lution (Fig. 1B) and from a glycerin solution (Fig. 1C) were similar. In the presence of sugar, besides crystallization (Fig. 1B), II may be partially reduced to I (Figs. 1B and 1D).

Prednisolone did not crystallize in any vehicle investigated. It decomposed fast in the presence of citrate buffer. The percent retained after 92 days was 62.9 (Table I). Some decomposition also was recorded in water and in 50% aqueous sucrose (Table I and Fig. 2D). Both 50% (v/v) glycerin in water and 50% aqueous sorbitol appear to be excellent I vehicles (Table I). The peak obtained from a I solution in sorbitol (Fig. 2C) compared better with the standard (Fig. 1A) than did the peak from a glycerin solution (Fig. 2B), which had a slight shoulder, perhaps a sign of slight decomposition. All solutions studied contained 10% (v/v) ethanol and 0.1% sodium benzoate.

The developed method also was suitable for the quantitative determinations of I, II, and hydrocortisone in commercial tablets (Table II). The results obtained with the blue tetrazolium method were very high, presumably due to interference from some inactive ingredients. When the active ingredients were extracted with ethanol without heat, inconsistent and sometimes low results were obtained. Therefore, the mixture was heated to extract the active ingredient quantitatively.

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Fluorometric Determination of Chlorzoxazone in Tablets and Biological Fluids

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Abstract □ A fluorometric determination for chlorzoxazone was developed based on the intrinsic drug fluorescence in chloroform using excitation and emission wavelengths of 286 and 310 nm, respectively. A calibration curve for chlorzoxazone in chloroform gave a linear working range of 0.027–2.3 μg/ml (r = 0.9999) with the minimum detectability at 27 ng/ml. The procedure was applied to chlorzoxazone analysis in spiked plasma and urine samples. Minimum detectable drug levels in these samples were 60 and 130 ng/ml, respectively. Data revealed that chlorzoxazone could be determined in plasma and urine even in the presence of 20-fold molar excesses of its major metabolite, 6-hydroxychlorzoxaxone, and acetaminophen. The method also was applicable to chlorzoxazone analysis in a commercial dosage form containing acetaminophen.

Keyphrases □ Chlorzoxazone—analysis, fluorometry, in commercial tablets and biological fluids, in presence of hydroxychlorzoxazone and acetaminophen □ Fluorometry—analysis, chlorzoxazone in commercial tablets and biological fluids, in presence of hydroxychlorzoxazone and acetaminophen □ Muscle relaxants—chlorzoxazone, fluorometric analysis in commercial tablets and biological fluids, in presence of hydroxychlorzoxazone and acetaminophen

Chlorzoxazone (I) is one of the most useful skeletal muscle relaxants in the treatment of painful muscle spasm, especially in combination with acetaminophen, a product that has been among the top 100 drug products in the United States in recent years. Studies of chlorzoxazone metabolism in humans have shown that its major metabolite is 6-hydroxychlorzoxazone (II) (1, 2). The drug is rapidly absorbed from the GI tract and essentially disappears from the blood in 7–8 hr. Less than 1% of the intact drug is excreted in the urine.

Among the analytical methods reported for I analysis have been spectrophotometry (1), titrimetry (3), and GLC and TLC (4–6). Direct application of many procedures to the determination of the drug in biological fluids is lacking. In other instances, parameters such as accuracy, linearity, sensitivity, and detection limit were not evaluated for the reported methodology.

In this paper, the intrinsic fluorescence of I is presented as a suitable analytical technique for the determination of the drug in biological fluids. The method also is useful for analysis of I in tablets. The method described is sensitive for I in the nanogram per milliliter range and is free from interference from II and acetaminophen. A previous report examined the analysis of I using derivatization with various fluorogenic reagents (7).

EXPERIMENTAL

Apparatus—Fluorescence measurements were made with a spectrophotofluorometer[1] equipped with a corrected spectra accessory. The following settings were used: excitation and emission slits, 10 nm; amplifier sensitivity coarse, 0.1; and measurement mode in true emission. Clear fused quartz cells (12.5 × 45 mm) were used as sample cells for dosage form analysis. Quartz microcells[2] (path length 5 mm) were used for the biological fluid assays.

