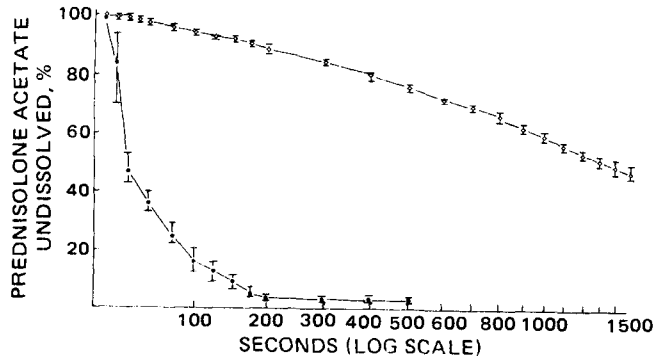

Figure 3—*Aqueous dissolution profiles of Fractions 1 (◇) and 4 (●) of prednisolone acetate suspensions from Run B. The points are the average of three experiments, and the bars are extremes of the three experiments.*

Fraction 5 consisted of washings from the elutriator chamber. Run C, which was conducted on a different lot of prednisolone acetate than Runs A and B, had insufficient washings to be collected. Hydrocortisone acetate, which was micronized and had few larger particles, also contained no particles within this fraction. All data for these two runs were extremely close, with a 7- or 8-μm increase in mean particle size from the previous fraction.

To demonstrate the nature of the shift of particle distribution from fraction to fraction, the particle distribution for Run B was plotted (Fig. 1). Five fractions were obtained, each with its own distinctive mean particle size. The only two fractions with heavy overlapping of particles were Fractions 1 and 2, which did show a shift of one channel. The other fractions all showed a shift of two or more channels.

Each fraction was distributed according to an apparent log-normal distribution. This fact is significant because of the widespread use of log-normal distributions in dissolution modeling (1–7, 11, 21, 22). Veng Pedersen (22), for example, assumed a log-normal distribution in successfully characterizing the dissolution of micronized glyburide using the cube root law.

To demonstrate the potential biological importance of the fraction differences, the aqueous dissolution rates of Fractions 1 and 4 of Run B were determined (Fig. 2). These two fractions provided two distinct particle-size distributions with essentially no overlapping of particle sizes.

The particle-size differences of these two fractions are dramatically demonstrated in the dissolution results (Fig. 3). These data indicate that 50% of Fraction 1 dissolved in less than 30 sec while 1400 sec elapsed before 50% of the large Fraction 4 dissolved.

The mixing of these fractions and the dissolution testing of other fractions would seem to be valuable tools in testing theories designed to predict dissolution kinetics from particle size. The design of sustained- or controlled-release suspensions from a mixing of fractions also may be another attractive possibility.

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Liquid Chromatography in Pharmaceutical Analysis IX: Determination of Muscle Relaxant–Analgesic Mixtures Using Normal Phase Chromatography

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Received June 1, 1977, from the *Department of Medicinal Chemistry, School of Pharmacy, University of Georgia, Athens, GA 30602.* Accepted for publication September 6, 1977.

Abstract □ High-pressure liquid chromatography was used to optimize the resolution of eight widely prescribed therapeutic agents commonly found in muscle relaxant–analgesic mixtures. The compounds were chromatographed on normal phase porous silica or cyanopropylsilane columns, using various solvent systems paired on the basis of Snyder's solvent selectivity scheme to give a polarity index for each system of 3.3. A carisoprodol, phenacetin, and caffeine mixture was selected to demonstrate the utility of the separation and quantification method. The mixture was chromatographed on a porous silica column, using tetrahydrofuran–toluene (50:50) at a flow rate of 2.0 ml/min. Each determination can be achieved in approximately 8 min with an accuracy of 3–5%.

