Callahan and Kosicki (5) observed that the use of lipids extracted from the same source as that of the malate dehydrogenase increased the activity and stability of this enzyme. They concluded that this action was due to hydrophobic interactions between the lipid and the enzyme and that any agent capable of participating in this type of interaction should serve to stabilize malate dehydrogenase.

This effect was not observed with the phospholipids (egg lecithin and cephalin) used in this study. Instead, a decrease in the activity and stability of the enzyme was observed; at concentrations approaching normal serum levels of phospholipid, total inhibition of activity resulted. This suggests that nonspecific hydrophobic interactions may not be the principal factors involved in the stability and activity of this enzyme. The enzyme probably has very specific requirements that must be met for participation in such an interaction. These requirements would be expected to be met by lipids from the same organ as the enzyme. Furthermore, high concentrations of lipid appear to be inhibitory (5).

The effect of self-association of the phospholipids, which might occur at the higher concentrations used in this study, must also be considered. By reducing the availability of interacting sites, self-association could reduce the possibility of a hydrophobic interaction between the lipid and the enzyme. This could eliminate the stabilizing effect observed by Callahan and Kosicki (5). However, it would not be expected to decrease enzyme activity below that observed in the absence of the lipids, particularly at the lower lipid concentrations where self-association would be minimal. Therefore, while this effect could be responsible for some experimental observations, self-association of the lipids could not in itself explain the reduced enzyme activity observed in this study.

In addition, it was observed that the enzyme did not lose any activity during the reaction, even after 15 min. This can be seen from the linearity of the absorbance *versus* time plots over the full course of the reaction (Figs. 1–3). But when the enzyme was incubated in water alone for 15 min prior to initiation of the reaction, the activity of the enzyme was decreased by about 20%. Furthermore, when the enzyme was incubated for 15 min in the presence of oxalacetic acid prior to the initiation of the reaction, the activity decreased to the same extent as was observed when the enzyme was incubated in water alone. When the experiment was repeated using NADH in place of oxalacetic acid, the same results were obtained. Thus, neither oxalacetic acid nor NADH alone stabilizes the enzyme in water, and the presence of both the coenzyme and the substrate is necessary for the stabilization of malate dehydrogenase. Since these two components are always present in the body along with the enzyme and the reaction is a continuous process, this interaction may be an important factor in maintaining stability of malate dehydrogenase *in vivo*. Whether high concentrations of lipid act to inhibit enzyme activity *in vivo* cannot be ascertained from this study. However, the possibility of such an effect should receive further consideration.

## REFERENCES


(1) L. Rothfield and D. Romeo, in "Structure and Function of Biological Membranes," L. I. Rothfield, Ed., Academic, New York, N.Y., 1971, pp. 252, 253.

(2) I. Sekuzu, P. Jurtshuk, and D. E. Green, *J. Biol. Chem.*, **238**, 975(1963).

(3) B. Fleischer, A. Casu, and S. Fleischer, *Biochem. Biophys. Res. Commun.*, **24**, 189(1966).

(4) M. L. Das and F. L. Crane, *Biochemistry*, **3**, 696(1964).

(5) J. W. Callahan and G. W. Kosicki, *Can. J. Biochem.*, **45**, 839(1967).

(6) A. W. Adamson, "Physical Chemistry of Surfaces," 2nd ed., Interscience, New York, N.Y., 1967, p. 26.

(7) L. Siegel and S. Englard, *Biochem. Biophys. Res. Commun.*, **3**, 253(1960).


