(5) F. Glenn, *Surg. Gynecol. Obstet.*, **134**, 249 (1972).
(6) L. Way, W. Admirand, and J. E. Dunphy, *Ann. Surg.*, **176**, 347 (1972).
(7) W. Walters and H. R. Wesson, *Surg. Gynecol. Obstet.*, **65**, 695 (1937).
(8) R. G. Danzinger, A. F. Hofmann, L. J. Schoenfield, and J. L. Thistle, *N. Engl. J. Med.*, **286**, 1 (1972).
(9) A. M. Molokhia, W. I. Higuchi, and A. F. Hofmann, *J. Pharm. Sci.*, **64**, 2029 (1975).
(10) T. Hisatsugu, H. Igimi, and M. Nishimura, *Jpn. J. Surg.*, **2**, 62 (1972).
(11) H. Igimi, *Igaku Kenkyu*, **42**, 55 (1972).
(12) L. D. Wright and J. A. Presberg, *Proc. Soc. Exp. Biol. Med.*, **115**, 497 (1964).
(13) W. M. Sperry and M. Webb, *J. Biol. Chem.*, **123**, 97 (1951).
(14) G. Saracco and E. S. Marchetti, *Ann. Chim.*, **48**, 1357 (1958).
(15) S. H. Yalkowsky, G. L. Flynn, and G. L. Amidon, *J. Pharm. Sci.*, **61**, 983 (1972).
(16) H. L. Spier and K. G. VanSenden, *Steroids*, **6**, 871 (1965).
(17) S. H. Yalkowsky, G. L. Flynn, and T. G. Slunick, *J. Pharm. Sci.*, **61**, 852 (1972).
(18) J. A. Ostrenga and C. Steinmetz, *ibid.*, **59**, 414 (1970).
(19) S. Prakongpan, W. I. Higuchi, K. H. Kwan, and A. M. Molokhia, *ibid.*, **65**, 685 (1976).
(20) V. G. Levich, "Physicochemical Hydrodynamics," Prentice-Hall, Englewood Cliffs, N.J., 1962.
(21) S. Prakongpan, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1974.

Sensitive Fluorescence Assay for *d,l*-Methadone

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Abstract □ *d,l*-Methadone forms a fluorophore when reacted with paraformaldehyde in concentrated sulfuric acid. Based on this reaction, a fluorescence assay suitable for quantitative *d,l*-methadone analysis from plasma and other tissues was developed. *d,l*-Methadone was extracted at pH 9.2 from the deproteinized filtrate of plasma or of aqueous tissue homogenate into an organic phase of 25% isobutanol in ethylene dichloride. After an aliquot of the organic phase was evaporated to dryness at 50–55° with an air jet, paraformaldehyde (0.1%, in concentrated sulfuric acid) was added, and fluorescence was read at 450 nm with excitation at 275 nm. By this method, *d,l*-methadone can be assayed in the presence of its metabolites, morphine, diacetylmorphine (heroin), codeine, and cocaine; however, amphetamine, meperidine, and quinine interfere.

Keyphrases □ Methadone—analysis, fluorescence assay, lung, liver, brain, serum, rats □ Narcotic analgesics—methadone, fluorescence assay, lung, liver, brain, serum, rats □ Fluorometry—analysis, methadone in lung, liver, brain, serum, rats

During the past decade, *d,l*-methadone has been extensively used in maintenance and rehabilitation programs for chronic diacetylmorphine (heroin) users (1). Most of the work on its distribution and metabolic disposition (2–4) has involved complex assays requiring labeled methadone. Other available methods are not sensitive enough to study *d,l*-methadone distribution following therapeutic doses (5–9). Recently, a highly sensitive GLC method (10) was reported.

When *d,l*-methadone is reacted with paraformaldehyde in concentrated sulfuric acid by heating at 100°, a fluorophore is formed (11). The purpose of this paper is to report a fluorescence assay based on this observation. This assay is applicable for methadone analysis from biological tissues in nanogram concentrations.

EXPERIMENTAL

Reagents—All reagents were prepared from reagent grade chemicals.

Zinc Sulfate Solution, 5%—Fifty grams of $ZnSO_4 \cdot 7H_2O$ was dissolved in glass-distilled water to a final volume of 1000 ml. The solution was filtered to remove any turbidity.