Keyphrases □ Muscle relaxants, various—high-pressure liquid chromatographic analyses in mixtures with analgesics □ Analgesics, various—high-pressure liquid chromatographic analyses in mixtures with muscle relaxants □ High-pressure liquid chromatography—analyses, various muscle relaxants and analgesics in pharmaceutical mixtures

Muscle relaxant–analgesic mixtures are widely prescribed drugs. The continued interest in these laboratories in the use of high-pressure liquid chromatography (HPLC) for the separation and quantitation of multicomponent

Table I—Solvent Groups Adjusted to a Common Polarity Index [a]

	Hexane, Group 0	Butyl Ether, Group 1	Carbon Tetrachloride, Group 7
Tetrahydrofuran, Group 3	80:20[b] (Solvent A)	65:35 (Solvent B)	50:50[c] (Solvent C)
Methylene chloride, Group 5	100:0[d] (Solvent D)	100:0[d] (Solvent D)	95:5 (Solvent E)
Chloroform, Group 9	75:25 (Solvent F)	60:40 (Solvent G)	60:40 (Solvent H)

[a] See Ref. 15. [b] Solvent ratio = row/column. [c] Tetrahydrofuran–carbon tetrachloride forms an unstable mixture, so toluene was substituted for carbon tetrachloride in this mixture. [d] One hundred percent methylene chloride has a PI value of approximately 3.3.

dosage forms[1] led to an investigation of muscle relaxant–analgesic mixtures. Among the drugs in these mixtures are meprobamate, methocarbamol, carisoprodol, chlorzoxazone, acetaminophen, aspirin, phenacetin, and caffeine.

Existing methods for meprobamate in dosage forms include formol titrimetry (1) and colorimetry (2). Methocarbamol can be analyzed using a spectrophotometric method (3); carisoprodol is determined in mixtures containing phenacetin and caffeine by quantitation in the near IR range (4). GLC and TLC methods (5, 6) were reported for chlorzoxazone. Acetaminophen has been analyzed by UV and visible spectrophotometry (7, 8), and aspirin has been analyzed by aqueous (9) and nonaqueous (10) titrimetry, GLC (11), and liquid chromatography (12) in mixtures with phenacetin and caffeine. Spectrophotometry is a method of determination for phenacetin (13), and caffeine can be determined by nonaqueous titrimetry (14).

A recent paper (15) introduced the concept of solvent selectivity and the polarity index as two measurable parameters for the selection of proper solvent mixtures for HPLC. In the present study, the Snyder scheme (15) was applied to the separation of muscle relaxant–analgesic mixtures on porous silica and chemically bonded cyanopropylsilane columns. The suitability of each column for the separation problem was evaluated.

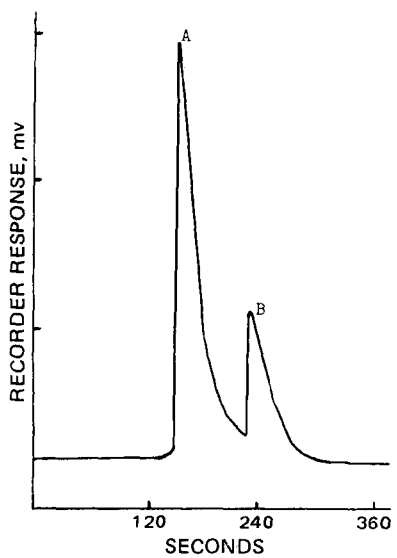

Figure 1—*Typical liquid chromatogram of a meprobamate–aspirin mixture in chloroform–carbon tetrachloride (60:40) on a cyanopropylsilane column at a flow rate of 2.0 ml/min. Key: A, aspirin; and B, meprobamate.*

[1] For other papers in this series, see S. J. Saxena, I. L. Honigberg, J. T. Stewart, and J. J. Vallner, *J. Pharm. Sci.*, **66**, 751 (1977).

Table II—Calibration Data for Standard Drug Solutions

Compound	Final Concentration, mg[a]	D/IS Ratio[b]	Slope	Intercept	$r \pm s_{y\cdot x}$
Carisoprodol	10.02	0.1667 ± 0.0605[c]			
	20.04	0.3572 ± 0.1291	0.0979	−0.0318	0.9999 ± 0.0024
	40.08	0.7539 ± 0.0732			
Phenacetin	7.97	0.5873 ± 0.0194			
	15.94	1.1714 ± 0.0542	0.3815	−0.0303	0.9999 ± 0.0103
	31.88	2.4067 ± 0.2444			
Caffeine	1.56	0.1186 ± 0.0201			
	3.12	0.2376 ± 0.0266	0.3706	0.0043	0.9999 ± 0.0013
	6.24	0.4662 ± 0.0414			

[a] Total milligrams per 5 ml of solution. [b] Data represent three replicate injections of standard solutions. D/IS is the ratio of the integrated area of the drug at some concentration divided by the integrated area of acetaminophen at a concentration of 55 mg/5 ml. [c] Confidence limits at $p = 0.05$.