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# Improved Methods for Quantitative Determination of Methadone


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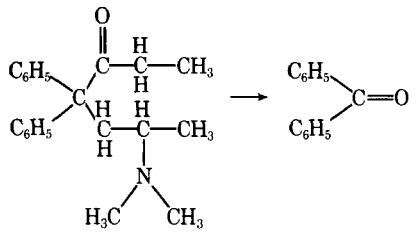
**Abstract** □ A spectrophotometric method is described that permits the rapid analysis of methadone in urine or tissues at concentrations corresponding to therapeutic, maintenance, or toxic doses of that drug. As little as 5 μg may be detected in a biological specimen. The procedure involves an alkaline extraction into *n*-hexane and subsequent back-extraction into a ceric sulfate–sulfuric acid solution. The acid extract is refluxed with *n*-heptane for 30 min, oxidizing methadone to benzophenone which, in contrast to the unchanged drug, has a high molar absorptivity in the UV region. A GLC method is also described. Both procedures require fewer manipulations and less analysis time than previously reported methods for determining methadone.



**Keyphrases** □ Methadone in urine and tissues—UV and GLC analyses □ UV spectrophotometry—analysis, methadone in urine and tissues □ GLC—analysis, methadone in urine and tissues


The low molar aborptivity of methadone renders spectrophotometric methods that determine unchanged methadone (1) unsatisfactory for analyzing the drug in biological specimens. Wallace *et al.* (2) recently developed a spectrophotometric method sufficiently sensitive to analyze therapeutic doses of methadone in urine and tissue specimens. The method is based upon the oxidation of methadone to benzophenone, a compound possessing a molar absorptivity approximately 34 times that of methadone.

This report describes an adaptation of the previously reported spectrophotometric method (2). The



*Scheme I—Chemical structure of methadone and benzophenone*

proposed method employs a different oxidant, requires fewer manipulative operations, and provides greater sensitivity in less time.

## EXPERIMENTAL[1]

**Reagents**—A 5.5 *M* sulfuric acid solution containing 25 mg of ceric sulfate[2]/ml was prepared by adding 76 ml concentrated sulfuric acid, very slowly and with constant stirring, to a large beaker containing 6.25 g ceric sulfate and 174 ml water. The solution is stable for 2 months at room temperature.

**UV Spectrophotometric Procedure**—Urine, 5–10 ml, or alkaline-digested (3) tissue, 5–10 g, was transferred to a separator, adjusted to pH 9–12, and extracted into spectroquality *n*-hexane in a 3-min manual extraction; the solvent volumes used in the extractions were 50 ml for urine and 200 ml for tissue specimens. The *n*-hexane layer was removed and filtered, and the recovered volume was recorded. Then 10 ml of 5.5 *M* sulfuric acid was added to the hexane, and this mixture was shaken for 3 min. Nine milliliters of the aqueous layer, 5 ml of spectrograde *n*-heptane, and 200–300 mg anhydrous ceric sulfate[2] were placed in a 250-ml round-bottom flask which was subsequently attached to a water-cooled reflux condenser. The mixture was refluxed for 30 min with constant magnetic stirring and a high reflux rate of approximately 200 drops/min. An alternative procedure was accomplished by extracting the hexane with 10 ml of a solution consisting of 25 mg ceric sulfate/ml of 5.5 *M* sulfuric acid. Nine milliliters of the aqueous acid extract was refluxed for 30 min with 5 ml heptane as already described.

After cooling, the heptane was read in the spectrophotometer from 215 to 360 nm against a similarly prepared *n*-heptane blank. Analysis at a single wavelength was achieved by determining the absorption at 247 nm. For a standard, 10 ml of an aqueous methadone solution containing 15 μg/ml was extracted and similarly determined. Alternatively, 9 ml of 5.5 *M* sulfuric acid containing 100 μg of methadone may simply be refluxed with heptane and ceric sulfate and the resulting absorbance may be adjusted for the previously determined mean recovery. The methadone concentration of an unknown is determined from the following equation:

$$\frac{\text{absorbance}_{\text{unknown}}}{\text{absorbance}_{\text{standard}}} \times \text{concentration of standard in heptane } (\mu\text{g/ml}) \times \frac{\text{ml hexane used in extraction}}{\text{ml hexane recovered}} \times \frac{\text{ml acid used for extraction}}{\text{ml acid used in reflux}} \times \frac{\text{ml heptane}}{\text{ml (g) specimen}} = \mu\text{g/ml} \quad \text{(Eq. 1)}$$

**GLC Procedure**—Five milliliters of urine and 1 ml of 0.5 *N* NaOH were mixed in a conical 15-ml test tube and centrifuged 10 min at 2100 rpm. Four milliliters of the supernate was transferred to a second tube, extracted with 0.25 ml of 5% isopropanol in chloroform, and again centrifuged 10 min at 2100 rpm; 5 μl of the solvent layer was injected into the chromatograph.

