Quantitative Analysis of Methadone in Biological Fluids Using Deuterium-Labeled Methadone and GLC–Chemical-Ionization Mass Spectrometry

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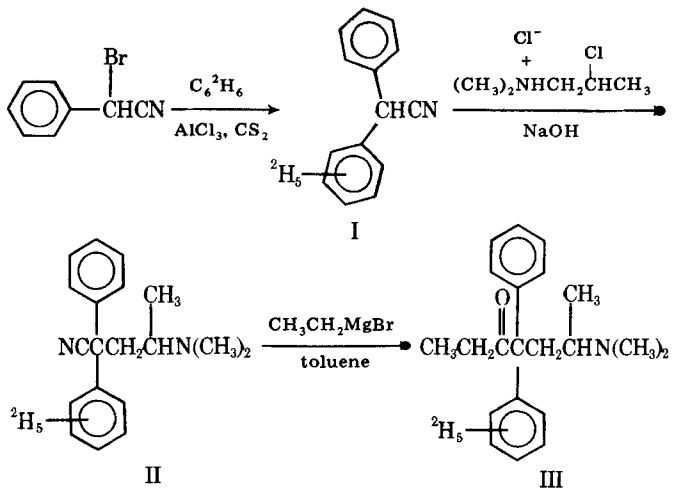
Abstract □ The (+)-, (−)-, and (±)-$^2H_5$-methadones, which contained five deuterium atoms in one aromatic ring, were synthesized for use in clinical pharmacological studies and as internal standards. GLC–chemical-ionization mass spectrometry was used to determine plasma and urinary methadone levels by an inverse isotope dilution assay. Plasma drug levels could be determined to 10 pmoles/ml, and urine levels could be measured to 5 pmoles/ml. Plasma methadone levels were examined in several patients undergoing methadone maintenance therapy. These levels generally ranged between 100 and 400 ng/ml (320–1300 pmoles/ml) after an average oral dose of 1 mg/kg/day. The methadone half-life was 28.8 ± 4.8 hr.

Keyphrases □ Methadone—GLC–chemical-ionization mass spectrometric analysis in plasma and urine, deuterium-labeled enantiomers and racemate synthesized □ GLC–chemical-ionization mass spectrometry—analysis, methadone in plasma and urine □ Deuterium-labeled compounds—(+)-, (−)-, and (±)-$^2H_5$-methadones synthesized □ Stereoisomers—(+)-, (−)-, and (±)-$^2H_5$-methadones synthesized □ Narcotic analgesics—methadone, GLC–chemical-ionization mass spectrometric analysis in plasma and urine, deuterium-labeled enantiomers and racemate synthesized

Low level quantitation of methadone in biological samples is most commonly done by GLC using the internal standard technique (1). This method, however, cannot detect quantities less than 10 ng/ml reliably and with high precision. Stable isotope-labeled compounds have recently proved useful as internal standards for quantitation of drugs and drug metabolites in biological fluids (2, 3). The technique, which involves adding a large excess of labeled compound directly to the biological sample, was first utilized to study the metabolism of nortriptyline (4). Recently, chemical-ionization mass spectrometry and deuterium-labeled morphine were used to monitor the possible abuse of narcotics by methadone maintenance patients (5).

1-$^2H_3$-Methadone and a methadone homolog were used to measure plasma levels and turnover rates of methadone simultaneously in human subjects (6). In this technique, ion intensities were measured by electron-ionization mass spectrometry, using the ion resulting from loss of a methyl group (M − 15). Quantitative data were obtained by comparing peak heights of methadone and 1-$^2H_3$-methadone to the internal standard, 2-dimethylamino-4,4-diphenyl-5-octanone. Since the chemical properties of deuterated drugs are virtually identical with those of the parent compound, this procedure overcomes problems caused by incomplete extraction and derivatization and also minimizes absorptive losses encountered on GLC. Chemical-ionization mass spectrometry offers a significant advantage in determining isotope ratios on compounds obtained from biological sources by improving the uniqueness of a particular ion and thus minimizing interferences from ions due to natural biological contaminants (7).

