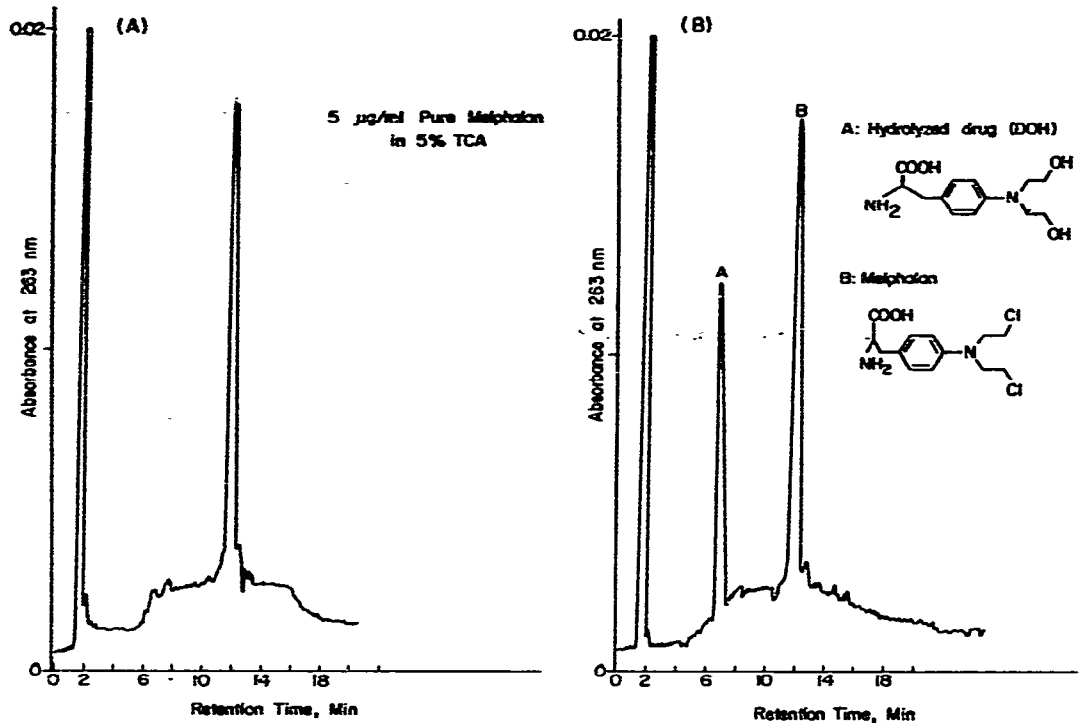

Fig. 1. HPLC analysis, using UV detection, of L-PAM and L-DOH "spiked" in human blood serum. Blood samples were extracted with 5% trichloroacetic acid (TCA) and 10 μl of the extract were injected on to the chromatograph (see Materials and methods). (A) L-PAM; (B) L-PAM plus L-DOH.

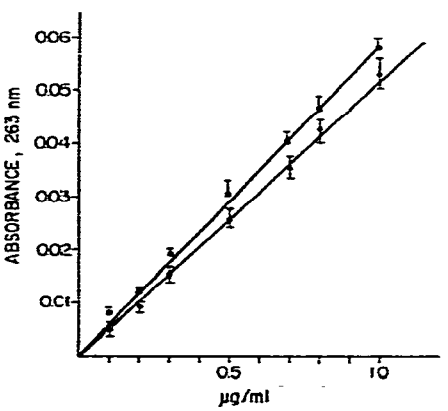

Fig. 2. Standard curves for L-PAM (•) and L-DOH (▪) in human blood samples. Points are the mean of three determinations ± S.D. (100-μl aliquots were injected on to the HPLC column at 0.002 a.u.f.s.).