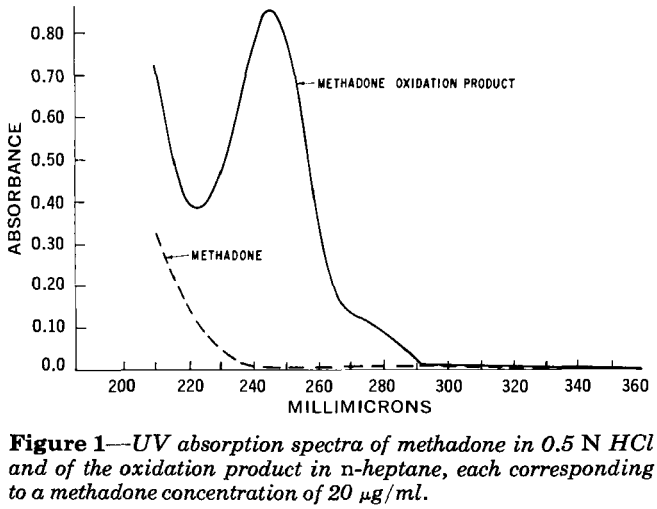



**Figure 1**—*UV absorption spectra of methadone in 0.5* N *HCl and of the oxidation product in* n*-heptane, each corresponding to a methadone concentration of 20 μg/ml.*

**Table I—Standard Curve of Methadone Oxidation Product**

| Methadone Concentration, μg/ml | Absorbance[a] | Absorbance Concentration, μg/ml |
|---|---|---|
| 5 | 0.282 | 0.056 |
| 10 | 0.565 | 0.056 |
| 15 | 0.816 | 0.054 |
| 20 | 1.112 | 0.056 |
| 30 | 1.662 | 0.055 |
| 40 | 2.221 | 0.056 |
| 60 | 3.173 | 0.053 |
| 80 | 4.180 | 0.052 |
| 120 | 6.132 | 0.051 |
| 200 | 10.129 | 0.051 |

[a] Absorbance, 247 nm, adjusted for blank and postreflux heptane dilution.

## RESULTS

The oxidation of methadone with ceric sulfate produced benzophenone (Scheme I). Both the methadone oxidation product and benzophenone exhibited identical UV and IR spectra, GLC retention times[3], and TLC $R_f$ values[4]. Oxidation to benzophenone results in a marked enhanced sensitivity (Fig. 1). The increase in molar absorptivity of benzophenone in *n*-heptane ($\epsilon$ = 18,713, $\lambda$ = 247 nm) over that of methadone in 0.1 *N* HCl ($\epsilon$ = 554, $\lambda$ = 292 nm) is approximately 34 times.

The absorption of the oxidation product adheres to the Beer-Lambert law as indicated in Table I. The data signify that the conversion of methadone to benzophenone is linear over the concentration range normally encountered in urinalysis. [In a clinical study[5] of a methadone maintenance program, 94% of the analyzed specimens (10 ml) resulted in a final heptane concentration of 4–40 μg/ml and 98.5% resulted in a concentration less than 46 μg/ml.] At concentrations less than 5 μg/ml, the wavelength of maximum absorption is displaced to slightly lower wavelengths.

