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APPLICATION OF A SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF MELPHALAN IN THE PRESENCE OF ITS HYDROLYSIS PRODUCTS

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SUMMARY

A procedure for the separation and quantitation of melphalan (L-PAM) and its hydrolysis products by high-performance liquid chromatography is described. The hydrolysis of L-PAM at 25 ± 0.1° and 41 ± 0.1° was studied between pH 3.0 and 9.0. The pattern of hydrolysis suggested that L-PAM decomposes via two consecutive pseudo first-order reactions. Pseudo first-order rate constants (k_1) were determined for the disappearance of L-PAM at various pH values in buffered solutions and in a formulated product. At both temperatures L-PAM solutions were found to be most stable at low pH. Chloride ion was found to reduce the rate of hydrolysis.

INTRODUCTION

Melphalan (L-PAM, NSC-8806, phenylalanine mustard, 4-[bis(2-chloroethyl)-amino]-L-phenylalanine) is an alkylating agent used clinically in the treatment of various neoplastic diseases[1,2]. Recent papers have described the treatment of melanoma and sarcoma of the extremities by an isolated perfusion technique sometimes in combination with heat at the perfusion site[3,4]. Inquiries to the Pharmaceutical Resources Branch of the National Cancer Institute have indicated a need for stability data on the formulated product (supplied as a kit by Burroughs Wellcome, London, Great Britain) at 41°[5]. Only a minimal amount of data is available on the hydrolysis of L-PAM. The disappearance of L-PAM has been measured in serum, plasma, whole

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blood[6–9], unbuffered distilled water[6] and bile[10]. However, the influence of pH or added electrolyte was not evaluated in a systematic manner.

The objective of this work is to develop a simple, precise reversed-phase high-performance liquid chromatographic (HPLC) procedure for the determination of L-PAM in the presence of its hydrolysis products 4-[2(-chloroethyl)(2-hydroxyethyl)-amino]-L-phenylalanine (L-MOH) and 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine (L-DOH) in aqueous solutions and to apply this method to study the kinetics of hydrolysis of the parent drug.

L-PAM has been determined in various media by gas–liquid chromatography (GLC)[11] and HPLC methods[7,8]. The GLC procedure[11] cited requires the derivatization of L-PAM and its hydrolysis products in a nonaqueous environment which if applied to aqueous solutions would require additional extractive workup of the sample. One of the HPLC procedures employs a more complex gradient technique[7]. A simple isocratic HPLC procedure for L-PAM has been reported[8] but it was not clear if the hydrolysis products are adequately identified and resolved.

EXPERIMENTAL

Apparatus and conditions

A modular high-performance liquid chromatograph (Model 3500B, Spectra-Physics, Santa Clara, Calif., U.S.A.), including a reciprocating piston pump with flow feedback control, delivered mobile phase at a constant rate (1 ml/min) to a stainless-steel column (250 × 3 mm) packed with 10-μm microporous silica bonded with octadecyl silane (Spherisorb 10 μm ODS, Spectra-Physics, Santa Clara, Calif., U.S.A.). A fixed wavelength (254 nm) ultraviolet detector (Model 8200, Spectra-Physics) with a sensitivity setting of 0.04 absorbance units full scale detected the eluted compounds. The detector output signal was recorded with a strip-chart recorder equipped with variable chart-speed controls (Model A5211-1, Omniscribe, Houston Instruments, Austin, Texas, U.S.A.). Samples were introduced to the column with a manual injection valve equipped with a 10-μl sample loop (Model CV-6-UHP-a-N60, Valco, Houston, Texas, U.S.A.).

The mobile phase consisted of 1 volume of methanol and 1 volume of 0.01 *M* NaH_2PO_4 adjusted to pH 3.0 with 0.66 *M* phosphoric acid. The column pressure was about 1400 p.s.i. at a flow-rate of 1 ml/min. All separations were affected isocratically at ambient temperature.

Quantitation was performed using an internal standard method. Standard curves constructed from the ratio of peak heights of L-PAM and L-DOH to the internal standard (I.S.) benzophenone *versus* concentration were linear ($r > 0.99$). The working range for L-PAM was 1–40 μg/ml (3.3–131 μM).

Reagents

Melphalan was supplied by the Division of Cancer Treatment, National Cancer Institute. Benzophenone (Aldrich, Milwaukee, Wisc., U.S.A.) was used as received. Methanol, HPLC grade (Fisher Scientific, Fair Lawn, N.J., U.S.A.) and distilled water were filtered through 0.5-μm and 0.8-μm solvent resistant filters, respectively (Millipore, Bedford, Mass., U.S.A.). L-DOH was prepared according to a procedure previously published[7]. The product was recrystallized from ethanol (m.p.

197–200°; lit. 196–200°[12]. All other chemicals were reagent grade and were used as received.