Barium Hydroxide Solution, 4.5%—Forty-five grams of $Ba(OH)_2 \cdot 8H_2O$ was dissolved in glass-distilled water to a final volume of 1000 ml. The solution was filtered to remove any turbidity and stored in tightly stoppered bottles.

Borate Buffer, pH 9.6—Fifty milliliters of 0.1 *M* boric acid in 0.1 *M* KCl was mixed with 36.85 ml of 0.1 *M* NaOH.

25% Isobutanol in Ethylene Dichloride—Isobutanol, 250 ml, was mixed with 750 ml of ethylene dichloride.

0.1% Paraformaldehyde in Sulfuric Acid—Paraformaldehyde, 25 mg, was dissolved in 25 ml of concentrated sulfuric acid. This reagent was prepared fresh just prior to use.

Procedure—Rats were pretreated with *d,l*-methadone and sacrificed by decapitation. Blood and other tissues such as lung, liver, and brain were removed. Plasma or serum was separated by centrifugation. Tissue samples were frozen over powdered dry ice and stored at −20° until assayed.

Just prior to assay, tissues were weighed and homogenized in distilled water (1:3). A 2-ml aliquot of the homogenate or 1 ml of serum (or plasma) diluted to 2 ml with distilled water was used for the assay. One milliliter of zinc sulfate solution was added to each sample, and the contents were thoroughly mixed on a vortex mixer before the addition of 1 ml of barium hydroxide solution. After thorough mixing, samples were centrifuged at 1000×*g* for 6 min[1].

Two-milliliter aliquots of the clear supernate were transferred into 15-ml glass-stoppered centrifuge tubes, and 0.4 ml of pH 9.6 borate buffer was added to give the final pH of 9.2. Six milliliters of 25% isobutanol in ethylene dichloride was added, and the tubes were shaken on a reciprocating shaker[2] for a minimum of 10 min to facilitate methadone extraction into the organic phase. The samples were centrifuged for 6 min at 1000×*g*, and the aqueous layer was removed by aspiration and discarded.

Three milliliters, or any other suitable aliquot of the organic phase, was then transferred to a set of test tubes, and the contents were evaporated to dryness under an air jet. The air was passed sequentially through a column of water and concentrated sulfuric acid. The test tubes were kept in a water bath maintained at 50–55°. Paraformaldehyde reagent, 0.1 ml, was added to each sample, and the tubes were transferred to a boiling water bath for 15 min.

After the samples were removed from the bath, 7.1 ml of distilled water was added to adjust the normality of the solution to 0.5. The contents were mixed and left at room temperature. Thirty minutes later, the fluorescence was read on a spectrophotofluorometer[3] with the emission monochromator rotary slit at 5, the excitation wavelength at 275 nm, and the emission wavelength at 450 nm (uncorrected). Tissue blanks, using tissues of untreated animals, and a set of standards were run simultaneously with each set of unknowns.

[1] Model UV, International Equipment Co.
[2] Eberbach model S1103.
[3] Model SPF, American Instrument Co.

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Table I—R_f Values of *d,l*-Methadone (M), Metabolite I (M-I), and Metabolite II (M-II) under Various Experimental Conditions [a]

Experimental Condition	R_f Value		
	d,l-Methadone	Metabolite I	Metabolite II
M + M-I + M-II	0.58	0.27	0.96
Lung homogenate (untreated) + M + M-I + M-II	0.58	0.27	0.97
Lung homogenate (treated, M, 20 mg/kg sc after 1 hr)	0.52	Not detected	Not detected
Liver homogenate (untreated) + M + M-I + M-II	0.46	0.21	0.98
Liver homogenate (treated, M, 20 mg/kg sc after 1 hr)	0.43	0.17	0.99
Ref. 14	0.55	0.15	0.95

[a] Gelman instant TLC media and a solvent system of *tert*-amyl alcohol–*n*-butyl ether–water (14:7:1) according to Ref. 14.

RESULTS AND DISCUSSION

Several homogenization media were evaluated, and the most desirable results were obtained with glass-distilled water. The aqueous homogenate or plasma was deproteinized by three different methods. When deproteinization was done with 5% $ZnSO_4$ and 4.5% $Ba(OH)_2$, the blank fluorescence values were the lowest and the recovery the highest and most consistent.