[1] A Staco variable transformer was used in conjunction with a CRC Multi-lectric Outlet to deliver 60 v ac to each of six 270-w Glas-Col heating mantles positioned on magnetic stirrers. A Beckman Acta CIII ratio-recording spectrophotometer with 10-mm cells was used for UV absorption measurements. A Shimadzu 4B-M gas–liquid chromatograph, equipped with dual flame-ionization detectors and 2.0-m × 4-mm i.d. glass columns, was utilized for the chromatographic assays. The injector and detector temperatures were 260°. A column temperature of 200° and a nitrogen flow rate of 45 ml/min were used with 3% OV-1 and 3% OV-17 on Gas Chrom Q, 100–120 mesh.

[2] Fisher, purified grade.

[3] Retention time 7.85 min on a U-shaped 1.83-m (6-ft) column, 2.5% SE-30 on Gas Chrom Q, 100–120 mesh, at a nitrogen flow rate of 45 ml/min and a column temperature of 210° (Barber-Coleman model 5000 flame-ionization detector with injector and detector temperatures of 220 and 230°, respectively, and flow rates of hydrogen and air of 20 and 200 ml/min, respectively).

[4] On silica gel G, 250 μm: *(a)* $R_f$ of 0.94 in ethyl acetate–methanol–ammonia (30:10:60), and *(b)* $R_f$ of 0.81 in acetic acid–methanol–water (85:10:5). Spots were developed by Dragendorff spray followed by sodium nitrite.

[5] J. E. Wallace, H. E. Hamilton, and J. T. Payte, unpublished data.

**Table II**—Evaluation of Conditions for Reflux and Relative Yields (Adjusted to 1.00 for Suggested Conditions)

| Amount of Ceric Sulfate, mg | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 100 | | 200–300 | | 350 | | 400 | | 750 |
| 0.86 | | 1.00 | | 0.97 | | 0.89 | | 0.74 |
| **Concentration of Sulfuric Acid, *M*** | | | | | | | | |
| 4.7 | | 5.0 | | 5.5 | | 6.0 | | 7.0 |
| 0.97 | | 0.99 | | 1.00 | | 1.01 | | 0.95 |
| **Duration of Reflux, min** | | | | | | | | |
| 10 | 15 | 20 | 25 | 30 | 40 | 50 | 60 | 70 |
| 0.73 | 0.79 | 0.98 | 0.99 | 1.00 | 1.01 | 0.99 | 1.02 | 0.85 |
| **Heat of Reaction, 30-min Duration[a]** | | | | | | | | |
| 55° | 65° | 75° | 85° | 95° | Reflux 50 v | | Reflux 60 v | Reflux 70 v |
| 0.02 | 0.09 | 0.38 | 0.54 | 0.76 | 0.97 | | 1.00 | 0.88 |
| **Volume of Heptane, ml** | | | | | | | | |
| 5 | | | 10 | | | | 15 | |
| 0.99 | | | 1.00 | | | | 0.97 | |
| **Volume of 5.5 *M* Sulfuric Acid, ml** | | | | | | | | |
| 5–10 | | | 15 | | | | 20 | |
| 1.00 | | | 0.94 | | | | 0.84 | |

[a] Data for 55–95° obtained by immersion of flask in a heating bath. Reflux values obtained by applying the indicated voltage to heating mantles.

The extraction of methadone and its primary metabolite was effectively achieved with both *n*-hexane and 5% isopropanol in chloroform. When using GLC, it was observed that both methadone and its metabolite exhibited optimal and similar extraction characteristics over the 9–12 pH range.

The effect of variations of reflux conditions (heat and duration of reflux, volume of heptane and sulfuric acid, amount of ceric sulfate, and concentration of sulfuric acid) upon the oxidation of methadone to benzophenone were investigated (Table II).