A stock solution of L-PAM (2 mg/ml) was prepared in methanol. Dilution of the stock solution with the appropriate buffer solution was made to a final concentration of 40 μg/ml. Sodium phosphate buffers (0.01 *M*) were used at pH 5.0 and pH 7.0. Equivalent concentrations of citric acid adjusted to pH 3.0 with 1 *M* sodium hydroxide and tris(hydroxymethyl)aminomethane base adjusted to pH 9.0 with 0.33 *M* phosphoric acid were used. The samples were stored at 25 $\pm$ 0.1° or 41 $\pm$ 0.1°. Periodically a 750-μl aliquot was removed and diluted with 250 μl of mobile phase containing benzophenone (60 μg/ml). These samples were analyzed chromatographically.

For the formulated product stability study the solution was prepared according to labeled directions supplied with the three component formulation kit. L-PAM, 100 mg, was dissolved in 1 ml of sterile ethyl alcohol (92%) containing 2% (w/v) hydrogen chloride which was supplied in the formulation kit. This solution of the drug was diluted to 10 ml with a solution consisting of 1.2% (w/v) K_2HPO_4, and 60% (w/v) propylene glycol in water for injection. Periodically a 400-μl aliquot was removed and diluted to 100 ml with distilled water. Immediately, a 750-μl aliquot of this diluted solution was withdrawn, combined with 250 μl of the internal standard solution and assayed chromatographically.

RESULTS AND DISCUSSION

Chromatographic behavior

L-PAM and its hydrolysis products were eluted from the column in order of their decreasing polarities; L-DOH, L-MOH and L-PAM. A representative chromatogram of a partially hydrolyzed L-PAM sample is presented in Fig. 1. The retention volumes (V_R), capacity factors (k') and separation factors (α) for L-PAM

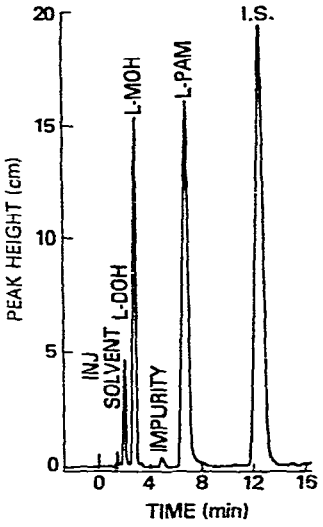

Fig. 1. Chromatogram of L-DOH, L-MOH, L-PAM and internal standard (I.S.) after 3 h at pH 9.0 and 25 $\pm$ 0.1°.

and its hydrolysis products are listed in Table I. Fractions containing L-PAM, L-MOH or L-DOH were collected as they eluted from the detector, and were reduced to dryness. The residues were silylated and then identified by GLC–mass spectrometry.

TABLE I

RETENTION VOLUMES (V_R), CAPACITY FACTORS (k') AND SEPARATION FACTORS (α)

Component	V_R *(ml)*	k'	α
L-DOH	2.04	0.50	1.91
L-MOH	2.66	0.96	4.00
L-PAM	6.56	3.82	2.09
I.S.	12.24	8.00	

A solution of L-DOH prepared by a previously published method[7] had retention characteristics identical to those of the L-DOH peak observed during the kinetic experiments.

Methanol, if present as more than 50% of the mobile phase or flow-rates greater than 1 ml/min, produced incomplete resolution of the L-DOH and L-MOH peaks. A 0.01 *M* sodium phosphate buffer adjusted to pH 3.0 was chosen as the aqueous component of the mobile phase in lieu of a previously published 1% acetic acid solution[8]. In our hands, L-MOH and L-DOH were not adequately resolved by the method of Chang *et al.*[8]. Also, another mustard formed acetate esters in a system containing less than 1% acetate[13].

Kinetic studies

Studies monitoring the disappearance of L-PAM from solution in aqueous buffers and a pharmaceutical dosage form were conducted at 25 ± 0.1° and 41 ±0.1°. Plots of log L-PAM concentration *versus* time were linear ($r > 0.99$) at all pH values and both temperatures, thus indicating that the disappearance of L-PAM was pseudo first-order. The rate constants and half-lives observed for the disappearance of L-PAM at 25 ± 0.1° and 41 ± 0.1° in a typical run in various media are presented in Table II. Duplicate or triplicate experiments were conducted to verify the results reported for each solution at each temperature. Rate constants and half-lives from replicate runs differed by no more than 4% in any case. The data indicate that L-PAM in the pH range studied, pH 3.0–9.0, is most stable at low pH (3.0) at either temperature. In addition, L-PAM is significantly more stable in the presence of increased chloride ion concentration.

The formulated product is apparently more stable than any of the other solutions studied (Table III). This is probably due in part to the high chloride ion and drug concentrations plus the presence of 60% propylene glycol. However, assessment of the kinetic behavior of the formulated product is especially difficult at 25° due to the formation of a precipitate which is first evident about 1 h after the L-PAM is dissolved. The disappearance of L-PAM however, is pseudo first-order up to 72 h. Points beyond 72 h deviated significantly from linearity. Examination of the chromatograms indicates qualitatively that much of the precipitated material is probably