EXPERIMENTAL[2]

Reagents and Chemicals—Powdered samples of meprobamate[3], methocarbamol[4], carisoprodol[3], chlorzoxazone[5], acetaminophen[6], aspirin[7], caffeine[6], and phenacetin[6] were used. All other chemicals and solvents were the highest quality commercially available.

Mobile Phases—The mobile phases consisted of varying proportions of chloroform mixed with carbon tetrachloride, hexane, or butyl ether; methylene chloride or methylene chloride mixed with carbon tetrachloride; and tetrahydrofuran mixed with toluene, hexane, or butyl ether such that the final composition of the mobile phase gave a polarity index (PI) value (15) of 3.3 (Table I). The solutions were prepared fresh daily.

Preparation of Drug Solutions—Solutions of each drug (5–10 mg/ml) were prepared by dissolving each powder in the appropriate mobile phase.

Internal Standard Solution—The stock internal standard solution (110 mg/10 ml) was prepared by dissolving acetaminophen[6] in tetrahydrofuran–toluene (50:50).

Standard Solutions for Calibration Curves—Separate stock solutions of carisoprodol (200.4 mg/10 ml), phenacetin (159.4 mg/10 ml), and caffeine (31.2 mg/10 ml) were prepared in tetrahydrofuran–toluene (50:50). Accurately pipetted volumes of 0.5, 1.0, and 2.0 ml of each stock solution were placed in 5-ml volumetric flasks. The internal standard stock solution, 1 ml, was added to each flask, followed by the addition of tetrahydrofuran–toluene (50:50) to volume.

The three concentrations of each drug were subjected to a linear regression analysis, and the slope and intercept were calculated (Table II).

Chromatographic Separation and Quantification—The degassed mobile phase was pumped through columns containing a small particle, fully porous silica packing material[8] or a monomolecular layer of a cyanopropylsilane chemically bonded to a small particle, fully porous support[9] at a flow rate of 2.0 ml/min at room temperature until a stable baseline was obtained. Replicate 40-μl injections of sample and standard solutions were made using a 100-μl syringe[10]. The chart recorder provided

[2] A Waters Associates liquid chromatograph (model ALC 202) equipped with an M-6000 pump, a differential RI detector, and an Infotronics integrator (model CRS-204) with digital printout, and Waters packed columns, 4 mm i.d. × 30 cm, were used.

[3] Wallace Laboratories, Cranbury, N.J.

[4] A. H. Robins, Richmond, Va.

[5] McNeil Laboratories, Fort Washington, Pa.

[6] Eastman Chemicals, Rochester, N.Y.

[7] Aldrich Chemicals, Milwaukee, Wis.

[8] μPorasil, ~10 μm, Waters Associates, Milford, Mass.

[9] μBondapak CN, ~10 μm, Waters Associates, Milford, Mass.

[10] Model B-110, Precision Sampling Corp., Baton Rouge, La.

Table III—Effect of Mobile Phase on Retention Times [a]

Mobile Phase Composition[b]	Meprobamate		Methocarbamol		Carisoprodol		Chlorzoxazone		Acetaminophen		Aspirin		Caffeine		Phenacetin	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
A	88	116	92	140	96	96	86	92	100	132	92	92	94	240	100	124
	(12)[c]	(12)	(8)	(20)	(8)	(8)	(8)	(8)	(4)	(16)	(10)	(8)	(8)	(16)	(4)	(12)
B	96	136	100	160	96	100	96	92	116	152	96	92	100	296	104	140
	(14)	(12)	(14)	(16)	(12)	(8)	(12)	(12)	(14)	(16)	(12)	(18)	(16)	(20)	(20)	(12)
C	96	172	92	208	96	108	96	96	96	170	92	94	106	300	116	146
	(8)	(16)	(12)	(19)	(8)	(8)	(16)	(6)	(12)	(12)	(6)	(8)	(16)	(32)	(12)	(10)
D	132	—[d]	120	—[d]	96	—[d]	129	—[d]	311	—[d]	112	—[d]	110	—[d]	108	—[d]
	(22)		(24)		(16)		(9)		(66)		(24)		(14)		(12)	
E	135	—[d]	125	—[d]	96	—[d]	141	—[d]	400	—[d]	123	—[d]	96	—[d]	117	—[d]
	(20)		(18)		(10)		(15)		(36)		(26)		(10)		(9)	
F	134	—[d]	126	—[d]	105	—[d]	162	700	111	—[d]	117	920	92	—[d]	120	1676
	(12)		(15)		(9)		(12)	(20)	(6)		(9)	(80)	(8)		(8)	(160)
G	129	—[d]	129	—[d]	112	—[d]	108	192	159	—[d]	88	200	104	—[d]	123	960
	(15)		(12)		(10)		(9)	(16)	(12)		(10)	(20)	(12)		(11)	(132)
H	236	—[d]	138	—[d]	107	—[d]	174	816	108	—[d]	168	1320	96	—[d]	129	700
	(18)		(15)		(9)		(20)	(52)	(6)		(18)	(108)	(8)		(9)	(200)

