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Sensitive Spectrophotometric Method for Determining Methadone in Biological Specimens

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Abstract □ The extensive use of methadone for the treatment of opiate addiction increases significantly the need for quantitative determination of methadone in urine and tissues. Existing spectrophotometric methods are limited in sensitivity by the low molar absorptivity of this compound (ϵ = 554 in 0.1 *N* HCl, λ = 292 nm.). Results demonstrate that a markedly enhanced sensitivity may be achieved by oxidizing the methadone to benzophenone (ϵ = 18,713 in *n*-heptane, λ = 247 nm.). Methadone is extracted into *n*-hexane at an alkaline pH and then back-extracted into dilute sulfuric acid. Refluxing the acid solution with barium peroxide and *n*-heptane oxidizes the methadone to benzophenone, which is immediately extracted into the heptane. The heptane layer is removed and washed, and the benzophenone is measured spectrophotometrically. The method is sufficiently sensitive to quantitate therapeutic levels of methadone in small volumes of urine.

Keyphrases □ Methadone in biological specimens—liquid–liquid extraction, barium peroxide–sulfuric acid oxidation, UV analysis □ Barium peroxide–sulfuric acid oxidation—analysis of methadone □ Benzophenone—UV analysis as methadone oxidation product □ UV spectrophotometry—analysis, methadone *via* oxidation to benzophenone

Methadone is an antitussive and analgesic agent (1, 2). In recent years, it has been used effectively as a treatment for narcotic abstinence syndromes (2–4). Several investigators claimed that the methadone maintenence program is the preferred treatment for heroin dependence and reported up to 75% success in achieving sustained rehabilitation (4).

It is apparent that opportunities for abuse of methadone will increase as methadone maintenance programs grow in popularity and number. Gardner (5) reported that the number of deaths resulting from methadone overdose has increased significantly since 1965, the year the maintenance programs were introduced (3). Baden (6), describing a number of deaths resulting from methadone abuse, observed that individuals have obtained large amounts of the drug by enrolling in several programs simultaneously. The increased use of methadone enhances the requirement for its quantitative determination in urine or tissue.

Spectrophotometric methods are available (7, 8), but sensitivity is less than satisfactory. GC and polarographic methods have been described (9–11); however, the degree of technical proficiency required to analyze methadone by these methods makes them unacceptable to many laboratories. This report describes a sensitive spectrophotometric method for determining methadone in biological specimens. Analysis is based upon oxidation of methadone to benzophenone, a compound that has a much greater molar absorptivity for UV radiations than does methadone.

EXPERIMENTAL[1]

Reagents—Methadone hydrochloride USP[2], in appropriate amounts, was dissolved in deionized water. Concentrated sulfuric acid[3] was diluted with water to obtain a 4.7 *M* solution. Barium

[1] A Staco variable autotransformer, Type 2PF1010, was used in conjunction with a CRC Multi-lectric Outlet, model 1035/72, to deliver, on the average, 57 v. to Glas-Col heating mantles. Water-cooled 400-mm. Allihn condensers were attached to 250-ml. flasks positioned in the heating mantles. Magnetic stirrers were positioned beneath the heating mantles to mix the reaction solution and to serve as a support for the entire reflux system. A Beckman DK-2A ratio-recording spectrophotometer with 10-mm. cells was utilized. (Any spectrophotometer capable of accurate measurements at 247 nm. is adequate.)
[2] Mallinckrodt Chemical Co.
[3] Baker analytical reagent.

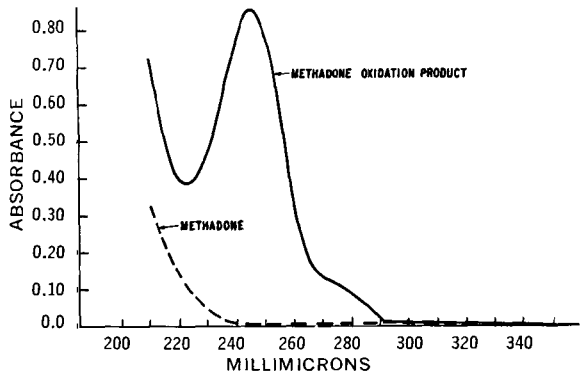


Figure 1—*UV absorption spectra of methadone, 20 mcg./ml. 0.1* N *HCl, and of the oxidation product in* n*-heptane derived from a sample of equivalent concentration.*

peroxide[4] was utilized as the primary oxidant. Thirty grams of anhydrous potassium hydroxide was dissolved in 100 ml. of water. *n*-Hexane and *n*-heptane[5] were utilized as extracting and refluxing solvents, respectively.