**Evaluation of Premixed Ceric Sulfate–Sulfuric Acid Solution**—The separate addition of sulfuric acid and oxidant to the reflux flask was compared to the use of a previously prepared ceric sulfate–sulfuric acid solution which allows the direct extraction of methadone from the hexane. For the separate addition of reagents, the mean absorbance at 247 nm for a concentration of 20 μg methadone/ml heptane, adjusted for blank, was 1.089 ± 0.094 *SD*, and it was 1.052 ± 0.062 ($n$ = 21) for the premixed solution. Comparisons were also made by using a blank urine pool to which 1.5 mg% methadone was added. Analysis of the urine by the two systems afforded an absorbance at 247 nm in heptane, adjusted for blank, of 0.954 ± 0.028 ($n$ = 8) for the separate addition of reagents and of 0.928 ± 0.050 ($n$ = 9) for the premixed solution. The absorbance obtained with the premixed oxidant solution was approximately 97% of that obtained with the separate addition of reagents for both aqueous standard solutions and extracted urines. A solution of the premixed oxidant was prepared and stored at room temperature in a clear glass flask; no attempt was made to protect the solution from normal laboratory illumi-

**Table III**—Interference of Compounds with the Determination of Methadone

| Compound | Absorbance[a] of Ceric Sulfate | Absorbance of Compound / Absorbance of Methadone | Absorbance[a] of Barium Peroxide | Absorbance of Compound / Absorbance of Methadone | Absorbance of Ceric Sulfate / Absorbance of Barium Peroxide |
|---|---|---|---|---|---|
| Methadone | 0.92 | 1.00 | 0.88 | 1.00 | 1.0 |
| Amitriptyline | 1.50 | 1.63 | 0.23 | 0.26 | 6.5 |
| Piperidolate | 0.58 | 0.63 | 0.33 | 0.38 | 1.8 |
| Hydroxyzine | 0.46 | 0.50 | 0.53 | 0.60 | 0.9 |
| Azacyclonol | 0.36 | 0.39 | 0.34 | 0.39 | 1.1 |
| Propoxyphene | 0.30 | 0.32 | 0.34 | 0.39 | 0.9 |
| Antazoline | 0.25 | 0.27 | 0.41 | 0.47 | 0.6 |
| Pipradrol | 0.25 | 0.27 | 1.21 | 1.37 | 0.2 |
| Diphenhydramine | 0.24 | 0.26 | 0.51 | 0.58 | 0.5 |
| Dimenhydrinate | 0.21 | 0.24 | 0.27 | 0.31 | 0.8 |
| Diphenylpyraline | 0.08 | 0.09 | 0.47 | 0.53 | 0.2 |
| Mephentermine | 0.05 | 0.05 | 0.47 | 0.53 | 0.1 |
| Phenmetrazine | 0.04 | 0.04 | 0.48 | 0.55 | 0.1 |
| Chlormezanone | 0.04 | 0.04 | 0.01 | 0.01 | |
| Atropine | 0.02 | 0.02 | | | |
| Amphetamine | 0.01 | 0.01 | 0.02 | 0.02 | |

**Compounds Exhibiting ≤ 0.01 Absorbance[a]**

| | | | | |
|---|---|---|---|---|
| Atropine | Chlordiazepoxide | Ephedrine | Imipramine | Nicotine |
| Bisacodyl | Chloroquine | Ethchlorvynol | Meperidine | Pheniramine |
| Caffeine | Chlorpheniramine | Ethinamate | Meprobamate | Phenobarbital |
| Cocaine | Chlorpromazine | Glutethimide | Methylphenidate | Tetracycline |
| Codeine | Diazepam | Hydantoin | Morphine | Trimethobenzamide |
| | | | | Triprolidine |
| | | | | Quinine |

[a] Absorbance at 247 nm for 20 μg/ml, adjusted for blank.