[a] Retention time is expressed as seconds measured as elapsed time between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using refractive index detection. Column I is cyanopropylsilane; II is porous silica. [b] Letters refer to solvent compositions in Table I. [c] Base peak width expressed as seconds. [d] Retention time greater than 1800 sec.

a record of drug elution from the column as peaks on a chromatogram. In all cases, the quantification of solute was determined by digital integration of the peak area[2].

RESULTS AND DISCUSSION

This study determined the operating conditions for HPLC that would optimize the resolution of chemically unrelated therapeutic agents commonly found in muscle relaxant–analgesic mixtures. Two operating parameters were studied: (*a*) variations in solvent selectivity with constant polarity, and (*b*) variations in polarity of the stationary phase. The effects of these parameters on retention times and base peak widths of the drugs are shown in Table III. The eluted peaks were monitored using differential refractive index detection since many of the muscle relaxants are aliphatic compounds and do not possess satisfactory UV absorption.

Preliminary data revealed that the polarity index (PI) of the solvent mixture should be approximately 3.3 to obtain a k' between 3 and 10. According to the Snyder (15) approach, PI measures the solvent strength of a particular mixture. This solvent strength influences the retention time or k' for a particular solute mixture. In addition, the separability of a solute is affected by its proton donor, proton acceptor, and dipole interactions with the mobile phase. These latter parameters affect solvent selectivity. Pure solvents can be grouped into approximately nine categories based on the distribution of the constitutive properties mentioned. After a suitable PI value has been obtained for a particular solute mixture, other solvent categories are intermixed to give the same PI values (same k' value) so as to change solvent selectivity and to maximize resolution. The solvent systems used in this study are listed in Table I.

The stationary phases investigated were limited to a porous silica support and a cyanopropylsilane layer chemically bonded to a porous silica support. Generally, in the solvent systems studied, the porous silica column either had too great an affinity for the components investigated or the compounds were too rapidly eluted with a change in solvent selectivity. In contrast, the cyanopropylsilane column showed lower affinity for the drugs but allowed for better comparisons of solvent selectivity. Experience with the Snyder solvent selectivity scheme has shown it to be a valuable tool for limiting the intuitive aspects of solvent selection, especially in normal phase chromatography.

The following observations were noted for the drugs examined. Meprobamate was eluted on the cyanopropylsilane column with all solvent systems employed; but on the silica support, only the tetrahydrofuran mixtures successfully eluted the drug in less than 1800 sec. Resolution of the meprobamate–aspirin combination was observed in Solvents F–H (Table III) on the cyanopropylsilane column and in Solvents A and C on the silica column. In Solvents A, C, and G, aspirin traveled close to the solvent front and was difficult to distinguish from other components present at the front. Figure 1 illustrates a chromatogram of a meprobamate–aspirin mixture using Solvent H on the cyanopropylsilane column.

Methocarbamol eluted on the cyanopropylsilane column with all solvents investigated; on the silica column, only the tetrahydrofuran mixtures eluted the drug in less than 1800 sec. Resolution of the frequently prescribed methocarbamol–aspirin combination was observed in Solvents G and H on the cyanopropylsilane column and in Solvents A–C on the silica column. Only Solvent H on the cyanopropylsilane column gave satisfactory resolution in which both components showed adequate retention times. Although a mixture of methocarbamol, phenacetin, and aspirin was resolved using Solvents A–C on the silica column, aspirin traveled near the solvent front and was indistinguishable from interfering substances present at the front.

