Table II—Summary of Theophylline Protein Binding Studies

Source	n	Procedure	Assay	Percent Bound	Reference
Rabbit plasma	—	Equilibrium dialysis: cellophane membrane	UV spectrophotometry	5–10	11
Bovine serum albumin (2%) solutions	—	Equilibrium dialysis: cellophane membrane	UV spectrophotometry	20	7
Plasma of normal dosed volunteers	7	Equilibrium dialysis: cellophane membrane	UV spectrophotometry	59 ± 3	4
Serum from dosed nursing mothers	4	Ultrafiltration: cellophane membrane	HPLC	42–69	5
"Spiked" normal adult plasma	21	Ultrafiltration: membrane cones[a]	LSC[b]	56.4 ± 3.8	6
"Spiked" cord plasma of full-term infants	21	Ultrafiltration: membrane cones[a]	LSC	36.4 ± 3.8	6
"Spiked" plasma of cirrhotic patients	4	Ultrafiltration: membrane cones[a]	LSC	32.3–40.2	8

[a] Amicon Centriflo cones (CF-50), Amicon Corp., Lexington, MA 02173. [b] LSC = liquid scintillation counting of ^{14}C-theophylline.

et al. (2) concluded that, since only 20% theophylline was protein bound (7), binding need not be considered when the volume of distribution was measured. However, Aranda *et al.* (6) reported decreased binding, which might be important when determining effective serum theophylline concentrations in premature infants with apnea. Piafsky *et al.* (8) found reduced binding in cirrhotic patients but did not determine if greater availability of free drug in the liver increased plasma clearance. In the present study, theophylline protein binding did not increase with age. Therefore, it is unlikely that it contributes to the decreased clearance rates found in older patients (9, 10).

Considerable variation in unbound serum theophylline could be possible in patients, based on the 29% range of protein binding found in this study. The effect of binding should possibly be considered in patients who do not have optimal bronchodilation from theophylline despite total serum theophylline concentrations of 10–20 μg/ml.

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Liquid Chromatography in Pharmaceutical Analysis X: Determination of Chlorzoxazone and Hydroxy Metabolite in Plasma

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Abstract □ A method for the high-pressure liquid chromatographic determination of chlorzoxazone and its hydroxy metabolite in human plasma samples is presented. The separation of the compounds is achieved on an octadecylsilane column with a mobile phase of absolute methanol–distilled water (40:60) at a flow rate of 2.0 ml/min (3100 psig). The chromatographic separation is achieved within 10 min. The overall analysis time is about 45 min, which includes extraction of the drug and metabolite from plasma followed by high-pressure liquid chromatographic separation and quantification. The accuracy of the procedure is in the 1–5% range.

Keyphrases □ Chlorzoxazone and hydroxy metabolite—high-pressure liquid chromatographic analysis in plasma □ High-pressure liquid chromatography—analysis, chlorzoxazone and hydroxy metabolite in plasma □ Relaxants, skeletal muscle—chlorzoxazone and hydroxy metabolite, high-pressure liquid chromatographic analysis in plasma

Chlorzoxazone (I) is one of the most useful agents in the treatment of painful muscle spasms, especially in combination with acetaminophen. It can be used as a potent long-acting central muscle relaxant (1, 2). Clinical studies (3) indicated its therapeutic utility and showed no significant side effects (4). Its metabolism was studied (5, 6), and the major metabolite was 6-hydroxychlorzoxazone (II). Chlorzoxazone is rapidly and completely absorbed from the GI tract. The intact drug essentially disappears from the human body in 7 hr, with less than 1% found in urine. Synthesis of II was reported previously (7).

Analytical methods previously reported for I in biological fluids are scarce. The initial and most commonly used method is a spectrophotometric assay (5), involving extraction of drug from biological fluid and reextraction into basic solution with the absorbance read at 289 nm. Other titrimetric (8), GLC (9, 10), and TLC (11) methods were reported for I but were not adapted to the analysis of the intact drug or metabolites in biological samples.