Fig. 3 represents three chromatograms of human blood by HPLC analysis. The first chromatogram (Fig. 3A) is of normal blood supplied by a blood bank. The second and third (Fig. 3B and C) are typical chromatograms of blood samples from patients, at days 0 and 6, respectively, undergoing treatment un-

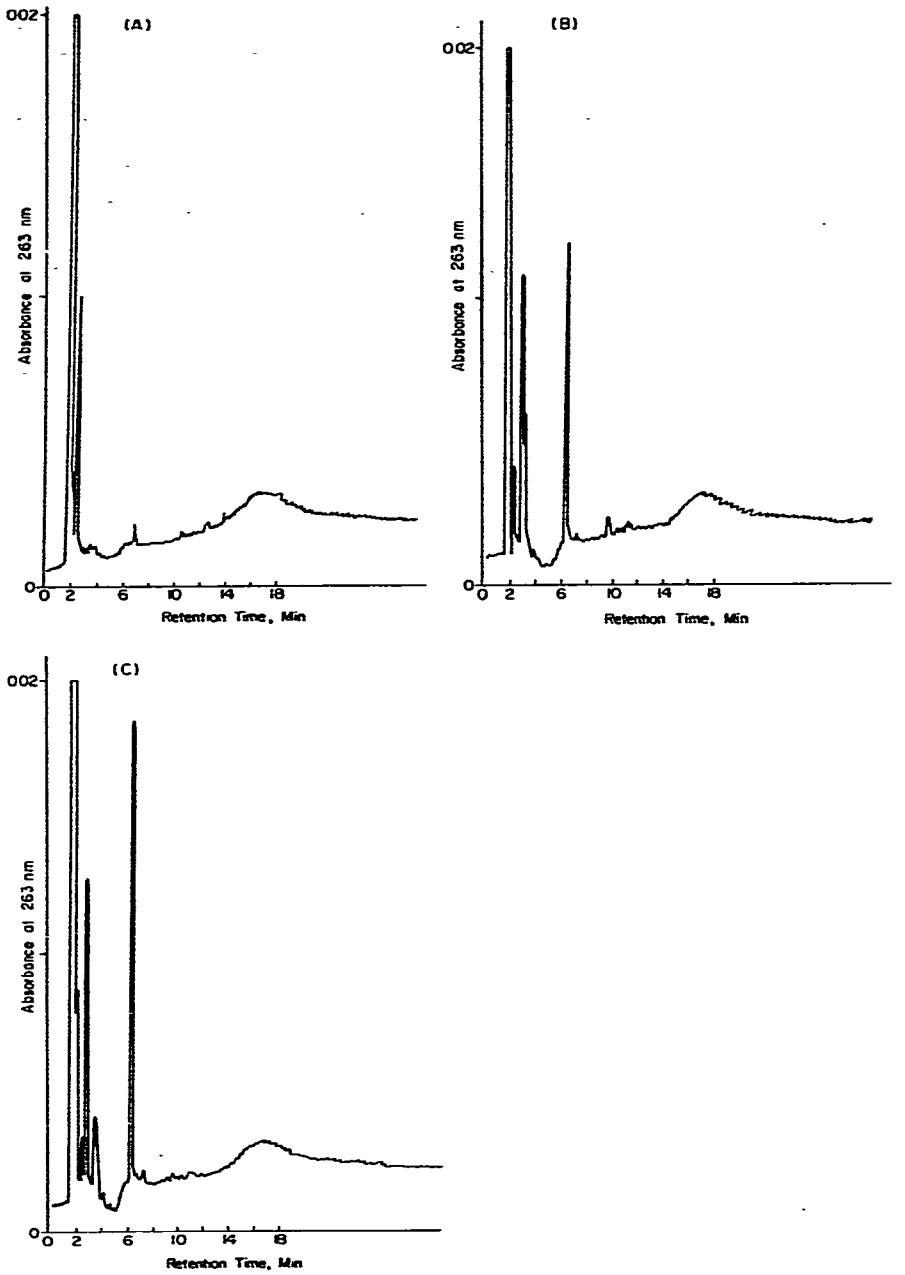

Fig. 3. Chromatograms of human blood samples. Typical chromatograms of: (A) normal, human blood; (B) from a patient at day 0 of L-PAM treatment; and (C) from a patient at day 6 of treatment.

