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A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF METHADONE IN HUMAN PLASMA AND URINE.

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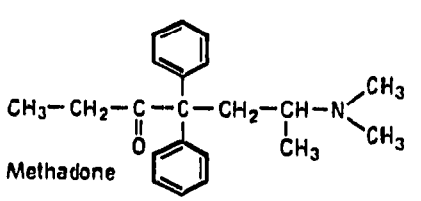
SUMMARY

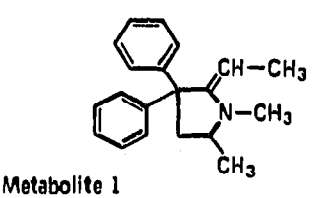
A method employing solvent extraction and gas–liquid chromatography is described which can detect as little as 0.015 μg of methadone per ml of human plasma. The method has been used to determine the concentration of methadone in plasma samples from normal volunteers given a single dose of methadone, from cancer patients receiving multiple doses for pathological pain and from former heroin addicts in a methadone treatment program. The concentration of methadone and its two metabolites in the urine of normal volunteers has also been determined.

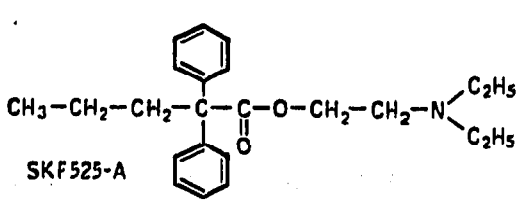
Following the intramuscular administration of 10 mg of methadone to three normal volunteers the drug disappeared from plasma with an average biologic half-life of 7.3 h.

INTRODUCTION

Methadone is a synthetic analgesic that is widely used in medicine for the relief of pain. The use of methadone in the stabilization treatment of narcotic addicts by DOLE AND NYSWANDER[1] has stimulated renewed interest in the pharmacodynamics of methadone in man. At present there is no information in the literature on the plasma concentrations in man after single or multiple doses of methadone. In human urine methadone and two biotransformation products have been identified (Fig. 1).







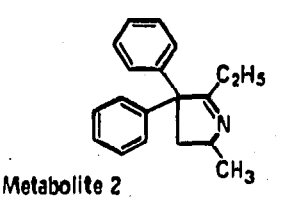


Fig. 1. Structural formulae of methadone, metabolites 1 and 2 and SKF 525-A, the internal standard.

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In 1968 BECKETT *et al.*[2] reported that 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (metabolite 1) is a metabolite of methadone in man. POHLAND *et al.*[3] recently identified and quantitated the levels of methadone and metabolite 1 in the urine collected for 24 h after administration of methadone to a human volunteer. They also reported a trace amount of 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (metabolite 2) in urine. Metabolites 1 and 2 are the result of mono- and di-N-demethylation, respectively, of methadone.

The purpose of this report is to describe a specific and sensitive gas chromatographic (GC) method for the measurement of methadone and its metabolites in biofluids and to demonstrate the results of the application of this method to samples of plasma and urine from individuals receiving methadone under experimental and therapeutic conditions.

MATERIALS AND METHODS

Chemicals and reagents

The *d* and *l* isomers of methadone were provided by Dr. J. M. MCGUIRE of the Lilly Research Laboratories (Indianapolis, Ind.). The metabolites of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine as the perchlorate salt (metabolite 1) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline·HCl (metabolite 2), were generously supplied by Dr. H. R. SULLIVAN of the Lilly Research Laboratories. The Internal standard for GC measurements, β-diethylaminoethyldiphenylpropylacetate·HCl (SKF 525-A) was a gift of Smith, Kline and French Laboratories (Philadelphia, Pa.). Methadone was administered as the *dl* racemic mixture (Dolophine®, Lilly).

The chloroform and *n*-hexane are spectral grade, all other solvents are reagent grade. The octyl alcohol is washed twice with 6 *N* HCl.

Stock solutions

Aqueous solutions of *l*-methadone, metabolites 1 and 2, each at a concentration of 4 μg/ml and SKF 525-A at a concentration of 20 μg/ml are prepared and kept refrigerated. The *d* and *l* isomers of methadone have identical properties in both the extraction and GC systems and therefore a stock solution can be prepared from either isomer or the racemic mixture.