Individual organic solvents as well as solvent mixtures were tested for their ability to extract methadone from aqueous solutions at various pH levels. At pH 9.0–10.0, over 90% of methadone was extracted from the aqueous phase into chloroform, ether, a mixture of 10% isopropanol in chloroform, or a mixture of 25% isobutanol in ethylene dichloride. In this investigation, a mixture of 25% isobutanol in ethylene dichloride was used. With 25% isobutanol in ethylene dichloride, *d,l*-methadone was nearly completely transferred from the aqueous into the organic phase, and an extraction period of 10 min was satisfactory.

By using the systematic multiple fractional extraction procedure (12), it was possible to demonstrate that at pH 9.6 up to 96% of methadone was extracted from the aqueous into the organic phase. Evaporation of the organic solvent by air could be achieved at 20–55°; however, it was best performed at 50–55°. At higher temperatures, inconsistent results were obtained. The recovery of *d,l*-methadone (0–50 μg) added to the aqueous phase just prior to the extraction was about 96%, while the recovery of methadone from tissue homogenate was between 75 and 80%.

The amount of paraformaldehyde reagent was not critical. The addition of 0.1 ml of 0.1% reagent was satisfactory for *d,l*-methadone concentrations ranging from 0.005 to 100 μg. Over the range of 0–50 μg of *d,l*-methadone/sample, the fluorescence intensity increased linearly with increasing concentration. The formation of the *d,l*-methadone fluorophore was complete after ~15 min (Fig. 1), and it was stable for 1 hr at room temperature. During this period, the fluorescence intensity decreased only by ~10%, and the decrease was of the same magnitude in both standard and unknown samples.

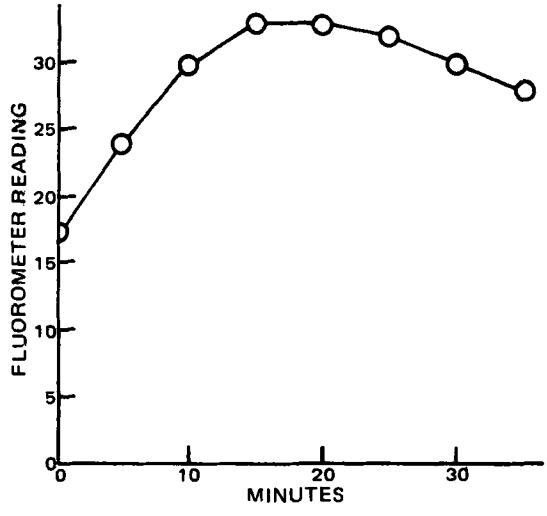

Figure 1—*Effect of heating time at 100° on the development of* d,l-*methadone fluorophore.*

Table II—Tissue *d,l*-Methadone Levels in Nontolerant Male Wistar Rats [a] after 20 mg/kg sc

Tissue	*d,l*-Methadone, μg/g of wet wt. (mean ± *SE*, *n* = 4)			
	0.5 hr	1 hr	2 hr	4 hr
Lung	54.2 ± 6.7	64.0 ± 6.7	52.6 ± 6.8	18.3 ± 4.1
Liver	25.1 ± 3.8	27.2 ± 4.5	20.0 ± 2.9	2.5 ± 0.2
Brain	4.1 ± 0.1	4.2 ± 0.2	3.0 ± 0.3	1.3 ± 0.3
Serum	1.3 ± 0.1	1.5 ± 0.04	0.9 ± 0.1	0.7 ± 0.1

[a] Average weight 200 g.

The last step, which consisted of diluting sulfuric acid with glass-distilled water up to 0.5 *N*, provided a way to measure *d,l*-methadone over concentrations from 0.005 to 50 μg/sample because the fluorescence output (arbitrary units per 100 ng of *d,l*-methadone) increased as the final sulfuric acid normality decreased. For example, at the final normality of 4.0, the fluorescence output was 0.8 unit/100 ng while at 0.5 *N* it was 83 units/100 ng.

The emission and excitation spectra of the "substance" extracted from the tissues of treated rats closely matched those obtained by using authentic *d,l*-methadone, indicating that the substance extracted was probably *d,l*-methadone.

Further confirmation of the identity was obtained by a method (13) that compares the distribution ratio patterns of a substance extracted at various pH levels from the biological samples to that of authentic chemical. If the distribution ratio patterns of the authentic and extracted substances are identical, then the two substances are considered to be identical. The distribution ratio pattern of "methadone" extracted from the tissues was compared to that of authentic *d,l*-methadone. In repeated experiments, the distribution ratios of authentic *d,l*-methadone and the material extracted from brain homogenate into 25% isobutanol in ethylene dichloride over pH 7.5–11.0 were almost identical (Fig. 2).