**Table IV**—Recovery of Methadone after Addition to Urine, Spectrophotometric Method

| Amount Added, μg/ml | Absorbance[a], 247 nm | Absorbance/ Concentration | Recovery, μg/ml |
|---|---|---|---|
| 2.5 | 0.229 | 0.092 | 2.16 |
| 5 | 0.466 | 0.093 | 4.40 |
| 10 | 0.875 | 0.088 | 8.27 |
| 20 | 1.772 | 0.089 | 16.74 |
| 30 | 2.750 | 0.092 | 25.98 |
| | Average percent recovery | | 85.5 |

[a] Each value represents the average of six determinations, adjusted for blank.

nation. Stability of the solution was established through periodic determinations performed over 10 weeks. There was no loss of oxidative activity during the initial 4 weeks; activity decreased to 96% of the freshly prepared reagent by the 8th week and to 83% by the 10th week, indicating the oxidant to be relatively stable for 2 months at room temperature.

**Interfering Species**—Several pharmacological agents were tested for interference with the spectrophotometric determination of methadone. The drugs tested included both common drugs of abuse and those having a chemical structure related to benzophenone and acetophenone. Most compounds exhibited negligible absorbance at 247 nm (Table III); several were oxidized to benzophenone or acetophenone, however, and amitriptyline was oxidized to anthraquinone. Only alkaline drugs are listed in Table III, since other compounds are eliminated in the extraction procedure. The barium peroxide and ceric sulfate reagents produced markedly different quantitative results with certain compounds, particularly amitriptyline and pipradrol. Less interference from other drugs was characteristic of ceric sulfate oxidation.

**Urine Recoveries**—Standard aqueous solutions of methadone hydrochloride were added to urine to provide concentrations ranging from 0.25 to 3.0 mg %. In the spectrophotometric analysis, heptane layers of the 2.0- and 3.0-mg % specimens were diluted 1:1 and 1:2, respectively, with nonrefluxed heptane prior to measurement of absorbance. Urine blanks were similarly determined, including the heptane dilution step. They were not significantly different from reagent blanks, 0.034 ± 0.011 ($n$ = 21). The urine recovery data for the spectrophotometric analyses are shown in Table IV; urine recovery data for the GLC analyses are shown in Table V.

**Application to Tissue Analysis**—Methadone hydrochloride in saline solutions, adjusted to pH 7.2, was administered subcutaneously in the rear leg of adult male rats at a dose level of 20 mg/kg. One hour following administration, the animals were sacrificed by chloroform inhalation; liver tissues were immediately removed, weighed, and subjected to alkaline digestion according to the procedure of Rickards *et al.* (3).

Spectrophotometrically, tissue blanks were 0.060 ± 0.008 ($n$ = 5), slightly higher than aqueous and urine blanks. Methadone concentrations determined by both techniques gave levels in the liver of 7.2 ± 2.0 ($n$ = 6) μg/g wet tissue.

**Urine Analysis**—Urine specimens obtained from patients receiving treatment in a methadone maintenance program were extracted with hexane for spectrophotometric analysis. The extracts were divided into two aliquots, one being analyzed by barium peroxide oxidation and the other by ceric sulfate oxidation. The mean ($n$ = 10) methadone concentrations were 0.87 and 0.86 mg %, respectively. The difference in concentration determined by the two methods was ≤0.05 mg % for eight of the 10 specimens.

**Table V**—Recovery of Methadone after Addition to Urine, GLC Method

| Amount Added, μg/ml | Peak Height[a] | Recovery, μg/ml |
|---|---|---|
| 2.5 | 1.51 | 2.27 |
| 5 | 2.88 | 4.32 |
| 10 | 5.56 | 8.34 |
| 20 | 10.01 | 15.02 |
| | Average percent recovery | 83.9 |

[a] Chromatographic conditions as given in the *Experimental* section utilizing 3% OV-17. Peak height expressed in inches at $10^3$ MΩ, 0.64 v. Each value represents the average of three determinations.