Sample preparation from plasma

This method for the extraction of methadone from plasma has been adapted and modified from a procedure described by WOLEN AND GRUBER[4] for the extraction from plasma of propoxyphene, a close structural analogue of methadone.

Into a siliconized centrifuge tube with a Teflon-lined screw cap, add 4 ml of plasma, 0.05 ml of the solution of the internal standard, SKF 525-A, 0.5 ml of DELORY AND KINGS' carbonate–bicarbonate buffer[5], 1 *M*, pH 9.8 (D/K buffer) and one drop of octyl alcohol. The sample is extracted with 5 ml of *n*-butyl chloride by shaking for 5 min in an automatic shaker and centrifuged for 5 min at 1500 r.p.m. The *n*-butyl chloride phase (upper) is carefully removed to avoid disturbing the interface and the sample is extracted with a second 5 ml of *n*-butyl chloride. The two *n*-butyl chloride phases are combined and extracted with 5 ml of 0.2 *N* HCl by shaking for 7 min. After 3-min centrifugation the *n*-butyl chloride (upper) phase is aspirated and dis-

carded. The acid phase is washed by shaking for 4 min with 5 ml of *n*-hexane followed by centrjfugation for 3 min. The *n*-hexane wash (upper) phase is discarded. The washed acid phase is made alkaline by the addition of three drops 60 % NaOH (pH should be at least 11 by indicator paper) and extracted by shaking with 8 ml of chloroform for 5 min. After centrifugation for 3 min the aqueous (upper) phase is aspirated and discarded. The chloroform is transferred to a 12-ml siliconized centrifuge tube and evaporated to dryness in a multiple flash evaporator with the bath at 50° (Evap-O-Mix, Buchler Corp., Fort Lee, N.J.). The sides of the tube are rinsed with 1 ml of chloroform and the sample taken to dryness. The sample is collected in the lower tip of the tube by rinsing with 0.15 ml of chloroform followed by evaporation to dryness. The sample is dissolved in 20 μl of chloroform and an appropriate volume up to 4 μl is injected into the gas chromatograph.

Sample preparation from urine

Methadone and metabolites may be recovered by the direct extraction of alkalized urine.

To 0.5–2.0 ml of urine add 0.25 ml of the solution of the internal standard, SKF 525-A, plus one drop of 60 % NaOH and 8 ml of chloroform. The extraction, rinsing with chloroform and collection of the sample are as described in the plasma method. The final sample is dissolved in 40 μl of chloroform and 2 μl are injected into the gas chromatograph.

Gas–liquid chromatography

The GC analysis is performed on a Varian Aerograph Model 1740 equipped with a hydrogen flame ionization detector. The column is a 6 ft. long glass spiral with a 2 mm I.D. The packing consisted of 3 % SE-30 on Gas-Chrom Q 80–100 mesh. The temperature of the detector and flash heater is 230°. The carrier gas is helium at a flow rate of 32 ml/min. Hydrogen and air flow are adjusted to give maximal detector response. Hydrogen flow was between 32 and 40 ml/min and air between 200 and 250 ml/min. Detector sensitivity is 4×10^{-11} A/mV at full scale.

The column oven temperature is 200° for plasma samples and 180° tor urine samples.

Quantitation and calibration curves

Quantitation is effected by drawing the base line and measuring the peak height from the mid-point of the base line to the apex of the peak. The ratio of the peak height of methadone or metabolite to that of the internal standard, SKF 525-A, is calculated. A standard calibration curve is constructed by plotting the peak height ratio against concentration (μg/ml) or amount (μg added) of methadone or metabolite. Each calibration curve was constructed from duplicate determinations of at least five different points. Over the range of values indicated below for the standard calibration curves for methadone and metabolites a graphical representation of the data is linear.

For plasma a standard calibration curve is established by adding *l*-methadone in the range of 0.1 to 1.2 μg to 4 ml of blood bank plasma and proceeding as described above for plasma. The ordinate of the calibration curve is expressed as concentration (0.025 to 0.300 μg/ml of plasma) so that the concentration of an unknown sample can be read directly provided the plasma volume remains at 4 ml.