Reagents and Chemicals—Chlorzoxazone[3], 6-hydroxychlorzoxazone[3], and acetaminophen[4] were obtained as pure powders and were used without further purification in the preparation of stock solutions. All other reagents and chemicals were commercially available and were used as received.

Determination of Chlorzoxazone in Dosage Form—A tablet[5] containing 250 mg of chlorzoxazone and 300 mg of acetaminophen was suspended in 15 ml of distilled water contained in a 50-ml centrifuge tube. After 25 ml of ethyl acetate was added, the solution was mixed[6] for 5 min. An aliquot of the ethyl acetate extract corresponding to 100 mg of chlorzoxazone was transferred to a 50-ml volumetric flask and diluted to volume with ethyl acetate. One milliliter of this solution was trans-

[1] Model MPF-4, Perkin-Elmer Corp., Norwalk, Conn.
[2] Part No. 018-0056, Hitachi, Ltd., Tokyo, Japan.
[3] McNeil Pharmaceuticals, Fort Washington, Pa.
[4] Eastman Organic Chemicals, Rochester, N.Y.
[5] Parafon-Forte, McNeil Pharmaceuticals, Fort Washington, Pa.
[6] Vortex-Genie mixer, Scientific Industries, Bohemia, N.Y.

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Table I—Determination of Chlorzoxazone in a Commercial Tablet [a]

Labeled Content in Aliquot, μg	Found, μg[b]	Percent of Theory
6.24	6.15 ± 0.23	98.61 ± 3.71
3.12	3.14 ± 0.12	100.75 ± 3.83
1.56	1.55 ± 0.07	99.57 ± 4.37
Average mean ± *SD*		99.64 ± 3.57
RSD, %		3.58

[a] Parafon-Forte, McNeil Laboratories, Fort Washington, Pa. [b] Based on triplicate samples.

ferred to a 25-ml volumetric flask, and the solution was adjusted to volume with ethyl acetate.

Individual aliquots of 19.5, 39, and 78 μl were each transferred to 5-ml volumetric flasks and evaporated to dryness with nitrogen. Chloroform, 5 ml, was added to redissolve each residue, and the chloroform solution fluorescence was determined using excitation and emission wavelengths of 286 and 310 nm, respectively. A calibration curve for chlorzoxazone in the 0.1–1.50-μg/ml range was determined concurrently using dilutions obtained from a stock solution of chlorzoxazone in chloroform. Slope and intercept data from the calibration data regression analysis were used to solve for the drug concentration in individual aliquots: fluorescence = (slope × concentration) + intercept.

Assay of Chlorzoxazone in Plasma or Urine—One milliliter of plasma or urine containing chlorzoxazone was added to a 15-ml centrifuge tube containing 0.5 ml of pH 2.5 phosphate buffer. Six milliliters of petroleum ether containing 1.5% isoamyl alcohol was added, followed by mixing[6] for 2 min and centrifugation for 5 min at 2000 rpm. Five milliliters of the organic phase was transferred to a clean 15-ml centrifuge tube, followed by the addition of 2.25 ml of 0.5 *N* NaOH. After mixing[6] (1 min) and centrifugation (3 min), the organic phase was aspirated and 1.25 ml of 1 *N* HCl and 5 ml of chloroform were added. The solution was mixed[6] for 1 min, and the aqueous phase was aspirated.

Anhydrous sodium sulfate (1 g) was added to the chloroform layer, and the mixture was centrifuged for 3 min. The chloroform was decanted into a clear 15-ml centrifuge tube and evaporated to dryness with a nitrogen stream. Chloroform, 1 ml, was added to redissolve the residue. Fluorescence measurements were made using excitation and emission wavelengths of 286 and 310 nm, respectively. A calibration curve for chlorzoxazone was performed concurrently using spiked plasma or urine samples[7] in the 1–10-μg/ml concentration range. Slope and intercept data from regression analysis of the calibration data were used to solve for the drug concentration in each sample: fluorescence = (slope × concentration) + intercept.