TABLE II

DISAPPEARANCE OF L-PAM IN VARIOUS MEDIA

Media	*pH*	*25 ± 0.1°*		*41 ± 0.1°*	
		$T_{1/2}$ *(h)*	k_1 (h^{-1})	$T_{1/2}$ *(h)*	k_1 (h^{-1})
Buffer	3.0	5.26	0.132	0.771	0.898
Buffer	5.0	4.93	0.140	—	—
Buffer	7.0	4.75	0.146	0.697	0.994
Buffer	9.0	3.93	0.176	0.630	1.10
Buffer plus NaCl (0.019 *M*)	7.0	6.74	0.103	—	—
Buffer plus NaCl (0.038 *M*)	7.0	8.33	0.0831	—	—
Buffer plus NaCl (0.075 *M*)	7.0	11.36	0.0610	—	—
Buffer plus NaCl (0.15 *M*)	7.0	15.97	0.0434	—	—

TABLE III

DISAPPEARANCE OF L-PAM IN THE FORMULATED PRODUCT

Temperature	$T_{0.9}$ *(h)**	$T_{1/2}$ *(h)*	k_1 (h^{-1})
25 ± 0.1°	18.92	124.50	0.0057
41 ± 0.1°	3.29	21.66	0.0320

* Time for disappearance of 10% of L-PAM.

L-MOH and L-DOH since those peaks are present in less than the expected amounts. It should be noted that the package insert provided with the formulation kit indicates that the solution will remain clear for "some time" but suggests that it be injected within 15–30 min. At 41° no precipitate is observed and the semilog plots of concentration versus time are linear throughout.

At 41 ± 0.1° the $T_{0.9}$ value of L-PAM disappearance in solutions prepared from the formulated product was about 3 h. Therefore, preheating time should be kept to a minimum in cases requiring infusion of a heated perfusate.

The inhibition of the hydrolysis of L-PAM by chloride ion as indicated in Table II may have some practical application. The enhanced stability of L-PAM might allow for therapy via a dilute infusion of drug in normal saline solution.

This HPLC method was also used to follow the appearance of L-MOH and L-DOH as well as the disappearance of L-PAM in solution. Fig. 2 shows a typical plot for the disappearance of L-PAM and the appearance of L-MOH and L-DOH at pH 7.0 and 41 ± 0.1°. Concentrations of L-MOH were obtained by difference after L-PAM and L-DOH were quantitated by comparison to standard solutions. Concentrations of L-PAM initially fall quite rapidly. L-MOH appears very early, reaches a maximum at about 75 min and decreases thereafter. L-DOH appears slowly at first and then more rapidly as the concentration of L-MOH nears a maximum. This type of plot as seen in Fig. 2 is consistent with a mechanism comprised of two consecutive pseudo first-order reactions, L-PAM $\xrightarrow{k_1}$ L-MOH $\xrightarrow{k_2}$ L-DOH, where k_1 and k_2 are pseudo first-

order rate constants. The rate constants k_2 were estimated by a method previously described[14,15] using dimensionless parameters and variables and were found to range from about 0.9 to 1.3 times k_1 for the buffered solutions at all of the pH values studied. In terms of antitumor activity k_2 is of little importance. Experimentally it has been determined[16] for a variety of nitrogen mustards including L-PAM that only the intact two-armed mustard has antitumor activity. One-armed analogs of several mustards were prepared and found to be devoid of activity.

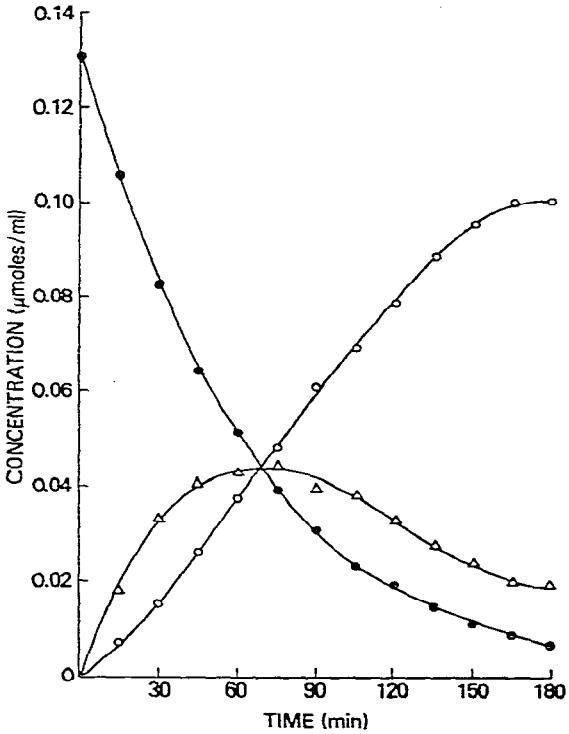

Fig. 2. Concentration *versus* time curves for L-DOH (○), L-MOH (△) and L-PAM (●) at pH 7.0 and 41 ± 0.1°.

This HPLC method has been shown to be convenient for the separation and quantitation of L-PAM and its hydrolysis products L-MOH and L-DOH in aqueous solution. No extraction procedures are necessary and more complex gradient HPLC systems are avoided. The method had adequate sensitivity for this study (1 μg/ml L-PAM) and was precise. The coefficient of variation for six equal and individually weighed samples over a period of eight weeks was 2.09% at 40 μg/ml of L-PAM.

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