Preliminary Extraction of Bile and Urine—Five to twenty milliliters of urine (containing 20–150 mcg. methadone) was placed in a 250-ml. separator and adjusted to pH 9–12 by the dropwise addition of 5 *N* sodium hydroxide. Fifty milliliters of *n*-hexane was added, the mixture was shaken vigorously for 3 min., and the aqueous layer was discarded.

Preliminary Extraction of Tissues—Tissue specimens were alkaline digested to release protein-bound methadone (12). Ten grams of tissue was combined with 10 ml. of 30% potassium hydroxide solution. A flask containing the mixture was immersed in a boiling water bath for 10–30 min. or until complete disintegration of the tissue was obtained. The solution was cooled, transferred to a separator, adjusted to pH 9–12, and extracted into approximately 5 volumes of *n*-hexane.

General Extraction—Hexane from the preliminary extractions was filtered through filter paper[6] into a 100-ml. stoppered graduate cylinder. The hexane was extracted with 10 ml. of 4.7 *M* sulfuric acid. Nine milliliters of the aqueous layer along with 5 ml. of spectrograde *n*-heptane and 325–350 mg. barium peroxide was added to a 250-ml. round-bottom flask, which was subsequently attached to a water-cooled reflux condenser. The mixture was refluxed for 45 min. with constant magnetic stirring, utilizing a high reflux rate of approximately 200 drops/min.

After cooling, the heptane was extracted with an equal volume of 1.0 *N* sodium hydroxide. The heptane was read in the spectrophotometer at 215–360 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, 9 ml. of 4.7 *M* sulfuric acid containing 100 mcg. of methadone was carried through the reflux step. The methadone concentration of an unknown is determined from the following equation:

$$\frac{\text{OD unknown}}{\text{OD standard}} \times \text{Concentration of standard in heptane (2.0 mg. \%)} \times \frac{\text{ml. hexane used for extraction}}{\text{ml. hexane recovered}} \times \frac{\text{ml. 4.7 } M \text{ } H_2SO_4 \text{ used for extraction}}{\text{ml. 4.7 } M \text{ } H_2SO_4 \text{ used in reflux}} \times \frac{\text{ml. heptane}}{\text{ml.(g.) specimen}} = \text{mg. \%} \quad \text{(Eq. 1)}$$

where OD = optical density. Satisfactory analysis was performed with as little as 5 ml. of urine and 2 ml. of heptane.

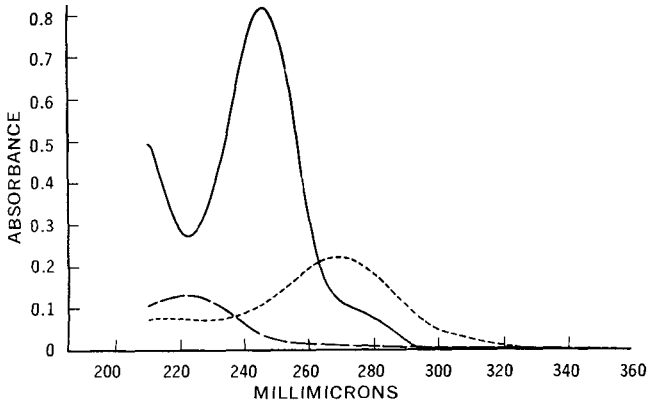


Figure 2—*UV absorption spectra of benzophenone* (——), *a typical urine blank* (– –), *and the barium peroxide–sulfuric acid reaction product* (- - -).