In continuing efforts to apply high-pressure liquid

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Table I—Effect of Mobile Phase Composition on Retention Times Using Octadecylsilane Column [a]

Mobile Phase[b]	Chlorzoxazone	Phenacetin	Hydroxy-chlorzoxazone
A	292 (32)[c]	220 (24)	139 (23)
	Rs[d] = 2.57	Rs = 3.45	
B	514 (50)	359 (38)	196 (27)
	Rs = 3.52	Rs = 5.02	
C	271 (22)	208 (22)	130 (18)
	Rs = 2.86	Rs = 3.90	

[a] Retention time expressed as seconds measured as elapsed time between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using UV detection. [b] Solvent composition: A, absolute methanol–distilled water (50:50); B, absolute methanol–distilled water (40:60); and C, acetonitrile–distilled water (35:65). [c] Base peak width expressed as seconds. [d] See Ref. 13.

chromatography (HPLC) in pharmaceutical analysis[1], an HPLC procedure for the separation and quantification of I and II in human plasma is reported. The chromatographic separation takes approximately 10 min. The overall analysis time is about 45 min, which includes extraction of the drug and its metabolite from plasma followed by HPLC separation and quantification.

EXPERIMENTAL[2]

Reagents and Chemicals—Powdered samples of chlorzoxazone[3] and 6-hydroxychlorzoxazone[3] were used in the analytical procedure and in the preparation of standard curves. All other chemicals and solvents were the highest purity of the commercially available materials.

Mobile Phase—Absolute methanol–distilled water (40:60) was used as the mobile phase and was degassed before use.

Internal Standard Stock Solution—Powdered phenacetin[4] was dissolved in absolute methanol to give a stock internal standard solution of 100 μg/ml.

Preparation of Stock Solutions for Calibration Curve—Separate stock solutions (5 μg/ml) of chlorzoxazone and 6-hydroxychlorzoxazone were prepared by dissolving 5 mg of each powder in 100 ml of absolute methanol followed by a 1:10 dilution of the resulting solution with methanol.

Determination of Calibration Curves for Plasma Levels—Into individual 15-ml centrifuge tubes were placed accurately pipetted volumes of 0.2, 0.5, and 1.0 ml of both chlorzoxazone and hydroxychlorzoxazone stock solutions. Then 0.1 ml of internal standard stock solution was added to each tube, and the contents were evaporated to dryness on a steam bath with a nitrogen stream.

Unspiked human plasma (1.0 ml) was added, and the mixture was shaken[5] for 1 min followed by the addition of 1 ml of 0.1 N H_2SO_4. Ammonium sulfate (0.5 g) was then added to each sample in four evenly divided portions, with shaking[5] and heating on a steam bath after each addition. Ether (5 ml) was added, and the mixture was shaken[5] and centrifuged at 5000 rpm for 5 min. The mixture was quick frozen with the aid of a dry ice–acetone solution in a Dewar flask. The ether layer was decanted and filtered through a 0.5-μm filter[6] into a clean 15-ml centrifuge tube and evaporated to dryness on a steam bath under a nitrogen stream. Absolute methanol (100 μl) was added to dissolve the residue, and a 20-μl aliquot was injected into the liquid chromatograph.

Determination of Chlorzoxazone and Hydroxychlorzoxazone in Human Plasma—Into individual 15-ml centrifuge tubes were placed accurately pipetted volumes of 0.25, 0.7, and 1.0 ml of both chlorzoxazone and hydroxychlorzoxazone stock solution. Then 0.1 ml of internal standard stock solution was added to each mixture. Each sample was then treated in the same manner as described under *Determination of Calibration Curves for Plasma Levels*. A control blank with unspiked human plasma also was performed. Replicate injections of 20 μl were made for each sample.