Elution of carisoprodol on the cyanopropylsilane column was achieved with all solvent systems. On the silica column, only Solvents A–C gave satisfactory retention times. The widely used mixture of carisoprodol,

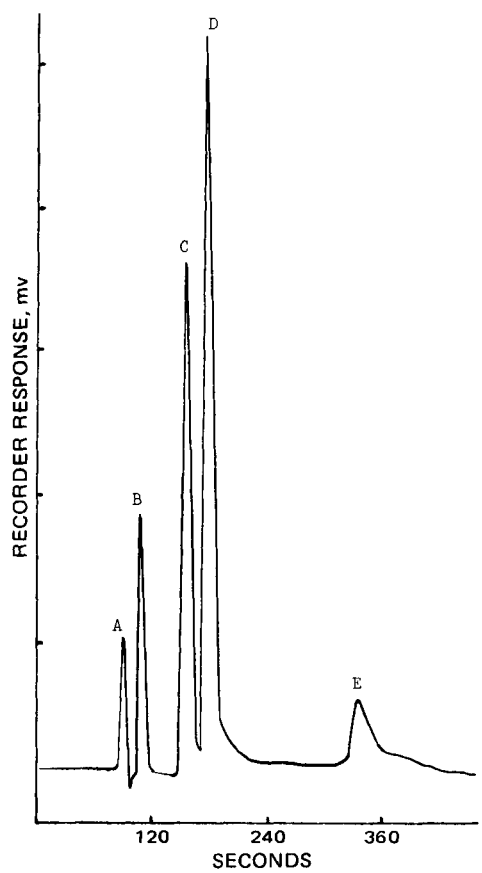

Figure 2—*Liquid chromatogram of a carisoprodol–phenacetin–caffeine mixture in tetrahydrofuran–toluene (50:50) on a porous silica column at a flow rate of 2.0 ml/min. Key: A, solvent front; B, carisoprodol; C, phenacetin; D, acetaminophen (internal standard); and E, caffeine.*

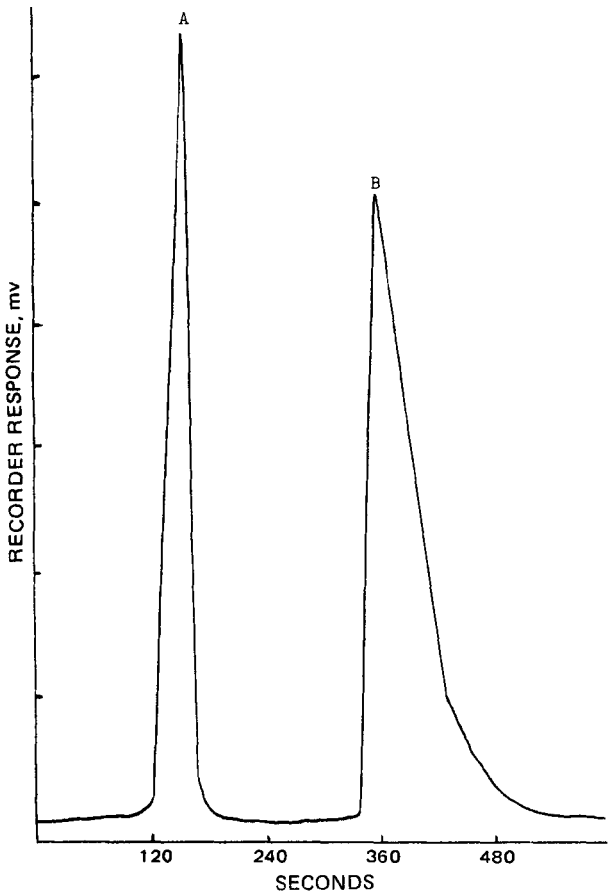

Figure 3—*Typical liquid chromatogram of a chlorzoxazone–acetaminophen mixture in methylene chloride–carbon tetrachloride (95:5) on a cyanopropylsilane column at a flow rate of 2.0 ml/min. Key: A, chlorzoxazone; and B, acetaminophen.*

phenacetin, and caffeine was separated using Solvent C on the silica column (Fig. 2). Other useful systems were Solvents C, F, and H on the cyanopropylsilane column and A and B on the silica column.