[4] Anhydrous powder reagent, Matheson Coleman and Bell.
[5] Spectroanalytical grade, Fisher Scientific.
[6] Schliecher & Schuell No. 589 or equivalent.

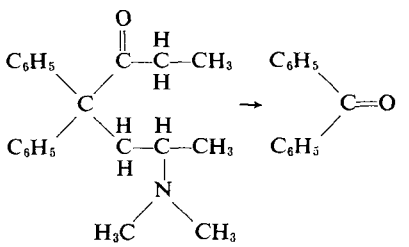
Table I—Standard Curve of Methadone Oxidation Product

Methadone Concentration, mcg./ml.	Oxidation Product Absorbance[a]	Absorbance/ Concentration
1	0.028	0.028
2	0.078	0.039
4	0.164	0.041
8	0.350	0.044
12	0.546	0.046
16	0.692	0.043
20	0.873	0.047
24	1.048	0.044

[a] Determined at 247 nm. in *n*-heptane.

RESULTS

Characteristics of Oxidation Product—The oxidation of methadone with barium peroxide produced benzophenone (Scheme I).



Scheme I—Oxidation of methadone to benzophenone by the barium peroxide system

This was established by the observation that identical UV and IR spectra were observed for benzophenone and the methadone oxidation product. The two compounds exhibited equivalent GC retention times and TLC R_f values. Oxidation to benzophenone results in a markedly enhanced sensitivity (Fig. 1). The increase in molar absorbance of benzophenone in *n*-heptane (ϵ = 18,713, λ = 247 nm.) over that of methadone in 0.1 *N* HCl (ϵ = 554, λ = 292 nm.) was approximately 34 times.The absorption of the oxidation product adheres to the Beer–Lambert law over a wide concentration range, as indicated by data in Table I. The data signify that the conversion of methadone to benzophenone is linear over the range of concentrations indicated. At concentrations greater than 25 mcg./ml., the oxidation is less than quantitative.

A second product is formed during the oxidation and must be removed by extracting the refluxed heptane with dilute alkali. It appears to be a barium peroxide–sulfuric acid product, which exhibits a relatively broad peak with maximum absorbance at 269 nm. (Fig. 4) and is very soluble in 0.1 *N* NaOH. A typical blank results in a UV maximum at 220–225 nm. with an absorbance of 0.03–0.04 at 247 nm. (Fig. 2).

Interfering Substances—Numerous drugs were investigated for interference with quantitative determination of methadone by the

Table II—Interference of Compounds[a] with the Barium Peroxide Oxidation Method for Determining Methadone

Compound	Absorbance[b]	Oxidation Product
Methadone	0.92	Benzophonone (B)
Pipradrol	1.25	B (chloro)
Hydroxyzine	0.57	B
Diphenhydramine	0.55	
Phenmetrazine	0.52	Acetophenone (A)
Diphenylpyraline	0.51	B
Mephentermine	0.51	A
Antazoline	0.45	A
Azacyclonol	0.38	B
Propoxyphene	0.38	A
Piperidolate	0.37	B
Amitriptyline	0.27	Unknown
Amphetamine	0.06	Nonspecific absorbance (NS)

Compounds Exhibiting ≤0.02 Absorbance[a]

Atropine	Chlorpromazine	Methylphenidate
Amobarbital	Ephedrine	Morphine
Bisacodyl	Ethchlorvynol	Nicotine
Caffeine	Ethinamate	Pheniramine
Cocaine	Glutethimide	Tetracycline
Codeine	Hydantoin	Trimethobenzamide
Chlordiazepoxide	Imipramine	Triprolidine
Chloroquine	Meperidine	Quinidine
Chlorpheniramine	Meprobamate	

[a] Each compound was extracted from urine and corresponds to a concentration of 20 mcg./ml. in *n*-heptane. [b] Absorbance at 247 nm.

method described in this report. Most of the compounds exhibited negligible absorbance at 247 nm., although several were oxidized to benzophenone or acetophenone (Table II). Amitriptyline exhibited significant absorbance but was oxidized to the unidentified product that is obtained after alkaline permanganate oxidation (13). Certain acidic compounds are oxidized by the barium peroxide–sulfuric acid system to UV absorbing species, but they afford no interference since they are eliminated in the initial extraction step.