For urine, standard calibration curves are established by addition to control urine of: (1) *l*-methadone in the range of 0.25 to 3.0 μg; (2) metabolite 1 in the range of 0.2 to 1.2 μg; (3) metabolite 2 in the range of 0.1 to 0.6 μg, and proceeding as described above for urine. The urinary calibration curves may be determined simultaneously or separately for each compound.

RESULTS AND DISCUSSION

The conditions for the preparation of plasma and urine samples are adjusted to favor the maximal recovery of basic drugs so that methadone and any basic metabolites present can be recovered and determined in the same sample. This is accomplished by buffering the biofluids to pH 9.8 with D/K buffer. Plasma buffered in the alkaline range often forms an emulsion during shaking with a nearly equal volume of organic solvents such as *n*-butyl chloride, benzene or methylene chloride. Emulsion formation can be completely prevented without resorting to any increase in the volume of the organic solvent by the addition of one drop of octyl alcohol.

The internal standard, SKF 525-A, was selected because of the similarity of its structure to that of methadone (Fig. 1) and its GC purity as evidenced by a single peak separated from methadone and the metabolites (see Figs. 2 and 3). The mean recovery

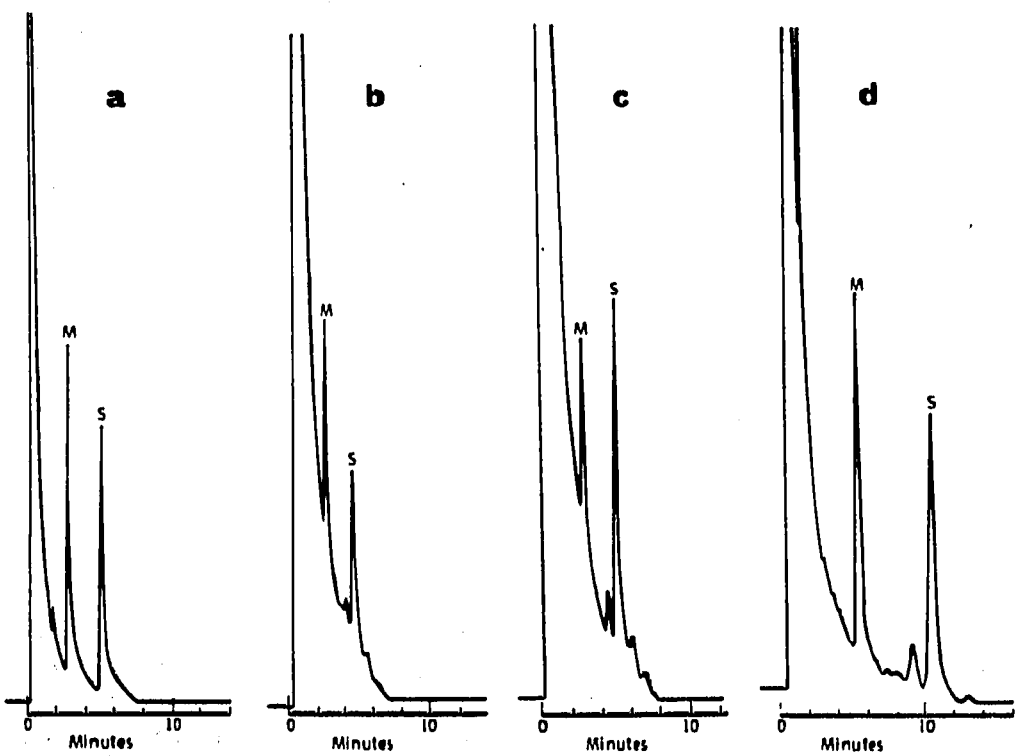

Fig. 2. Chromatograms of standards and human plasma extracts. (a) Standards dissolved in chloroform of M (methadone) at 0.05 μg and S (the internal standard, SKF 525-A) at 0.08 μg. Retention times: M, 2.8 min; S, 4.7 min. (b) Extract of plasma from volunteer B taken 2 h after the i.m. administration of 10 mg of methadone. Retention times as in (a). (c) Extract of plasma from cancer patient (AM) taken 1 h after the i.m. administration of 10 mg of methadone. Retention times as in (a). (d) Extract of plasma from a patient (RT) in a methadone-treatment program taken 3 h after the p.o. administration of 100 mg methadone. Retention times: M, 5.4 min; S, 10.9 min. For (a), (b) and (c), GC conditions as described in METHODS. The GC conditions for (d) are in RESULTS AND DISCUSSION.