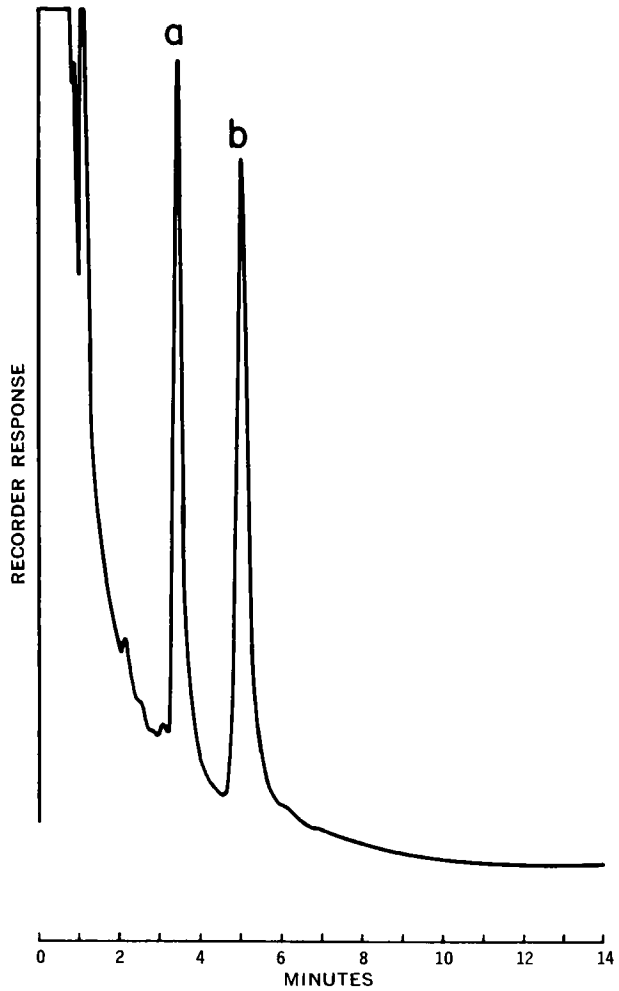



**Figure 2**—*Gas–liquid chromatogram of urine extract (3% OV-1 column). Key: a, 1,5-dimethyl-3,3-diphenyl-2-ethylidenepyrrolidine (methadone metabolite); and b, unchanged methadone.*

Additionally, urine specimens obtained from patients receiving methadone were divided into two aliquots and examined by the ceric sulfate spectrophotometric method and by GLC. A rapid and simple extraction procedure which did not require back-extraction, buffer washes, or solvent evaporation was utilized for the GLC study. This procedure provided well-defined chromatograms (Fig. 2) and excellent separation of the unchanged methadone and its primary metabolite, 1,5-dimethyl-3,3-diphenyl-2-ethylidenepyrrolidine (4, 5). Retention times for methadone and its metabolite were 4.9 and 3.4 min, respectively, with the OV-1 column (relative retention time of methadone/metabolite = 1.44) and 6.1 and 4.3 min, respectively, with the OV-17 column (relative retention time = 1.42).

Regression analysis of the data comparing the analysis of urine by the spectrophotometric method with the GLC technique provided a correlation of 0.6 if the combined peak areas of methadone and the metabolite from the chromatogram were used. This variation in the analysis of urine samples was the result of a lack of proper concentration of the metabolite for the standard.

**Application to Methadone Metabolites**—The ceric sulfate–sulfuric acid oxidation also converts the primary metabolite to benzophenone.

Methadone was extracted from urine and subjected to TLC separation according to the procedure of Wallace *et al.* (6). The positions corresponding to methadone and its major metabolite [$R_f$ 0.59 and 0.52, respectively, in $n$-butanol–acetic acid–water

(4:1:2)] were scraped off, eluted into *n*-hexane, back-extracted into the ceric sulfate–sulfuric acid solution, and examined by the oxidation–UV method. The resulting UV absorption spectra from both the unchanged methadone and the metabolite were identical to that of benzophenone. GLC examination of the heptane following reflux further substantiated that benzophenone was formed from both the parent drug and the metabolite.