Chlorzoxazone was eluted by all solvent systems on the cyanopropylsilane column and by all solvents except D and E on the silica column. A chlorzoxazone–acetaminophen combination was satisfactorily separated on the cyanopropylsilane column with Solvent E (Fig. 3). Other useful systems were A and F–H on the cyanopropylsilane columns and A–C on the silica column. However, solvent systems in which one component is eluted in close proximity to the solvent front present problems in dosage form analysis because of the presence of inert ingredients that tend to elute at the front.

All of the solvent systems eluted acetaminophen on the cyanopropylsilane column. Solvents A–C were the only satisfactory solvents for the drug on the silica column.

Aspirin eluted from the cyanopropylsilane column with all of the solvents investigated. Solvents A–C and F–H eluted aspirin on the silica column in less than 1800 sec. Aspirin was eluted close to the solvent front

Table IV—Approximate Resolution [a] (R_s) Values for Carisoprodol–Phenacetin–Caffeine Mixture

Drug Mixture[b]	Cyanopropylsilane Column Mobile Phase[c]		Silica Column Mobile Phase		
	F	H	A	B	C
Caffeine and carisoprodol	1.5	1.3	—	—	—
Carisoprodol and phenacetin	1.8	2.4	2.8	4.0	4.2
Phenacetin and caffeine	—	—	8.3	9.8	7.3

[a] See Ref. 16. [b] On the cyano column, the drugs were eluted in the following order: caffeine, carisoprodol, and phenacetin. The order of elution from the silica column was carisoprodol, phenacetin, and caffeine. [c] Letters refer to solvent compositions in Table I.

Table V—Analysis of Carisoprodol, Phenacetin, and Caffeine in Known Mixture

Compound	Amount Added[a], mg	Amount Found[b], mg	Accuracy, %
Carisoprodol	20.04	20.77 ± 1.53[c]	3.64
Phenacetin	15.94	15.32 ± 0.92	3.89
Caffeine	3.12	2.98 ± 0.58	4.49

[a] Total milligrams per 5 ml of solution. [b] Based on six replicate determinations of known mixture. [c] Confidence limits at $p = 0.05$.

in approximately 50% of the solvents tested or had extremely long retention times in about 25% of the solvents.

All solvents were satisfactory for the elution of caffeine on the cyanopropylsilane column, and A–C were permissible to use with the silica column.

Phenacetin eluted on the cyanopropylsilane column using all solvents studied; on the silica column, Solvents A–C and F–H showed satisfactory elution.

Satisfactory quantitation of a multicomponent dosage form is dependent on adequate resolution of the components. The value R_s (16), a theoretical parameter, is a reasonable measurement of the separation of two species on a column. It can be approximated from any two drugs included in this investigation from information in Table III. A mixture of carisoprodol, phenacetin, and caffeine was selected to exemplify this situation. Table IV shows calculated R_s values for the drug combination in various solvent systems on both the cyanopropylsilane and silica columns. Solvent C was chosen for the quantitation since it represented the system in which good overall resolution was achieved and it allowed the use of acetaminophen as a convenient internal standard.

Figure 2 illustrates a chromatogram of the assayed drugs. Various concentrations of standard solutions of each drug dissolved in tetrahydrofuran–toluene (50:50) were chromatographed using the silica column. Acetaminophen was added to each solution as the internal standard. The area under the curve for each peak on the chromatograms was determined with a digital integrator. The ratio of each peak area to the area of the internal standard was calculated for each chromatogram. A linear regression line of these data for each drug gave the slope, intercept, and correlation coefficient for each calibration curve (Table II).

A known mixture containing a quantity of each drug present in the ratios found in a commercial dosage form[11] was chromatographed, and the ratios of drug peak areas/internal standard peak areas (D/IS) were calculated for each drug. The constants (slope and intercept) for the linear regression equation shown in Table II were used to solve for drug concentration [D/IS = slope (concentration) + intercept]. The calculations were performed on a programmable calculator. The data in Table V demonstrate the quantitative results obtained for the simulated dosage form. The utility of HPLC in the analysis of a carisoprodol–phenacetin–caffeine mixture is clearly demonstrated with an accuracy of 3–5%.

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High-Pressure Liquid Chromatographic Analysis of Melphalan in Plasma

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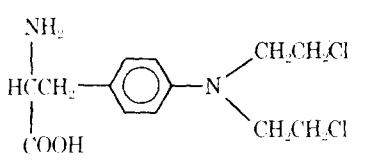
Received April 22, 1977, from the *Section of Hematology and Oncology, Departments of Internal Medicine and Pharmacology, and the Cancer Center, College of Medicine, University of Arizona, Tucson, AZ 85724.* Accepted for publication September 1, 1977.