RESULTS AND DISCUSSION

Fluorometric examination of I in water at pH 2–14 showed maximum fluorescence at pH < 6 using excitation and emission wavelengths of 286 and 310 nm, respectively. The fluorescence was essentially quenched at pH ≧ 10. A comparison of chlorzoxazone fluorescence in various organic solvents (water, ethanol, isobutanol, tetrahydrofuran, ethyl acetate, chloroform, carbon tetrachloride, and petroleum ether containing 1.5% isoamyl alcohol or 2% isobutanol) indicated that the drug was more fluorescent in the polar solvents. However, sensitivity was limited by the influence of Raman scatter of the various solvents, especially at low I concentrations.

Data showed that the chloroform Raman peak was less intense than those of the other solvents studied. Chloroform also gave less interference at the I emission wavelength while showing adequate solubility for the drug. Thus, chloroform was selected as the solvent for the fluorescence measurements. A calibration curve for I in chloroform showed a linear working range from 0.027 to 2.3 μg/ml (r = 0.9999) with minimum detection at 27 ng/ml (signal/noise = 2).

Chlorzoxazone was extracted from water samples using various organic solvents. The percent recovery of I from water using chloroform was 100.0 ± 4.4% (n = 6). Optimal extraction was at pH < 7. Other solvents such as ethyl acetate, isobutyl or isoamyl alcohol, ethyl acetate–isobutanol (3:2), and petroleum ether containing 1.5% isoamyl alcohol or 2% isobutanol also were useful extraction solvents. However, when these solvents were applied to chlorzoxazone extraction in plasma or urine samples, petroleum ether containing 1.5% isoamyl alcohol yielded the highest percent recovery in terms of chlorzoxazone fluorescence in chloroform.

Inclusion of isoamyl alcohol in petroleum ether serves two purposes: (*a*) it increases the solvent system polarity, and (*b*) it prevents drug adsorption on the glass walls of the containers (8). Increasing the isoamyl alcohol concentration in the petroleum ether from 1.5 to 10% or adding isobutanol (2–10%) did not significantly increase the percent recovery of I but gave higher blank readings. In general, higher blank readings were obtained for biological fluids extracted with polar solvents, probably due to extraction of endogenous plasma and urine components that absorb strongly at 280 nm in the vicinity of the I fluorescence excitation wavelength. Such substances also could reduce chlorzoxazone fluorescence, a phenomenon known as the inner-filter effect (9).

The petroleum ether–isoamyl alcohol extraction solvent minimized these interferences as well as those of II and acetaminophen, potentially interfering substances likely to be present with I in biological samples. Calculated percent recoveries of II and acetaminophen from aqueous solution using the solvent mix were 0.2 and 0.12%, respectively. Little or no interference in the chlorzoxazone assay in plasma or urine was observed even in the presence of a 20-fold excess of II and/or acetaminophen (Table II).

In the assay for I in biological fluids, chlorzoxazone extraction with

Table II—Typical Analysis of Chlorzoxazone at 1.7 μg/ml in Spiked Plasma and Urine Samples [a]

Mixture	Amount Found[b], μg/ml		Percent of Theory	
	Plasma	Urine	Plasma	Urine
Chlorzoxazone and hydroxychlorzoxazone[c]	1.698 ± 0.035	1.735 ± 0.028	99.88 ± 2.19	102.06 ± 1.72
Chlorzoxazone and hydroxychlorzoxazone[d]	1.733 ± 0.042	1.679 ± 0.010	101.94 ± 2.45	98.76 ± 0.01
Chlorzoxazone and hydroxychlorzoxazone[e]	1.614 ± 0.092	1.597 ± 0.026	97.62 ± 1.61	93.94 ± 1.67
Chlorzoxazone and acetaminophen[e]	1.63 ± 0.020	1.70 ± 0.095	97.03 ± 1.80	100.06 ± 5.66

[a] Samples were spiked by addition of microliter aliquots of stock chlorzoxazone, hydroxychlorzoxazone, and acetaminophen solutions in ethanol to 1.0-ml blank plasma or urine samples. [b] Based on quadruplicate samples. [c] Hydroxy metabolite was present in equimolar concentration to that of chlorzoxazone. [d] Hydroxy compound present in fourfold molar excess. [e] Compound present in 20-fold molar excess.