The present paper reports synthesis of deuterium-labeled methadone and its use in measuring picomolar quantities of methadone in biological fluids by GLC–chemical-ionization mass spectrometry. In addition, the synthesis of (+)-$^2H_5$-methadone and (−)-$^2H_5$-methadone also is described. These two compounds will be useful in studies of the pharmacological behavior of individual methadone enantiomers.



Scheme I

EXPERIMENTAL

Melting points were determined using standard techniques, and the uncorrected values are reported unless noted differently. Proton NMR spectra were obtained in deuterochloroform at 220 MHz[1]. The data are presented in parts per million (ppm) relative to tetramethylsilane. Optical rotations were measured using a polarimeter[2]. GLC analyses were carried out using a 1.83-m × 1-mm i.d. glass column packed with 10% W-98 on 80-100-mesh Gas Chrom Q[3].

Electron-impact mass spectra were run at a source temperature of 150° using an ionization potential of 30 ev[4]. Chemical-ionization mass spectra were obtained at a source pressure of 0.4 torr methane or isobutane and a source temperature of 150°[5]. Isotope ratios were measured using the stable isotope ratiometer–multiple-ion detection system described elsewhere (8).

Synthesis—2-Phenyl-2-($^2H_5$-phenyl)acetonitrile (I) was prepared by alkylation of $^2H_6$-benzene with α-bromophenylacetonitrile according to a procedure described by Schultz *et al.* (9) and suitably modified by using carbon disulfide as the solvent for the Friedel–Crafts reaction (Scheme I). Thus, from 50 g (0.595 mole) of $^2H_6$-benzene[6], 42 g (0.212 mole, 36% yield) of I was obtained as pale-yellow crystals. The product was pure by GLC analysis.

(±)-, (+)-, and (−)-4-Dimethylamino-2-phenyl-2-($^2H_5$-phenyl)-valeronitriles (II)—Compound I, 42.0 g (0.212 mole), and 47.5 g (0.300

[1] Model HR-220, Varian Analytical Instruments Division, Palo Alto, CA 94303.
[2] Model M4 QIII, Carl Zeiss, West Germany.
[3] Model 1440 gas chromatograph, Varian Aerograph, Walnut Creek, CA 94598.
[4] Model 270 mass spectrometer, Perkin-Elmer, Norwalk, CT 06852.
[5] Biospect quadrupole chemical-ionization mass spectrometer, Scientific Research Instruments, Baltimore, MD 21207.
[6] Containing 99 atom % ^2H, Aldrich, Milwaukee, WI 53233.

mole) of 1-dimethylamino-2-chloropropane hydrochloride were combined in 30 ml of water containing 24 g (0.0600 mole) of sodium hydroxide (10) (Scheme I). The reaction mixture was stirred at 88° for 6 hr and then cooled, and the product was isolated in ether. The ethereal solution was washed with 0.1 *N* NaOH, and the labeled product was extracted into 3 *N* HCl. The aqueous solution was washed twice with ether to remove neutral products.

The free base was obtained by adjusting the pH to 12 with sodium hydroxide, extraction into ether, and drying over magnesium sulfate. The ethereal solution was clarified with activated charcoal, filtered, and then evaporated to dryness. The oily product was crystallized three times from hexane to give 23.8 g (0.084 mole, 40% yield) of (±)-II. The racemic product was resolved into pure enantiomers by crystallization of the tartrate salt as described by Pohland *et al.* (11). For this reaction, 23.8 g (0.084 mole) of (±)-II and 6.65 g (0.045 mole) of (+)-tartaric acid were combined in acetone and refluxed. Sufficient water was added to dissolve the reagents. Crystallization was induced by evaporating the solvent in the cold and allowing the product to crystallize at −10° overnight. The (−)-II bitartrate salt was recrystallized once more from acetone.