Abstract □ A new sensitive and rapid high-pressure liquid chromatographic determination of melphalan in plasma was developed. Recovery of 1 μg added to 1 ml of plasma at 23° was 94% but was greatly reduced at higher temperature. The method has been applied to plasma determinations of melphalan in rats and humans and is currently being utilized for human pharmacokinetic studies.

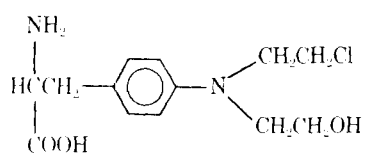
Keyphrases □ Melphalan—high-pressure liquid chromatographic analysis in plasma □ High-pressure liquid chromatography—analysis, melphalan in plasma □ Antineoplastic agents—melphalan, high-pressure liquid chromatographic analysis in plasma

Since melphalan [L-phenylalanine mustard, 4-bis(2-chloroethyl)amino-L-phenylalanine, NSC 8806] was synthesized (1), it has become an important drug for the treatment of various cancers, including multiple myeloma and ovarian and breast carcinoma (2–4). Little information is available concerning its pharmacokinetic properties in humans or animals because of its rapid hydrolytic degradation, which is common to alkylating agents (5–8).

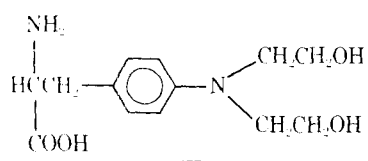
The colorimetric assays utilizing nitrobenzylpyridine (7, 9–11) and the spectrofluorometric method (8) for the determination of melphalan (I) in biological fluids are not sensitive enough for the study of its pharmacokinetic properties. The fluorometric method for biological samples is not practical because of background interference and its lengthy nature (8). These methods are not specific; they measure the total concentration of alkylating agents including one hydrolysis product of I. A GLC method for I (12) has not been applied to biological samples. Recently, Furner *et al.* (13) reported a high-pressure liquid chromatographic (HPLC) method for the determination of I in animal serum.



I



II



III

The present paper reports a sensitive and rapid HPLC method for the determination of I in biological fluids. Practical application of the developed method is demonstrated by *in vivo* determinations of the plasma I concentration in animals and humans. *In vitro* plasma recovery studies also are presented.

EXPERIMENTAL

Materials—Methanol[1] was used as received. Dansylproline[2] was used as an internal standard. The standard I[3] dissolved in methanol containing 2% acetic acid and the internal standard dissolved in methanol were stored at −20° at all times between usage. Aqueous solvents were filtered with a 0.45-μm cellulose acetate filter[4]. Methanol and other organic solvents were filtered with a fluoropore[4] prior to use.

Instrument Conditions—HPLC was performed with an apparatus consisting of two pumps[5], a solvent programmer[6], a detector[7], and a reversed-phase column[8]. An isocratic solvent system of water and methanol (1:1) with 1% acetic acid was delivered at the rate of 2 ml/min, and I was detected at 254 nm. The total analysis time per sample was approximately 20 min.

Extraction of I from Plasma—Five micrograms of internal standard was added to 1 ml of human plasma prior to extraction of I for *in vivo* studies. For *in vitro* studies, 2 μg of internal standard was added for every 1 μg of I. Two milliliters of methanol (0°) was added to 1 ml of plasma and mixed vigorously on a vortex mixer for 20 sec. The sample was then cooled at −60° (acetone and dry ice) for 3 min. The plasma–methanol mixture was centrifuged at 3000 rpm for 3 min on a clinical centrifuge. The clear methanolic solution, which contained the internal standard and I, was injected directly onto the column. Calculation of I was as follows:

[1] Burdick and Jackson Co., Muskegon, Mich.
[2] Pierce Chemical Co., Rockford, Ill.
[3] Obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[4] Millipore Corp., Bedford, Mass.
[5] Waters 6000, Waters Associates, Milford, Mass.
[6] Waters 600, Waters Associates, Milford, Mass.
[7] Waters 440, Waters Associates, Milford, Mass.
[8] Waters micro C_{18} column, Waters Associates, Milford, Mass.