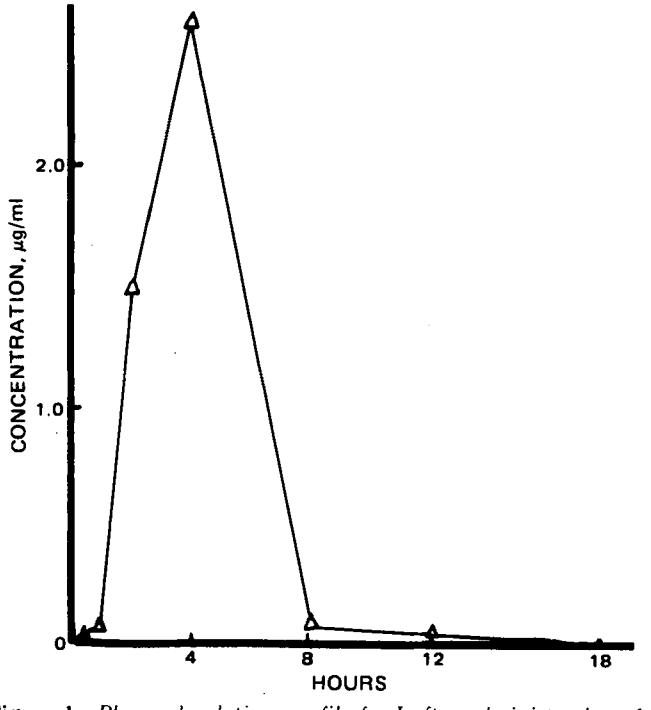

Figure 1—*Plasma level–time profile for I after administration of a single 300-mg oral dose to a healthy adult male.*

[7] Samples were spiked by addition of 2.5, 5.0, 10.0, and 20.0 μl of a 3.4-mg/10-ml ethanolic solution of I to 1.0 ml of blank plasma or urine samples.

petroleum ether–isoamyl alcohol is followed by a reextraction of the drug into aqueous sodium hydroxide, adjustment to pH 6.8 with acid, and extraction into chloroform for the final fluorometric measurement. With this procedure, percent recoveries of chlorzoxazone from plasma and urine compared to unextracted drug were 86.63 ± 1.66 (n = 8) and 95.37 ± 2.42 (n = 8), respectively. The linear working ranges for I in plasma and urine were 0.06–3.2 (r = 0.9993) and 0.13–3.0 (r = 0.9995) μg/ml, respectively, with minimum detectable levels at 60 and 130 ng/ml, respectively.

To demonstrate the application of the fluorometric I analysis, drug determinations in a commercial dosage form and in spiked plasma and urine samples were performed (Tables I and II). Plasma and urine data for spiked samples of I at 0.85, 3.4, and 6.8 μg/ml in the presence of varying concentrations of II and acetaminophen gave similar results. In addition, a plasma level–time profile for I (Fig. 1) illustrated the utility of the assay in determining plasma drug levels after oral administration of a single 300-mg dose to a healthy adult male. The peak plasma I level was obtained within 4 hr, with the drug essentially disappearing from plasma in 8 hr. Analysis of I in urine samples taken over 24 hr revealed that 116 μg of unchanged drug (<0.1% of administered dose) was excreted. These findings are comparable to those of previous metabolic studies (2).

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Acetazolamide Binding to Two Carbonic Anhydrase Isoenzymes in Human Erythrocytes

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Received July 13, 1978, from *Alza Corporation, Palo Alto, CA 94304.* Accepted for publication August 18, 1978.