der the SWOG L-PAM regimen. Identical preparation techniques were used for blood samples in all analyses so that the chromatograms could be compared exactly. In the day 0 chromatogram (Fig. 3C) no L-PAM peak appears, but the presence of L-DOH (t_R = 6.5 min) is clearly detected. In the day 6 chromato-

gram (Fig. 3C) the L-PAM peak is again absent, but the peak corresponding to L-DOH is considerably larger than in the day 0 chromatogram. These findings are consistent with the results of previous investigations which demonstrated that L-PAM disappears within a few hours following oral administration [14]. The present investigation, however, indicates that L-DOH remains in the blood for considerably longer periods of time than L-PAM; in fact, the L-DOH peak is detectable in blood samples collected more than three weeks after the last L-PAM ingestion.

Further identification of the L-DOH peak was achieved through chemical ionization (CI) mass spectrometry. HPLC fractions corresponding to the L-DOH peak were collected, evaporated to dryness, dissolved in acetonitrile, and analyzed by direct-probe ammonia CI process, using a Finnigan 3200 quadruple gas chromatograph—mass spectrometer. The M + H ion peak (taken as the base peak) and the peaks of other ions characteristic in CI spectra of L-DOH were identical to those of an L-DOH authentic sample.

The detection of L-DOH in patients' blood long after L-PAM administration triggered the study of comparative levels of L-PAM and L-DOH in rats following a single oral dose. Fig. 4 illustrates the results of analyses of animal blood samples over a period of 72 h following L-PAM administration. The concentration of L-PAM reaches its peak in about 2 h, decreases sharply within 4 h, then drops below 1 μg/ml. The concentration of L-DOH, however, ranges from 2 to 5 μg/ml and does not vary significantly over a 72-h period.

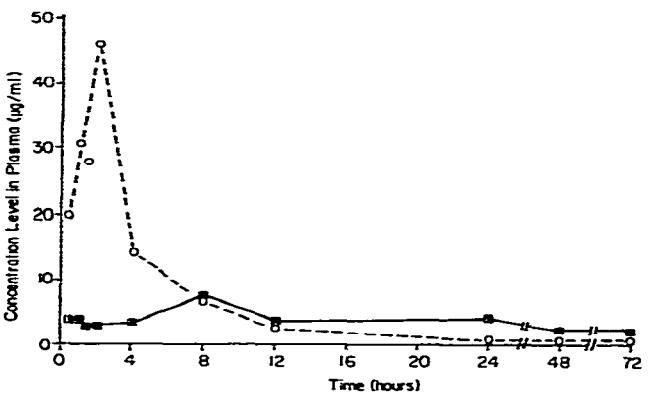

Fig. 4. Levels of L-PAM (○) and L-DOH (■) in blood samples over a period of 72 h. Each point represents the mean of three animals.

DISCUSSION

The determination of drug metabolites is essential for the understanding of the mechanism of action of the parent molecule. Previous studies on L-PAM, however, have neglected the determination of melphalan metabolites and their roles in the mechanism of action of L-PAM. The quantification of L-PAM metabolites has been impeded by difficulties in the analytical procedure. Chang et al. [8] reported that quantitative analysis of L-PAM hydrolytic products in biological samples is impossible by their HPLC method because of interference

from the biological matrix background. The HPLC methods of Furner et al. [12] and Flora et al. [10] present similar problems of interference.

There are several significant advantages of the HPLC method reported here; it accomplishes, by comparatively simple means, the distinct separation and quantification of L-PAM and L-DOH in biological samples with no background interference, and with a high degree of sensitivity accuracy, and reproducibility. The sensitivity of detection is consistently as low as 0.1 ppm, and the linearity of the standard curve in human blood serum persists at concentrations as high as 20 μg/ml.