The free base, obtained after neutralization with hydroxide, was crystallized twice from hexane. The yield was 8.02 g (0.028 mole, 67% yield) of white prisms, mp 97.5–99.0° [lit. (12) mp 99–101°]. The specific rotation of the product was $[\alpha]_D^{23.7}$ −50.9° (1-dm cell, 1.0205 g/ml) [lit. (11) $[\alpha]_D^{25}$ −50.0°]. The mother liquors obtained from the resolution were combined, and the free base [primarily the (+)-enantiomer] was recovered. Resolution of the (+)-enantiomer was attempted by treatment of the base with (−)-tartaric acid in the manner just described, but crystallization could not be induced. The free base was recovered and crystallized twice from hexane to give 11.6 g of (+)-II, mp 87.5–90.0° (corrected) [lit. (9) mp 97–100°].

The specific rotation was determined in ethanol, $[\alpha]_D^{24.0}$ +31.3° (1-dm cell, 1.087 g/ml) [lit. (11) $[\alpha]_D^{25}$ +50.0°]. This specific rotation represents an 81% enantiomeric purity. The electron-impact mass spectrum (30 ev) showed a base peak at *m/e* 72 [$M^+ - \cdot CH_2C(C_6H_5)(C_6{}^2H_5)CN$], a weak molecular ion at *m/e* 283 (M^+), and a characteristic ion at *m/e* 197 [$M^+ - \cdot CH_2CH(CH_2)N(CH_3)_2$]. The material was pure by GLC analysis.

(±), (+)-, and (−)-$^2H_5$-Methadones (III)—Compound (−)-II, 8.021 g (0.028 mole), was dissolved in 100 ml of anhydrous toluene and treated with ethylmagnesium bromide (0.060 mole) at reflux temperature under nitrogen. The intermediate imine was hydrolyzed with 3 *N* HCl at reflux temperature for 1 hr. Following isolation in ether, (−)-III was recrystallized twice from methanol to give 7.064 g (0.022 mole, 79%) of white prisms, mp 96.5–97.0° (corrected) [lit. (12) mp 98.7–99.0°]. The product was analyzed by GLC on a 1.83-m × 1-mm i.d. glass column packed with 3% W-98 on 80–100-mesh Gas Chrom Q at 190° and exhibited a single peak at 288 sec (the nitrile had a retention time of 232 sec under these conditions).

The electron-impact mass spectrum (30 ev) showed a base peak at *m/e* 72 [$M^+ - \cdot CH_2C(C_6H_5)(C_6{}^2H_5)COCH_2CH_3$], a weak molecular ion at *m/e* 314 (M^+, 0.4%), and several low intensity ions of diagnostic importance. These ions are *m/e* 172 [$M^+ - CH_2CHCO - CH_2CH(CH_3)N(CH_3)_2$], 228 [$M^+ - \cdot CH_2CH(CH_3)N(CH_3)_2$], and 299 [$M^+ - \cdot CH_3$]. The chemical-ionization mass spectrum (0.4 torr methane, source temperature 130°) exhibited an intense pseudomolecular ion at *m/e* 315 (MH^+), a base peak at *m/e* 72 [$MH^+ - CH_2C(C_6H_5)COCH_2CH_3$], and a minor ion at *m/e* 270 [$MH^+ - HN(CH_3)_2$].

The NMR spectrum (0.1 *M* in deuterochloroform) showed proton resonance signals at δ 0.47 (d, J = 6.5 Hz, C_7 methyl), 0.83 (t, J = 7 Hz, C_1 methyl), 2.10 (s, *N*-methyl protons), 2.32 (m, C_2 methylene and C_6 methine protons), and 7.15–7.40 (m, aromatic protons) ppm. The NMR spectrum was identical with the published spectrum (13). Integration of the aromatic proton signal indicated a 50% deuterium content. The isotope composition was determined by methane chemical-ionization mass spectrometry on a probe sample using the protonated molecular ion and contained 1.2% $^2H_0$, 1.6% $^2H_1$, 2.9% $^2H_2$, 8.9% $^2H_3$, 16.8% $^2H_4$, and 68.6% $^2H_5$ species. Since the long-term stability of methadone is poor, it was converted to the hydrochloride salt in ethanolic hydrochloric acid. The hydrochloride was recovered in 97% yield after two crystallizations from ethanol.