Abstract □ Acetazolamide binding to high activity and low activity carbonic anhydrase isoenzymes in red blood cells was studied. Inhibitory constants of 0.041 and 2.72 μ*M* and maximum binding capacities of 17.2 and 155 μ*M*, respectively, were found.

Keyphrases □ Acetazolamide—binding to two carbonic anhydrase isoenzymes, human erythrocytes, *in vitro* □ Carbonic anhydrase inhibitors—acetazolamide, binding to two carbonic anhydrase isoenzymes, human erythrocytes, *in vitro* □ Erythrocytes—acetazolamide binding to two carbonic anhydrase isoenzymes, *in vitro*

Acetazolamide, a carbonic anhydrase inhibitor widely used in the treatment of glaucoma, acts directly on the carbonic anhydrase in secretory cells of the ciliary body to reduce aqueous production and to lower intraocular pressure (1). The decrease in aqueous humor formation is thought to result from a decrease in bicarbonate flux from secretory cells (2, 3). Acetazolamide also acts indirectly to lower intraocular pressure by eliciting systemic acidosis (1). With the administration of therapeutic acetazolamide doses, renal carbonic anhydrase is inhibited, decreasing reabsorption of renally excreted bicarbonate (4).

BACKGROUND

Most carbonic anhydrase is in the red blood cells, with extravascular sources contributing an additional 10% or less (1). Two types of carbonic anhydrase have been identified: high activity type C and low activity type B (5). Extravascular carbonic anhydrase, including that found in the ciliary body, is generally the high activity type C isoenzyme (5). In humans, red blood cells are the major source of low activity type B isoenzyme (4). The ratio of low activity isoenzyme to high activity isoenzyme in human erythrocytes was reported as 6:1 (6).

Acetazolamide binding to human erythrocytes was studied previously (7). Intracellular and extracellular acetazolamide concentrations were determined by GLC following drug equilibration between the red blood cells and the external medium (either plasma or buffered saline). Rosenthal plots were constructed from the bound intracellular and extracellular concentrations to obtain the dissociation constants (inhibitory constant) of the enzyme–drug complex and the maximum binding capacity of the enzyme.

The inhibitory constant reported for the high activity isoenzyme was approximately an order of magnitude larger than that reported for the low activity isoenzyme (5, 6). Therefore, if *in vitro* binding studies are performed such that a wide range of intracellular acetazolamide concentrations is obtained, a definite curvature in the Rosenthal plot should be seen. Resolution of the curved plot into linear segments should permit the determination of maximum binding capacities and inhibitory constants for both isoenzymes.

Rosenthal plots constructed from *in vitro* equilibration studies performed in this laboratory exhibit a pronounced curvature. Inhibitory constants determined from the resolution of the curve were used as parameters in a nonlinear pharmacokinetic model for acetazolamide described previously (8).

EXPERIMENTAL

Human blood was drawn into heparinized tubes and immediately centrifuged to isolate the red blood cells. The cells were washed twice with two volumes of pH 7.4 buffered saline (0.15 *M* NaCl and 0.01 *M* phosphate), which were discarded. The cells were then suspended in the same buffered saline, and the hematocrit of the reconstituted red blood cell suspension was determined.

Acetazolamide was dissolved in methanol (0.5 μg/μl), and aliquots containing 1–200 μg of the drug were added to 20-ml disposable vials. Methanol was evaporated with a nitrogen stream. Red blood cell suspension was added to the vials (6 ml to vials containing <6 μg of acetazolamide and 3 ml to vials containing >6 μg of drug). The contents were incubated for 2 hr at 37° with gentle swirling in a reciprocating shaker. All incubations were performed using the suspension prepared from cells obtained from a single subject.

After incubation, the artificial blood medium was centrifuged. The acetazolamide concentration in 1–3-ml aliquots of the supernate was determined using a previously described high-pressure liquid chromatographic (HPLC) method (9).

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