of the internal standard added to 4 ml of plasma was 69.8 % ± 4.1 S.D. for ten determinations. The recovery of SKF 525-A was independent of the presence of methadone.

Table I contains the results of a determination of the recovery of known concentrations of methadone added to 4 ml of plasma. The amount recovered as measured by GC was independent of the methadone concentration in the range tested from 0.2 to 1.2 μg with a mean recovery of 96.7 % ± 3.3 S.D. These results were confirmed using

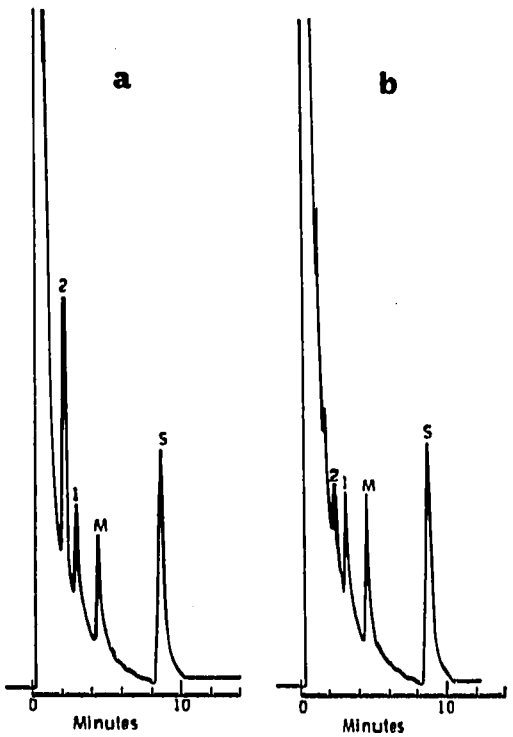

Fig. 3. Chromatograms of human urine extracts. (a) Standards of M (methadone), S (the internal standard, SKF 525-A) and metabolites 1 and 2 (1, 2) recovered from urine. Retention times: M, 4.7 min; S, 8.8 min; 1, 3.2 min; 2, 2.3 min. (b) Extract of urine from volunteer A collected during 8- to 12-h period after methadone. GC conditions as described in METHODS.

TABLE I

DETERMINATION OF METHADONE ADDED TO HUMAN PLASMA

Added (μg)	*Recovered* (μg)	*Recovery* (%)
0.200	0.189	94.4
0.200	0.187	93.8
0.400	0.368	92.1
0.400	0.400	100.0
0.600	0.590	98.3
0.600	0.554	92.3
0.800	0.800	100.0
0.800	0.788	98.5
1.200	1.172	97.6
1.200	1.296	100.8

Mean = 96.7 ± 3.3 S.D.

^{14}C-labeled methadone (*d*-6-dimethylamino-4,4-diphenyl-3-heptanone·HBr, 5.2 mCi/mmole labeled at carbon No. 2). The mean recovery of 1.0 μg of [^{14}C]methadone added to 4 ml of plasma was 94.9 % ± 1.5 S.D. for six determinations.

Examples of chromatograms obtained under the conditions described in METHODS are presented in Figs. 2 and 3. The multiple extraction procedure for plasma samples results in an extract that is free of any interfering peaks (see Figs. 2b and c). In most cases it was possible to introduce samples 12 to 15 min apart.

In Fig. 2d the GC conditions were altered in an attempt to determine whether the metabolites can be detected in plasma. The column oven temperature was reduced to 180° and gas flow adjusted to increase the retention time of methadone. Under these conditions the retention times of metabolites 1 and 2 are 4.0 and 2.6 min, respectively. In this sample and in several other plasma samples from individuals receiving large daily doses of methadone we were unable to detect any evidence of either metabolite (see Fig. 2d). Standards of the metabolites added to blood bank plasma can be recovered by the method.