Compound (+)-III was prepared from 6.013 g (0.021 mole) of the corresponding nitrile [(±)-II] in 64% yield. The crystalline product, mp 95–96° (corrected) [lit. (12) mp 98.7–99.0°], was pure by GLC and exhibited mass spectra identical with those obtained for the (−)-enantiomer. The free base, 3.389 g (0.0107 mole), was converted to the hydrochloride salt in 98% yield. Racemic methadone, (±)-III, was obtained by combining equimolar amounts of (+)- and (−)-III.

Standard Solutions—Methadone hydrochloride and $^2H_5$-methadone hydrochloride were dissolved in water to give a nominal concentration of 1 mg/ml. In these studies, either the racemate or one enantiomer was used. Stock solutions of urine and plasma, obtained from healthy, drug-free adult donors, were made by serial dilution of a 1-ml aliquot of the aqueous methadone hydrochloride stock solution with urine or plasma. Concentrations of these solutions ranged from 0.3 pmole to 289 nmoles/ml of unlabeled methadone. An aqueous solution of $^2H_5$-methadone hydrochloride (3.074 nmoles/ml) was prepared for use in adding the labeled internal standard to the biological samples.

Table I—Linear Regression Analysis Data for Urine and Plasma Dilution Curves[a]

Curve	Slope ± *SD*	Intercept ± *SD*	*r*
Urine[b]	0.9986 ± 0.0091	0.2221 ± 0.0129	0.9999
Urine	0.9975 ± 0.0211	0.2364 ± 0.0302	0.9982
Plasma	1.0107 ± 0.0153	0.1793 ± 0.0221	0.9992
Plasma	1.0352 ± 0.0140	0.2040 ± 0.0200	0.9993

[a] Regression data were calculated using the log of the difference in the isotope ratio between dilution standards and blank values *versus* the log of the molar ratio of methadone present in the sample to the internal standard added to the sample. [b] Determined using 3.85 nmoles of internal standard. All other curves were prepared using 1.54 nmoles of internal standard.

Sample Preparation—Plasma or urine, 1 ml, was treated with the appropriate amount of internal standard and allowed to equilibrate at room temperature for 1.5 hr. The sample was adjusted to pH 9 with 2 ml of 0.5% ammonium hydroxide, and the solution was extracted with 5 ml of ethyl acetate. The aqueous layer was discarded, the organic phase was extracted a second time with 0.5% ammonium hydroxide, and the aqueous phase was discarded. Then the methadone was back-extracted into 2 ml of 0.05 *N* HCl, and the organic phase was discarded.

The aqueous phase was adjusted to pH 9 and extracted with 5 ml of ethyl acetate. The ethyl acetate extract was dried over sodium sulfate and evaporated under nitrogen. The samples were dissolved in 20 μl of ethyl acetate to give a nominal concentration of 25 ng of methadone/μl. A 5-μl aliquot was analyzed by GLC–chemical-ionization mass spectrometry.

Mass Spectrometric Analysis—Methadone samples were analyzed using isobutane chemical-ionization mass spectrometry (0.5 torr source pressure and 160° source temperature). Samples (about 50–100 ng each) were introduced through the GLC inlet system and were analyzed on a 1.8-m × 1-mm i.d. glass column packed with 10% W-98 on 80-100-mesh Gas Chrom Q at a temperature of 180° and a helium flow rate of 9.2 ml/min. Under these conditions, methadone had a retention time of 7.3 min and the pyrrolidine metabolite had a retention time of 4.3 min. Isotope ratios were measured using the stable isotope radiometer–multiple-ion detection system described by Klein *et al.* (8).

Standard Dilution Curves—Urine dilution curves were routinely prepared by treating 1 ml of urine stock solutions with 1.75–3.85 nmoles of $^2H_5$-methadone hydrochloride. Plasma dilution curves were prepared by treating 1 ml of plasma stock solutions with 1.75 nmoles of $^2H_5$-methadone hydrochloride. In all cases, a plasma or urine blank value also was determined.