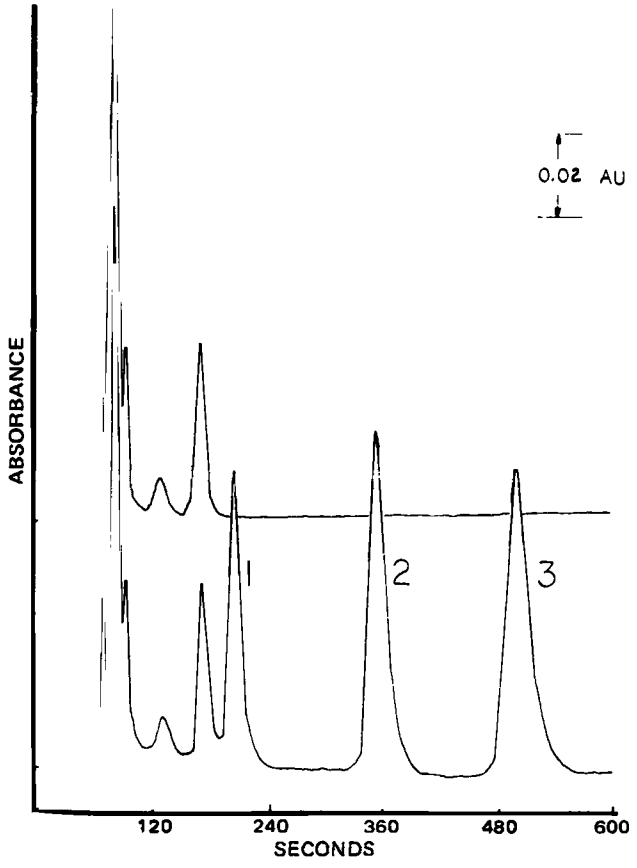

Figure 1—*Liquid chromatogram of chlorzoxazone and 6-hydroxychlorzoxazone from a spiked plasma sample at a flow rate of 2.0 ml/min on an octadecylsilane column. The mobile phase was absolute methanol–distilled water (40:60). The initial drug and metabolite concentration in plasma was 5 μg/ml. Key: A, plasma blank; and B, spiked plasma sample containing 6-hydroxychlorzoxazone (1), phenacetin (2) (internal standard), and chlorzoxazone (3).*

Chromatographic Separation and Quantification—The degassed mobile phase was pumped through an octadecylsilane column[7] at a flow rate of 2.0 ml/min (3100 psig) at room temperature (23 ± 2°) until a stable baseline was obtained. Replicate 20-μl injections of sample and standard solutions were made using a 100-μl syringe[8]. A record of drug elution from the column as peaks on a chromatogram was provided by a chart recorder. The wavelength of the UV detector was set at 280 nm. Each solute was measured by digital integration of the peak area[1].

RESULTS AND DISCUSSION

The HPLC analysis of a chlorzoxazone–hydroxychlorzoxazone mixture and its application to plasma extracts necessitated the examination of three separate problems: (*a*) the development of a set of HPLC operating parameters that would separate the two compounds without peak overlap, (*b*) the detection and quantification of each compound at the levels found in plasma (1–10 μg/ml), and (*c*) the determination of the contribution of the extracted plasma blank to the chromatograms.

Initial chromatographic separation was attempted using a cyanopropylsilane[9] column and the Snyder concept (12) of solvent selectivity and polarity index (PI) as two measurable parameters for the selection of a proper solvent mixture in HPLC. Preliminary data revealed that the polarity index of the solvent mixture should be in the 1.9–2.3 range to obtain a k' for each component between 3 and 10. Various solvents in Snyder's nine solvent categories were intermixed to give the same PI value but change solvent selectivity and maximize resolution of the solute

[1] For other papers in this series, see I. L. Honigberg, J. T. Stewart, and M. Smith, *J. Pharm. Sci.*, 67, 675 (1978).

[2] A Waters Associates liquid chromatograph (model ALC 202) equipped with an M-6000 pump and a U6K injector, a Perkin-Elmer model LC-55 variable wavelength UV–visible detector, a Spectra-Physics autolab minigrator with digital printout, and Waters packed columns (30 cm × 4 mm i.d.) were used.

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

[4] Matheson, Coleman and Bell, East Rutherford, N.J.

[5] Vortex Genie mixer, Scientific Industries, Bohemia, N.Y.

[6] Catalog No. FHLP01300, Millipore Corp., Bedford, MA 01730.

[7] Waters packed <10-μm μBondapak C_{18} columns, Waters Associates, Milford, Mass.