Evaluation of Conditions for Reflux—The extraction efficiency was optimal over the 9–12.5 pH interval, decreasing slightly from pH 9 to 7 and falling off more sharply thereafter (<10% optimum recovery at pH 4). In strongly alkaline media (pH>13), methadone is reversibly converted to a water-soluble form and extraction recoveries are negligible. Emulsions are avoided if at least two volumes of hexane per one volume of urine are utilized.

A 3-min. extraction of 10 ml. urine with 50 ml. hexane results in a mean methadone recovery of 85%, as opposed to 81% with 25 ml. of hexane. The combination of two successive extractions provides a 95% recovery of methadone. The efficiency of the method to recover known amounts of methadone added to urine is summarized in Table III. Standard aqueous solutions of methadone hydrochloride were added to urine to provide concentrations ranging from 0.10 to 2.50 mg.%. The volume of urine extracted was 5 ml. for specimens containing greater than 1.5 mg.%, 10 ml. for those containing 0.5–1.5 mg.%, and 20 ml. for specimens containing less than 0.5 mg.%.

Table III—Recovery of Methadone after *In Vitro* Addition to Urine

Amount Added, mcg./ml.	Recovery, Mean ± *SD*[a], mcg./ml.
1.0	0.86 ± 0.02
2.5	2.09 ± 0.02
5.0	4.25 ± 0.03
7.5	6.39 ± 0.06
10.0	8.45 ± 0.07
15.0	13.40 ± 0.07
20.0	16.91 ± 0.20
25.0	21.20 ± 0.20
Average percent recovery	85.5
20.0	18.98 ± 0.70 (double extraction)[b]
Average percent recovery	94.9

[a] Each value represents the average of five determinations. [b] Aqueous specimen was extracted twice with *n*-hexane.

Table IV—Evaluation of Conditions for Reflux: Relative Yields, Adjusted to 1.00 for Optimum Conditions

Amount of Barium Peroxide, mg.							
300	325–350[a]		375	400		600	800
0.93	1.00		0.94	0.89		0.85	0.79
Concentration of Sulfuric Acid, *M*							
4.0	4.4	4.6	4.7	4.8	5.0	7.0	9.0
0.82	0.88	0.90	1.00	0.90	0.88	0.83	0.18
Duration of Reflux, min.							
20	20	40	45	50	60	80	100
0.62	0.86	0.90	1.00	0.98	0.96	0.95	0.87
Voltage Setting, Heating Mantle Rheostat, v.							
40		50		60	70		80
0.88		0.94		1.00	0.99		0.96
Volume of Heptane, ml.							
2.5		5		10	20		30
0.92		0.98		1.00	0.99		0.75
Volume of 4.7 *M* H_2SO_4, ml. (1.00 for the Volume of 10 ml., Arbitrarily)							
2.5		5		10	15		20
0.95		1.04		1.00	0.96		0.93

[a] Mean of data for 325, 333, 337.5, 341, and 350 mg.

Hexane, heptane, and octane were examined for use as possible reflux solvents. No benzophenone was detected in octane, and the amount of benzophenone in hexane was approximately 20% of that obtained with heptane.

Production of benzophenone was maximal with 325–350 mg. of barium peroxide and a 4.7 *M* sulfuric acid concentration. Optimum conditions for the oxidation of methadone are presented in Table IV. Oxidation of methadone by the method gives a 78% yield of benzophenone.

***In Vivo* Urine Studies**—Urine specimens were obtained from adult male patients receiving treatment in a methadone maintenance program. The daily oral dosages of methadone ranged from 50 to 110 mg., with a mean dosage of 78 mg./patient. Urine was collected for 24 hr. following medication, and the mean methadone concentration was 1.56 mg.% during the initial 12 hr. and 0.75 mg.% during the second 12 hr. The total amount of unchanged methadone recovered during the 24-hr. period after ingestion varied from 8.1 to 17.8%, with a mean recovery of 11.6%. Beckett *et al.* (11) and Steinberg (14), following oral administration of methadone to human subjects and utilizing a GC technique, recovered 1–20% of unchanged methadone with a mean of 9.6%. The low recoveries reported by Beckett *et al.* (11) and confirmed by our studies reflect that there is extensive *in vivo* metabolism of methadone and that methadone is eliminated by more than one route.