Isotope ratios were obtained using isobutane chemical ionization and by monitoring the protonated molecular ions at *m/e* 310 and 315 for methadone and $^2H_5$-methadone, respectively. Dilution curves were constructed by plotting the log of the difference in the isotope ratio between the dilution standard and the plasma or urine blank against the log of the mole ratio of methadone to the internal standard.

RESULTS AND DISCUSSION

The most attractive position for labeling methadone with deuterium is on one of the aromatic rings. These positions are chemically inert and offer the advantage that several deuterium atoms may be introduced into the molecule from an inexpensive precursor ($^2H_6$-benzene). Although methadone and its pyrrolidine and pyrroline metabolites may be hydroxylated in the aromatic ring, this metabolic pathway is minor and does not interfere with the use of III for quantitative purposes.

To relate the observed isotope ratio to the mole composition of an isotopic mixture, standard isotope dilution curves were routinely prepared for both plasma and urine methadone standards by adding a known amount of internal standard (typically 2–4 nmoles) to each sample. The samples were allowed to stand at 4° for 1.5 hr to permit equilibration of protein-bound methadone with the internal standard. Samples were then extracted and analyzed. In both cases, plasma and urine blank values also were determined.

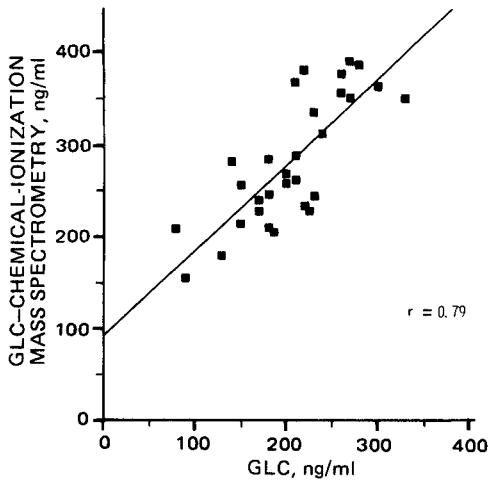


Figure 1—*Comparison of plasma methadone levels by GLC and by GLC–chemical-ionization mass spectrometry* (r = *0.79 and slope = 0.93*). *These data were obtained over 6 months from several individuals undergoing maintenance therapy.*

The protonated molecular ions observed at *m/e* 310 and 315 in the isobutane chemical-ionization mass spectra for methadone and $^2H_5$-methadone, respectively, were used to measure the isotope ratios. Standard isotope dilution curves were used to relate the observed integrated ion intensity ratio of methadone to $^2H_5$-methadone (I_{310}/I_{315}) present in a sample to the mole ratios of methadone to $^2H_5$-methadone (M_{2H_0}/M_{2H_5}) in that sample. The logarithm of the difference in the isotope ratio between the dilution sample and the plasma or urine blank (log *D*):

$$\log D = \log [(I_{310}/I_{315})_{\text{sample}} - (I_{310}/I_{315})_{\text{blank}}] \qquad \text{(Eq. 1)}$$

was plotted on the ordinate.

The logarithm of the mole ratio of methadone, M_{2H_0}, present in a sample to the amount of internal standard, M_{2H_5}, added to the sample (log *M*):

$$\log M = \log \left[\frac{M_{2H_0}}{M_{2H_5}}\right] \qquad \text{(Eq. 2)}$$

was plotted on the abscissa.

The technique of using the difference in isotope ratios between the standard and the unknown yields more reproducible data by minimizing small daily variations in mass spectrometer performance. Plasma and urine methadone isotope dilution curves were prepared for concentrations ranging from 0.3 pmole to 289 nmoles/ml. Both curves covered a useful linear range of 5 pmoles/ml to 289 nmoles/ml. These concentrations are typical of those encountered in samples obtained from patients receiving methadone maintenance therapy at doses of 40–100 mg/day. Occasionally, there was interference in the plasma methadone assay from a component in blood that obscured both the GLC peak and the ion at *m/e* 310. This contaminant was difficult to remove completely, and it interfered with the plasma assay below 10–15 pmoles/ml. Back-extraction of methadone into dilute hydrochloric acid, as described, partially eliminated this interference.