## DISCUSSION

The extended linearity of the spectrophotometric method permits the use of a single aliquot of the specimen to encompass most urine methadone concentrations encountered in patients receiving methadone maintenance. Analysis by the barium peroxide method required varying specimen volumes since linearity was achieved up to a concentration of 25 μg/ml.

A comparison of the procedures and results of the ceric sulfate and barium peroxide methods indicates several advantages of the former in addition to the extended linearity and elimination of the required alkaline wash. Oxidation of methadone by the method of this report gives a 84.6 ± 3.2% yield of benzophenone as opposed to a 77.8 ± 3.3% yield obtained with the barium peroxide method. In addition to the time saved through the elimination of an extraction step, the proposed spectrophotometric method requires a shorter reaction time; *e.g.*, 20 min reflux with barium peroxide provided only a 48% yield of benzophenone compared to an 82% yield obtained with ceric sulfate. Additionally, reflux conditions were less critical since no significant change in percent yield was observed as the amount of primary oxidant was varied from 200 to 300 mg, the molarity of the sulfuric acid was varied from 5 to 6, or the duration of reflux was varied from 25 to 50 min. Similar variations in the barium peroxide method resulted in significantly decreased yields; changes in the amount of acid, solvent, and heat of reflux produced similar effects in both methods.

The proposed methods provide rapid and sensitive quantitative methods for determining methadone at therapeutic levels in biological specimens. Since other diphenyl-substituted drugs are susceptible to cerium sulfate, utilization of the GLC technique in conjunction with the spectrophotometric method provides a highly specific analytical methodology for methadone in biological specimens. The methods are particularly useful as a quantitative test in suspected methadone overdose cases and as a mechanism to monitor pharmacokinetic studies. In methadone maintenance programs the techniques can be used in collaboration with TLC to confirm on a relative basis that patients are receiving, within broad limits, proper dosages of the drug.

## REFERENCES


(1) S. J. Mule, *Anal. Chem.*, **36**, 1907(1964).

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

(3) J. C. Rickards, G. E. Boxer, and C. C. Smith, *J. Pharmacol. Exp. Ther.*, **98**, 380(1950).

(4) A. H. Beckett, J. F. Taylor, A. F. Casy, and M. M. A. Hassan, *J. Pharm. Pharmacol.*, **20**, 754(1968).

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

(6) J. E. Wallace, J. D. Biggs, J. H. Merritt, H. E. Hamilton, and K. Blum, *J. Chromatogr.*, **71**, 135(1972).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# Methionine Substitutes in Ruminant Nutrition I: Stability of Nitrogenous Compounds Related to Methionine during *In Vitro* Incubation with Rumen Microorganisms


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**Abstract** □ Thirty-one nitrogenous compounds with possible methionine activity were included as potential nitrogen sources in media for *in vitro* incubation of rumen microorganisms. Compounds not supporting cellulose digestion were included in studies of ammonia release and specific analyses designed to confirm resistance of the compounds to microbial deamination. *N*-Acetyl-DL-methionine (IV) and DL-homocysteine thiolactone hydrochloride (XIV) were highly stable when added individually and were selected for more intensive study. When added in combination with urea, XIV was more stable than IV as indicated by cellulose digestion, ammonia release data, and specific GLC analysis for the compounds. Compound XIV was also tested in aqueous buffers at pH 6.8 and 2.3. Little destruction was observed within 24 hr at either pH. These results indicate that dietary XIV has good potential for escaping rumen destruction in a form suitable for subsequent conversion to methionine.



**Keyphrases** □ Methionine substitutes—ruminant nutrition, stability of 31 nitrogen compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nitrogen sources—methionine substitutes in ruminant nutrition, 31 compounds compared to methionine, *in vitro* incubation with rumen microorganisms □ Nutrition, ruminant—31 nitrogen compounds examined to circumvent rumen microbial amino acid deamination


Ruminant animals depend on mixtures of microbial and dietary proteins for their amino acid nutrition (1–5). Dietary proteins may vary greatly in both content and availability of amino acids, and limitations