Tissue Studies—Tissue specimens (liver, lungs, kidneys, stomach, and intestines of adult male rats) containing no methadone gave a mean blank absorbance of 0.035 ± 0.008 (17 determinations) as compared to a mean blank absorbance for urine specimens of 0.037 ± 0.009 (nine determinations). The mean recovery of methadone after addition to intact liver, stomach, and intestines was 78.7 ± 1.3% (10 determinations). Methadone hydrochloride in physiological salt solution (0.9%) was administered subcutaneously in the rear leg of adult male rats at a dose level of 20 mg./kg. For one group of rats, the pH of the methadone solution was not adjusted (pH 4.6) and only the liver was examined. For a second group, the pH of the injected solution was adjusted to 7.2 and liver, lung, and kidney tissues were examined. After 1 hr. the animals were

Table V—Distribution of Methadone[a] in the Rat

Tissue	Methadone, mcg./g. (Dry Weight) Range of Values	Mean ± *SD*
Liver	33.6–49.6	41.7 ± 8.0
Lung	250.8–364.5	301.2 ± 48.1
Kidney	68.2–87.3	77.0 ± 9.6

[a] Methadone was administered at a dose of 20 mg./kg., and the animals were sacrificed after 1 hr.

sacrificed and the tissues were immediately removed, weighed, and subjected to alkaline digestion according to the procedure of Rickards *et al.* (12). The level of methadone in the liver of rats injected with its physiological salt solution of methadone at pH 4.6 was 13.2 ± 6.6 mcg./g. of liver (dry weight), whereas the liver concentration of the drug in animals injected with the near neutral solution of methadone was 41.7 ± 8.0 mcg./g. (dry weight). Methadone levels in several tissues of the rat are shown in Table V.

DISCUSSION

Barium peroxide, other than being employed as a catalyst, has received little interest in the field of oxidative organic chemistry; Baldeon (15) used barium peroxide and sulfuric acid as the basis of a semiqualitative colorimetric test for differentiating between phenols, amines, esters, and organic acids. Preliminary studies in this laboratory indicate that the barium peroxide–sulfuric acid oxidizing system is a highly suitable oxidant for substituted diphenyls. Alkaline permanganate oxidation of methadone resulted in less than 30% of the yield obtained with barium peroxide–sulfuric acid oxidation.

The extraction efficiency of *n*-hexane for methadone compares favorably with other solvents. Several existing methods (8) use ethyl ether or chloroform, but three to five extractions are required followed by concentration of the combined extracts through solvent evaporation. Mule (7) reported a 92% recovery of methadone using 25% isobutanol in ethylene dichloride, but the procedure required extensive (45 min.) mechanical agitation.

The *in vitro* tissue recovery is sufficiently reproducible to permit the use of a correction factor to adjust results for incomplete tissue recovery. The elevated tissue absorption and distribution experienced at pH 7.2 as compared to injection of methadone at pH 4.6 agree with the observations of Way and Adler (16).

With the possible exception of propoxyphene and several antihistamines, most drugs capable of interfering with methadone quantitation may be rarely encountered in the methadone user. TLC screening can be used to preclude or identify the presence of interfering substances. By one TLC technique, *in vivo* methadone specimens result in a characteristic TLC pattern consisting of two similarly colored spots[7]. The two compounds correspond to unchanged methadone and its cyclic metabolite (11). The metabolite is soluble in hexane; therefore, it is determined by the proposed method.

The procedure is applicable to tissue as well as to urine analysis; however, low circulating levels do not permit an accurate assay of therapeutic levels of methadone in blood.

[7] Unpublished data from this laboratory.

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The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care," published by the National Academy of Sciences–National Research Council.

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