The third method used in the identification of the extracted substance was TLC as described previously (14). Comparison of pure methadone added to lung or liver homogenates and the substance extracted from treated tissues showed nearly identical R_f values (Table I). These data further support the claim that the substance assayed was *d,l*-methadone.

The two major methadone metabolites (2, 3) are 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (Metabolite I) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (Metabolite II). TLC studies indicated

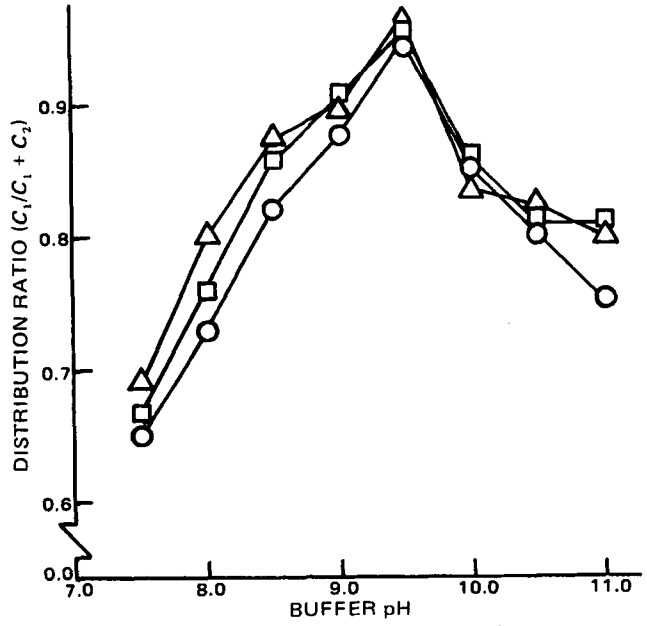

Figure 2—*Distribution ratios of 25 μg of* d,l-*methadone between borate buffer of various pH levels and 25% isobutanol in ethylene dichloride. Key:* ○, *tissue homogenate of treated animals;* △, d,l-*methadone added to tissue homogenate of untreated animals; and* □, d,l-*methadone from aqueous solution. Organic phase is* C_1, *and buffer phase is* C_2.

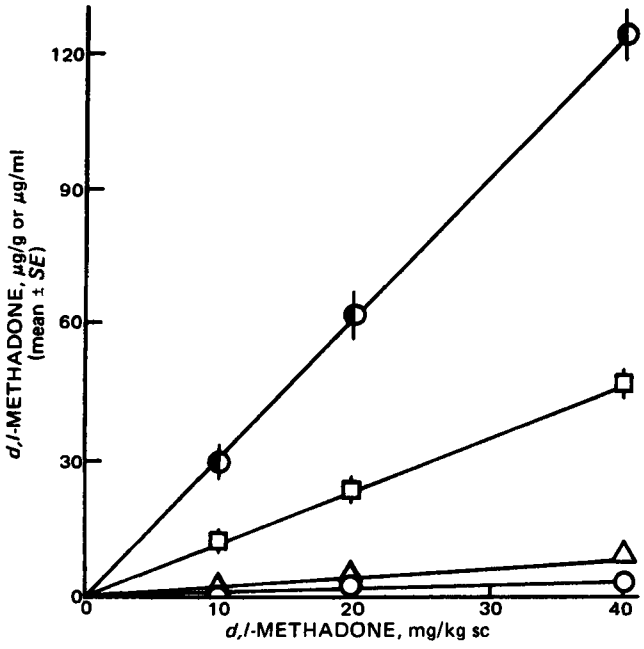

Figure 3—*Tissue* d,l-*methadone levels in the rat 60 min after subcutaneous administration of various* d,l-*methadone doses. Key:* ◐, *lung;* □, *liver;* △, *brain; and* ○, *serum.*

that these metabolites are extracted into the organic phase. However, they do not form fluorophores with paraformaldehyde. Also, morphine, diacetylmorphine (heroin), and codeine do not react with paraformaldehyde to form fluorophores. Cocaine forms a fluorophore but is not extracted from the aqueous phase (pH 9.2) under the conditions employed. Therefore, cocaine probably will not interfere in the methadone assay. Meperidine, amphetamine, and quinine form fluorophores with paraformaldehyde, and they are extracted into the organic phase. To remove the interference from these drugs, separation prior to their extraction into the organic phase is necessary.