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

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

Table II—Typical Calibration Data for Standard Solutions

Compound	Final Concentration, μg[a]	D/IS Ratio[b]	Slope[c]	Intercept	$r \pm s_{yx}$[d]
Chlorzoxazone	1.0	0.2097 ± 0.0179			
	2.5	0.5159 ± 0.0341	0.2613	−0.0846	0.9962 ± 0.0377
	5.0	1.2417 ± 0.0032			
6-Hydroxychlorzoxazone	1.0	0.1036 ± 0.0088			
	2.5	0.2438 ± 0.0122	0.1128	−0.0203	0.9977 ± 0.0126
	5.0	0.5502 ± 0.0100			

[a] Total micrograms per 100 μl of solution. [b] Data represent triplicate injections of duplicate samples of standard solutions. D/IS is the ratio of the area of the drug at some concentration divided by the area of phenacetin at a concentration of 100 μg/100 μl. [c] Repetition of calibration curve data on different days ($n = 4$) revealed that relative standard deviations of the slope for chlorzoxazone and hydroxychlorzoxazone were 1.25 and 4.3%, respectively. [d] The r is the correlation coefficient calculated from regression analysis, and s_{yx} is the standard error of the estimate of y (D/IS) on x (concentration).

Table III—Determination of Chlorzoxazone and Hydroxy Metabolite in Human Plasma

Mixture	Components	Amount Added, μg	Amount Found, μg[a]	*RSD*, %	Accuracy, %
A	Chlorzoxazone	1.25	1.30 ± 0.06	4.6	4.0
	Hydroxychlorzoxazone	1.25	1.22 ± 0.06	4.9	2.4
B	Chlorzoxazone	3.5	3.47 ± 0.05	1.4	0.86
	Hydroxychlorzoxazone	3.5	3.52 ± 0.04	1.1	0.57
C	Chlorzoxazone	5.0	4.78 ± 0.13	2.7	4.4
	Hydroxychlorzoxazone	5.0	5.08 ± 0.28	5.5	1.6

[a] Based on triplicate injections of duplicate plasma samples.

mixture. This approach was unsuccessful because of the lack of satisfactory resolution of the mixture caused by band spreading of II on the column.

Further investigations revealed that reversed-phase chromatography on an octadecyl column was a more successful approach to the separation of I and II. The effect of mobile phase composition on the retention time of each component on the column is shown in Table I. Although adequate separation could be achieved using any of the solvent systems studied, as evidenced by the resolution (*Rs*) values (13), the compounds were most propitiously separated with absolute methanol–distilled water (40:60). This solvent mix allowed the separation of drugs from endogenous plasma components (Fig. 1) as well as the use of phenacetin as a suitable internal standard.

A flow rate of 2.0 ml/min (3100 psig at room temperature) was the most satisfactory since separations could be obtained in less than 10 min. The void volume of the column was 2.4 ml. UV detection at 280 nm was employed since both I and II have UV absorption maxima close to this value and the detector would, therefore, provide maximum sensitivity for the low concentration levels present in the plasma. Acetaminophen does not interfere with the assay since it elutes close to the solvent front in the mobile phase employed.

The solutions used in the determination of the standard curves as well as the plasma samples were extracted with ether. The percent recoveries for I and II using the described procedure were 96.1 ± 3.0 and 83.2 ± 5.2%, respectively[10] (mean ± *SD*). The internal standard (percent recovery was 83.2 ± 2.6%) was added to the original samples prior to extraction to improve precision. The drug concentrations used in the preparation of the standard curve and plasma were in the range of those reported for plasma (6). The minimum detectability of both the drug and its metabolite with the chromatographic procedure was 80 ng at two times the noise level.

Calibration curves for plasma of I and II were obtained as follows. Digital integration of the area under the curve for each peak on the chromatograms was performed. The ratios of each compound to the area of the internal standard were calculated for each chromatogram. Regression analysis of these data at the various concentrations of each component gave the slope, intercept, and correlation coefficient for the plasma calibration curve (Table II).

A liquid chromatogram of I and II from a spiked plasma sample is shown in Fig. 1. No interference from blank plasma was noted. The ratios of drug peak areas to internal standard peak areas (D/IS) were calculated for both components. The constants (slope and intercept) shown in Table II for the linear regression equations were used to solve for drug concentration in the plasma sample: [D/IS = (slope × concentration) + intercept]. Calculations were performed on a programmable calculator[11].

The data in Table III demonstrate the utility of HPLC in the analysis of I and II at plasma concentration levels.

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[10] Percent recoveries of chlorzoxazone and the hydroxy metabolite were determined by comparing the HPLC peak area ratios of equivalent quantities of extracted and unextracted compound to unextracted internal standard. Triplicate injections of three individual samples of drug and metabolite were performed.

[11] Olivetti-Underwood Programma 101.