To preserve the accuracy of isotope ratio measurements throughout the calculations (the coefficient of variation is typically 5% at an isotope ratio of 100:1), the data were treated as logarithms. Slopes and intercepts were calculated using standard linear regression techniques (14). The linear regression data (Table I) were obtained over 6 months and show excellent long-term reproducibility. The slight difference observed in the slopes and intercepts between plasma and urine curves was not statistically significant. The correlation coefficients indicate a tight fit of the data to the regression line.

The GLC–chemical-ionization mass spectrometric method presented was used to determine urinary methadone levels in a sample obtained immediately *postpartum* from an infant whose mother was maintained on methadone during pregnancy. Two samples (500 and 100 μl) were analyzed. The samples contained 19.0 ± 1.1 and 19.6 ± 1.1 μg/ml (22.6 μg/ml by GLC), respectively, for the runs, indicating that the determination was independent of sample size.

Plasma methadone levels in several individuals receiving a daily oral maintenance dose (1 mg/kg) of methadone were examined by a GLC method (15) and by the described procedure. Figure 1 shows the correlation between plasma drug levels measured by these two methods. Linear regression analysis of these data gives a slope of 0.93 (r = 0.79 and n = 30) and an intercept of 90 ng/ml. These data suggest that the difference from zero of the intercept may be due either to overestimation by the GLC–chemical-ionization mass spectrometric procedure or to underestimation by the GLC procedure. In the former case, the presence of a background ion at *m/e* 310 would raise the estimated mole ratio and give inflated values for methadone present in the sample. Since this effect did not occur in those direct recovery studies conducted on aqueous, urinary, and plasma standards, this contaminant possibility is not the cause.

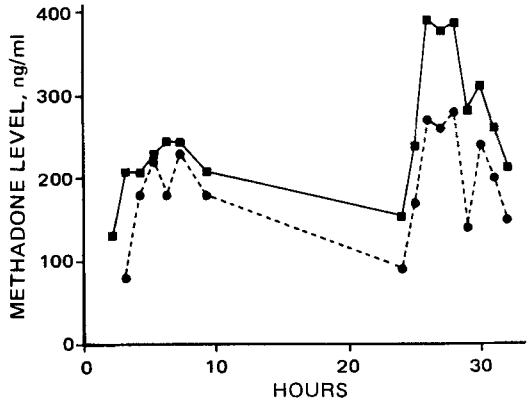


Figure 2—*Plasma methadone levels observed in an individual receiving 80 mg/day (1 mg/kg/day) of methadone. Levels were measured by GLC* (●) *and GLC–chemical-ionization mass spectrometry* (■).

Methadone is extensively bound to both albumin and globulin fractions of plasma proteins (16). Presumably, solvent extraction of a drug causes denaturation of plasma proteins and allows equilibration of free and bound methadone. However, using ethyl acetate, the extent of protein denaturation is not known and may be incomplete. Methadone may also be occluded in the denatured protein matrix that occurs at the interface between the organic and aqueous phases and may not be completely extracted. Quantitative studies of plasma methadone levels by GLC involve adding a small mass (~1–5 ng) of ^3H-methadone to the sample to serve as a recovery marker in the extraction of methadone from plasma[7]. It is assumed that the radiotracer is fully equilibrated with the 100–400 ng of methadone present and bound to plasma proteins and that all