Fig. 3b gives an example of the identification of methadone and metabolites 1 and 2 in a 1-ml aliquot of the urine sample collected during the 8- to 12-h period following the administration of methadone to volunteer A.

Fig. 4 represents the cumulative urinary excretion of methadone and metabolite 1 for the 96 h following 10 mg of methadone intramuscularly-(i.m.) (data for volunteer A). The most rapid rate of urinary base excretion occurred during the initial 24 h after methadone administration with 18.5 % of the total dose appearing in the urine as either methadone or metabolite 1. For this subject *ca.* 60 % of the total base excreted was as methadone and 40 % as metabolite 1 for each 24-h period during the four days of the study.

Additional data on the urinary excretion of methadone are presented in Table II. The concentrations of methadone and metabolites 1 and 2 were measured in an aliquot of the pooled urine collected for 96 h after the i.m. administration of 10 mg of methadone to three volunteers. *Ca.* 40 % of the total dose administered could be acccunted for as methadone and metabolite 1. A very small but measurable amount of metabo-

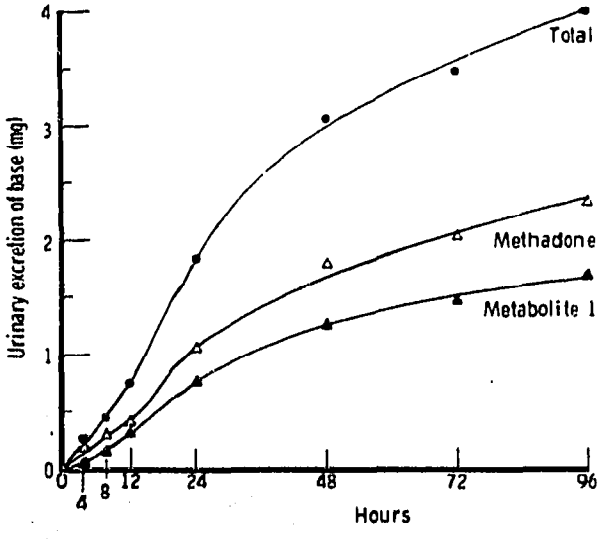

Fig. 4. Urinary excretion of methadone and metabolite 1 after the i.m. administration of 10 mg methadone to volunteer A.

TABLE II

TOTAL URINARY EXCRETION OF METHADONE AND METABOLITES IN THE 96 h FOLLOWING THE I.M. ADMINISTRATION OF 10 mg OF METHADONE

Subject	*mg of base*			
	Methadone	*Metabolite 1*	*Metabolite 2*	*Total base recovered*
Volunteer A	2.36	1.69	0.067	4.12
Volunteer B	3.22	0.69	trace	3.91
Volunteer C	3.14	0.70	0.089	3.93

lite 2 was present in the urine of A and C with only a trace present in B. We have also detected methadone and both metabolites 1 and 2 in urine samples from patients in a methadone-treatment program. Incubation of urine samples from volunteers or patients in a methadone-treatment program with the enzyme glucuronidase indicates that neither methadone nor metabolite 1 are present in urine as the glucuronide conjugate.

In comparing our preliminary data (Table II) on the urinary excretion of methadone and metabolite 1 with the limited data in the literature (see refs. 3 and 7) it is interesting to note that after i.m. administration we observe a methadone to metabolite ratio of 1.5 to 4.5 while POHLAND *et al.*[3] and BECKETT[7] report data on the urinary excretion following oral administration of methadone from which ratios that are much less than 1 can be calculated. BECKETT has demonstrated that the recovery of unchanged methadone varies greatly with the pH of urine, being favored by an acid urine. In our experiments the pH of the urine samples was not measured. However, in addition to any effects that urinary pH may have had on the ratio of methadone to metabolite 1, the preferential distribution of methadone to the enzymes of biotransformation in the liver following oral (p.o.) *versus* i.m. administration may also account in part for the predominance of metabolite 1 over methadone seen in the experiments of BECKETT[7].

The plasma levels of methadone in normal volunteers are presented in Fig. 5.