Table II gives the levels of *d,l*-methadone in lung, liver, brain, and serum at various time intervals after 20 mg of *d,l*-methadone/kg was administered to rats subcutaneously. Figure 3 shows that the tissue levels were dose dependent.

REFERENCES

(1) V. P. Dole and M. E. Nyswander, *Br. J. Addict.*, **63**, 55 (1968).

(2) A. H. Beckett, M. Mitchard, and A. A. Shihab, *J. Pharm. Pharmacol.*, **23**, 347 (1971).

(3) A. Pohland, H. E. Boaz, and H. R. Sullivan, *J. Med. Chem.*, **14**, 194 (1971).

(4) A. L. Misra, S. J. Mule, R. Bloch, and N. L. Vadlamani, *J. Pharmacol. Exp. Ther.*, **185**, 287 (1973).

(5) A. E. Robinson and F. M. Williams, *J. Pharm. Pharmacol.*, **23**, 353 (1971).

(6) H. W. Elliott, F. N. H. Chang, I. A. Abdou, and H. H. Anderson, *J. Pharmacol. Exp. Ther.*, **95**, 494 (1949).

(7) J. C. Rickards, G. E. Boxer, and C. C. Smith, *ibid.*, **98**, 380 (1950).

(8) E. L. Way, C. Y. Sung, and W. P. McKelway, *ibid.*, **97**, 222 (1949).

(9) J. E. Wallace, H. E. Hamilton, J. T. Payte, and K. Blum, *J. Pharm. Sci.*, **61**, 1397 (1972).

(10) C. E. Inturrisi and K. Verebely, *J. Chromatogr.*, **65**, 361 (1972).

(11) E. J. McGonigle, *Anal. Chem.*, **43**, 966 (1971).

(12) M. T. Bush and P. M. Densen, *ibid.*, **20**, 121 (1948).

(13) B. B. Brodie and S. Udenfriend, *J. Biol. Chem.*, **158**, 705 (1945).

(14) A. L. Misra, N. L. Vadlamani, and S. J. Mule, *J. Chromatogr.*, **67**, 379 (1972).

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Bioavailability of Chlorothiazide Tablets in Humans

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Received January 8, 1979, from the *Division of Biopharmaceutics and Pharmacokinetics, Department of Pharmaceutics, College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.* Accepted for publication March 2, 1979. *Present address: Clinical Pharmacology Department, A. H. Robins Company, Richmond, VA 23220.

Abstract □ A urinary excretion bioavailability study was conducted in 12 healthy male subjects to evaluate three 250-mg and three 500-mg chlorothiazide tablet products. The study was a crossover design, and urine samples were collected 1, 2, 3, 4, 6, 8, 12, and 24 hr after administration of each dose. The resulting data were statistically analyzed for significant differences in cumulative percent of dose excreted at each sampling time, total drug recovery after 24 hr, maximum excretion rate, and time of maximum excretion rate. No statistically significant differences were found between the three 250-mg tablets tested. The urinary drug recovery after administration of one of the 500-mg products was significantly ($p < 0.05$) lower than that from the other two 500-mg tablets. The total mean recovery from each product ranged from only 11 to 20%, indicating that in general chlorothiazide was not well absorbed following oral administration. Attempts at correlating the urinary excretion data with the dissolution rate determinations were not successful.

Keyphrases □ Chlorothiazide—bioavailability, tablets, humans, urinary recovery □ Diuretics—chlorothiazide, tablets, bioavailability, humans □ Bioavailability—chlorothiazide tablets, humans

The Food and Drug Administration recently implemented a bioequivalence requirement for chlorothiazide tablets (1), and available information indicates that oral chlorothiazide dosage forms might exhibit bioavailability problems. The drug solubility is <1 mg/ml at pH ≤ 7 (2–4), and the usual oral dose is 0.5–1.0 g.

On the basis of urinary excretion studies, chlorothiazide appears to be incompletely absorbed from the GI tract of animals and humans (4–6). These studies reported 10–58% urinary recoveries of the oral dose administered to humans. The low urinary recovery of unchanged drug is not thought to be the result of other elimination routes since no metabolites have been identified and the urinary recovery of intravenously administered chlorothiazide approaches 100% (4, 6, 7).

In view of the potential for oral chlorothiazide dosage

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