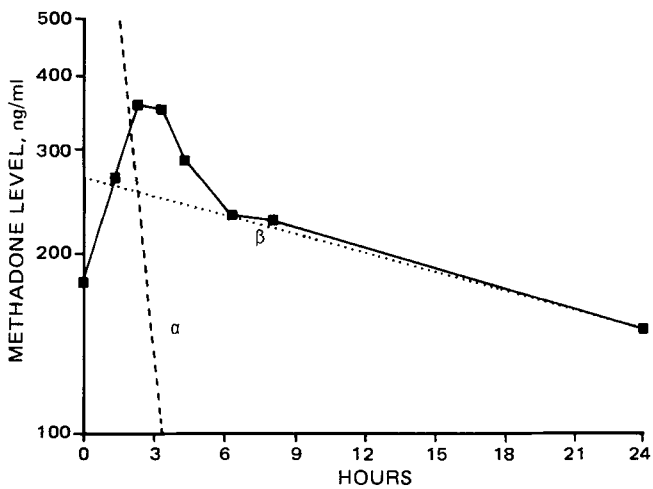


Figure 3—*Pharmacokinetic analysis of plasma methadone levels in an individual receiving 100 mg of methadone/day po. Methadone levels were measured by GLC–chemical-ionization mass spectrometry. The rapidly changing curve* (- - -) *represents the distribution phase* (α) *of the plasma methadone level–time profile* ($T_{1/2\alpha}$ = *0.79 hr*). *The slowly changing curve* (· · ·) *represents the elimination phase* (β) *of the drug* ($T_{1/2\beta}$ = *28.8 hr*).

[7] Recovery of radiolabeled methadone through the extraction and back-extraction procedure described ranges from approximately 55 to 65% for plasma and from approximately 85 to 90% for urine (M. J. Kreek, C. L. Gutjahr, J. W. Garfield, D. V. Bowen, and F. H. Field, *Ann. N.Y. Acad. Sci.*, in press). The remainder of the radioactivity is left in the aqueous, protein-containing fraction.

binding sites are equivalent, which may not be the case (17). Incomplete equilibration of the small mass of radiolabeled methadone with various protein-bound fractions of methadone may result in an artificially high estimate of drug recovery based on recovery of the radioactive marker. If this were true, the apparent dilution would respond to changes in the specific activity of the radioactive marker. These studies are currently in progress.

Data presented in Fig. 2 show the plasma methadone levels in an individual maintained on 80 mg of methadone/day. Plasma methadone levels were measured for 2 successive days following administration of the maintenance dose at 0 and 24 hr. Plasma drug levels generally ranged between 100 and 400 ng/ml and were consistent with those obtained previously (18, 19). The GLC curve showed consistently lower plasma levels than the GLC–chemical-ionization mass spectrometric curve, even though the profiles of the plasma disappearance curves were very similar.

Data obtained using the GLC–chemical-ionization mass spectrometric procedure are sufficiently accurate and reproducible to permit pharmacokinetic studies in human subjects. Figure 3 shows the plasma methadone disappearance curve for a study conducted in a patient receiving 100 mg of methadone/day. These data were fitted to a two-component exponential function [$y = 1876 \exp(-0.87t) + 268 \exp(-0.024t)$] by a weighted nonlinear, least-squares computer program. The half-life for the elimination phase (β) of the plasma disappearance curve was 28.8 ± 4.8 hr. Methadone clearance, defined as the sum of all hepatic and renal excretory routes and biotransformation pathways (20), was 125 ml/min in this patient[8].

The described procedure has a lower detection limit of about 5 pmoles/ml in urine and about 10 pmoles/ml in plasma. Since the required sample volume is only 1 ml, the assay is actually about five times more sensitive than the procedure described by Sullivan *et al.* (6), which requires 4 ml of plasma. The limiting factor in their procedure is the low abundance of the ion [m/e 294 $(M - 15)^+$] used for quantitative ion ratio measurements. The procedure described here uses one of the most intense ions in chemical-ionization mass spectrum of methadone [m/e 310, $(MH)^+$].

The sensitivity of the present method is also about a factor of 15–20 better than GLC procedures (19, 21), which require a larger volume of plasma. This method will permit more accurate measurement of low methadone levels present in adolescent heroin addicts on methadone maintenance therapy who are receiving lower dosages (5–40 mg/day) and in newborn infants whose mothers are maintained on methadone. This method will also permit studies in animal models in which plasma methadone levels are lower and turnover rates are much faster than in humans.

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ˣ To whom inquiries should be directed.

[8] *In vivo* metabolic tracer studies with deuterium-labeled methadone [M. J. Kreek and D. L. Hachey, *Clin. Res.*, **23,** 571A (1975)] indicate the drug half-life for the elimination phase of the plasma disappearance curve was 31.5 ± 2.9 hr. A more detailed report of methadone pharmacokinetics will be presented elsewhere.