This method allows the detection and quantification of L-DOH in patients receiving L-PAM chemotherapy. The observation that L-PAM was not detected in blood of patients 24 h after the last dose is in agreement with previous pharmacokinetic studies [14]. These studies suggested that, in humans following an oral administration, L-PAM quickly disappeared with a half-life of 67 min. Although the fate of L-DOH was not reported in their studies, Tattersall et al. [14] indicated that there is a prolonged terminal phase in the disappearance of label from the plasma of patients receiving an oral dose of L-[^{14}C]PAM. The delayed detection of L-DOH observed in our studies agrees with this observation and suggests that L-DOH is the major component of the label. Albert et al. [15] reported that no L-DOH was detected 24 h following an oral administration of L-[^{14}C]PAM. In their studies, however, they utilized HPLC methods described by Chang et al. [8] and Furner et al. [12], where the quantitation of L-DOH seems to be impossible due to the interference from biological materials.

Our animal studies also indicated a delayed elimination of L-DOH where 2—3 μg/ml was detected 72 h following the administration of a single oral dose. Vistica et al. [16] reported that L-PAM is transported across the cellular membrane by an amino-acid carrier transport system. Therefore delayed elimination of L-DOH in humans and rats may be due to its reabsorption in the kidney tubules by the same mechanism responsible for the active transport of amino acids. Although the role of L-DOH in L-PAM chemotherapy is not yet known, the rapid degradation of L-PAM compared with the persistence of L-DOH suggests that its role, which needs to be clarified, may be an important one.

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REFERENCES

1 A.E. Ahmed, P.J. Bour and K. Patel, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 31
2 R. Alexanian, D.E. Bergsagel, P.J. Migliore, W.K. Vaughn and C.D. Howe, Blood, 31 (1968) 1—10.
3 B. Clarkson, in A.C. Sartorelli and D.G. Johns (Editors), Antineoplastic and Immunosuppressive Agents, Vol. I, Springer-Verlag, New York, 1974, p. 172.
4 B. Fisher, P. Carbone, S.G. Economon, R. Frelick, A. Glass, H. Lerner, C. Redmond, M. Zelen, P. Band, D.L. Katrych, N. Wolmark and E.R. Fisher, N. Engl. J. Med., 292 (1975) 117—122.

5 R.B. Livingston and S.K. Carter, Single Agents in Cancer Chemotherapy, Plenum Press, New York, 1970, pp. 99—111.
6 T.H. Wasserman, R.L. Comis, M. Goldsmith, H. Handelsman, J.S. Penta, M. Slavik, W.R. Soper and S.K. Carter, Cancer Chemother. Rep., 6 (Part 3) (1975) 399—419.
7 S.Y. Chang, D.S. Alberts, D. Farquhar, L.R. Melnick, P.D. Walson and S.E. Salmon, J. Pharm. Sci., 67 (1978) 682—684.
8 S.Y. Chang, D.S. Alberts, L.R. Melnick, P.D. Walson and S.E. Salmon, J. Pharm. Sci., 67 (1978) 679—682.
9 S.Y. Chang, T.L. Evans, D.S. Alberts and I.G. Sipes, Life Sci., 23 (1978) 1697—1701.
10 K.P. Flora, S.L. Smith and J.C. Cradock, J. Chromatogr., 177 (1979) 91—97.
11 R.L. Furner, R.K. Brown and G. Duncan, Cancer Treat. Rep., 61 (1977) 1637—1646.
12 R.L. Furner, L.B. Mellet, R.K. Brown and G. Duncan, Drug Metab. Dispos., 4 (1976) 577—583.
13 J.T. Goras, J.B. Knight, R.H. Iwamoto and P. Lim, J. Pharm. Sci., 59 (1970) 561—563.
14 M.H.N. Tattersall, M. Jarman, E.S. Newlands, L. Holyhead, R.A.V. Milstead and A. Weinberg, Eur. J. Cancer, 14 (1978) 507—513.
15 D.S. Alberts, S.Y. Chang, H.S.G. Chen, T.L. Evan and T.E. Moon, Clin. Pharmacol. Ther., 26 (1979) 737—745.
16 D.T. Vistica, J.N. Toal and M. Robinobitz, Biochem. Pharmacol., 27 (1978) 2865—2870.