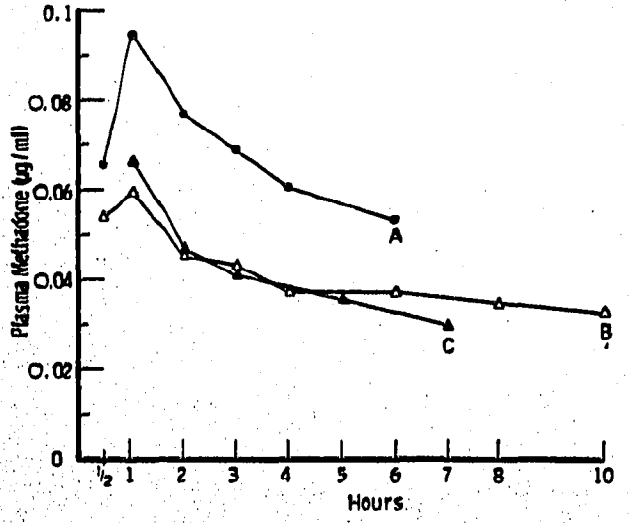

Fig. 5. Plasma levels of methadone in man. 10 mg of methadone were administered i.m. to the volunteers A, B and C.

The volunteers were healthy males not receiving any other drugs for a least two months prior to the experiment. The age and weight of each volunteer were: (A) 30 years and 84 kg, (B) 33 years and 77 kg, and (C) 28 years and 84 kg. Each volunteer received a single 10-mg dose of methadone i.m. and then 10-ml venous blood samples were withdrawn as indicated. The citrated blood was centrifuged and the plasma collected and extracted immediately or stored at −15°. Fig. 5 indicates that following the administration of methadone the peak plasma concentration occurred in the 1-h sample (curves A and C) and in each case the plasma methadone concentration declined steadily in subsequent samples. These results are of particular interest in view of the report of BEAVER *et al.*[6] that in man the analgesic effects of methadone (8 or 16 mg i.m.) peak at 1 h after drug administration and decline steadily thereafter. When the data in Fig. 5 are plotted semilogarithmically (not shown) the half-life for the elimination of the methadone from plasma can be calculated. These calculations yield very similar half-life values: for (A) 7.6 h, (B) 6.7 h, and for (C) 7.5 h. However, studies that include additional late hour samples are required to substantiate a simple first order elimination model.

Table III contains values of the plasma concentrations of methadone at various times after drug administration. These data are presented to indicate that this method can be used to quantitate methadone in the plasma of individuals in both experimental and therapeutic situations. The pre-administration samples were taken just prior to the administration of methadone. The cancer patients receive methadone i.m. every 4 to 6 h as required for the relief of pain. The methadone-stabilized patients had been maintained for several months on a single daily oral dose of methadone. The surgical patient and normal volunteers had not received methadone prior to the dose reported in Table III. The data for normal volunteers are taken from Fig. 5 and are included for comparison with the data from patients receiving multiple doses.

TABLE III

PLASMA CONCENTRATIONS OF METHADONE

Subject	*Dose (mg)*	*Route*[b]	*Plasma concentration (μg/ml)*			
			Pre-administration	*Hours after methadone*		
				1	*3*	*4*
Cancer patient (AM)	10	i.m.	0.457	0.668	—	0.621
Cancer patient (HJ)	15	i.m.	0.042	0.120	—	0.043
Surgical patient (DS)	10	i.m.	none	0.032	—	0.025
Methadone-stabilized[a] (RP)	80	p.o.	—	—	0.531	0.645
Methadone-stabilized (RT)	100	p.o.	—	—	0.513	1.030
Volunteer (A)	10	i.m.	—	0.095	0.069	0.061
Volunteer (B)	10	i.m.	—	0.060	0.043	0.038
Volunteer (C)	10	i.m.	—	0.067	0.042	—

[a] Methadone-stabilized = patient in a methadone-treatment program.
[b] i.m. = intramuscular; p.o. = oral.

The ability to measure the differences in the plasma concentration of methadone after single *versus* multiple doses should provide the means for a more complete understanding of the pharmacodynamics of methadone in man.

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