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NEW GAS CHROMATOGRAPHIC ASSAY FOR THE QUANTIFICATION OF METHADONE

APPLICATION IN HUMAN AND ANIMAL STUDIES

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SUMMARY

A new gas chromatographic assay utilizing 2-dimethylamino-4,4-diphenyl-5-nonanone as the internal standard was developed for the quantification of methadone. The method involved extraction of methadone with 1-chlorobutane from tissue at pH 9.8, re-extraction of an aliquot of the organic solvent with 0.5 *M* sulphuric acid, alkalinization and final extraction into chloroform. The assay was used to determine the concentration of methadone (i) in whole blood samples from a normal volunteer following a single 9.4-mg oral dose of *d*-methadone hydrochloride, (ii) in whole blood, saliva and gastric juice from a methadone addict maintained on 90 mg of *dl*-methadone hydrochloride per day, (iii) in mouse liver microsomes incubated with methadone, and (iv) in the perfusate of the isolated perfused rat liver.

INTRODUCTION

Since the introduction of methadone by Dole and Nyswander[1] for the rehabilitation of heroin addicts several assays have been developed for quantification of the drug. Thin-layer chromatography (TLC) was used to quantitate methadone in human urine samples[2,3]. UV spectrophotometry was used to determine methadone after oxidation to benzophenone with cerium sulphate[4] or barium peroxide[5]. Hartvig and Näslund[6] improved the sensitivity of the oxidation method using gas chromatography (GC) with electron capture detection to quantitate the benzophenone produced. Sullivan *et al.*[7] have developed a mass fragmentographic assay for methadone. The poor sensitivity of TLC techniques, non-specificity of oxidation and time-consuming nature of mass fragmentography have made GC with flame ionization detection the most popular method for the quantification of methadone in blood and urine samples[8–15].

This report presents a rapid, specific and sensitive GC assay for methadone. An ideal internal standard was synthesized which had several advantages over those previously described: unlike hydrocarbons and other compounds not containing a

basic center, the internal standard could be added at the beginning of the extraction procedure; the internal standard had a high partition coefficient in the solvent system used and was, therefore, quantitatively extracted; and the internal standard was chemically similar to methadone, differing only by the presence of two methylene units. The method had the added advantage that no evaporation step was required. The assay had a broad application in the determination of methadone concentrations in whole blood, urine, gastric juice, and saliva of humans. It was also useful in drug metabolism and tissue distribution studies of methadone in animals and in *in vitro* preparations such as incubations of the drug with liver microsomes or studies in the isolated perfused rat liver.

A detailed comparison of the partition coefficient of *d*-methadone, *l*-methadone and the racemic drug at different pH values using 2,2,4-trimethylpentane, 1-chlorobutane, or 1-octanol was undertaken in order to re-evaluate the significance of the report by Misra and Mulé[16] that the two isomers of methadone had different partitioning characteristics and to establish the rationale of the new extraction procedure for the assay of methadone.

MATERIALS AND METHODS

Reagents and animals

The solvents used were 1-chlorobutane and 2,2,4-trimethylpentane from Burdick and Jackson (Muskegon, Mich., U.S.A.), and chloroform (nanograde), diethyl ether (anhydrous), 1-bromobutane (Analytical Reagent) and xylene (Analytical Reagent) from Mallinckrodt (St. Louis, Mo., U.S.A.). 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine hydroiodide was obtained from Applied Science Labs. (State College, Pa., U.S.A.). Methadone hydrochlorides (*d*-, *dl*- and *l*-) were gifts from Eli Lilly (Indianapolis, Ind., U.S.A.). *dl*-[^{14}C]Methadone hydrochloride was obtained from California Bionuclear (Sun Valley, Calif., U.S.A.). The circular dichroism spectra demonstrated that the optical purity of the *d*- and *l*-isomers was greater than 98%. Male Swiss-Webster mice (20–30 g) and male Sprague-Dawley rats (200–300 g) were purchased from Simonsen Labs. (Gilroy, Calif., U.S.A.).

Synthesis of the internal standard

1-Bromobutane (29.6 g) in diethyl ether (200 ml) was added to 5.25 g of iodine activated magnesium turnings under a dry nitrogen atmosphere over a period of 1 h. After the formation of the Grignard reagent was complete, a solution of 4-dimethylamino-2,2-diphenylvaleronitrile (20 g) in xylene (60 ml) was added and the diethyl ether distilled off until the temperature of the reaction mixture had reached 85°. After 3 h under reflux at 85°, 6 *N* hydrochloric acid (60 ml) was added to the reaction mixture and refluxing continued for an additional 3 h. Without the additional refluxing time an intermediate compound, presumably 2-dimethylamino-4,4-diphenyl-5-iminononane, was isolated. It had GC characteristics similar to those of the desired product and a mass spectrum with a molecular ion at *m/e* 336 and fragment ions at *m/e* 251 and 72. After the hydrolysis was complete the reaction mixture was cooled and hot water (800–1000 ml) was added. The slurry was adjusted to pH 9 with 10 *N* sodium hydroxide and extracted with diethyl ether. The diethyl ether was dried over anhydrous magnesium sulfate and distilled to give an oily residue (20 g). Distillation of the oil under

reduced pressure (140° at 0.003 mm Hg) did not sufficiently purify the product for use as an internal standard. The oil was then purified by preparative GC to give 2-dimethylamino-4,4-diphenyl-5-nonanone (63%). Stock solutions of the internal standard were prepared in 1-chlorobutane and stored at −20°.

The electron impact mass spectrum of 2-dimethylamino-4,4-diphenyl-5-nonanone (Fig. 1) was obtained on a DuPont Model 21-491 B gas chromatograph–mass spectrometer under the following conditions: accelerating potential, 1.8 kV; ionizing potential, 70 eV; trap current, 300 μA; and source temperature, 200°. A 6 ft × 2 mm I.D. glass column packed with 1.5% OV-101 on Gas-Chrom Q 100–120 mesh was used for the GC. The oven temperature was programmed from 150–280° at 10°/min. The molecular ion was at *m/e* 337 with major fragments at *m/e* 322 and 251 caused by loss of $-CH_3$ and $-CH_2CH(NMe_2)CH_3$. The base peak at *m/e* 72 was due to the fragment ion, $CH_3CH(NMe_2)^+$. The new internal standard had a similar fragmentation pattern to methadone, which had a base peak at *m/e* 72, a weak molecular ion at *m/e* 309, and fragment ions at *m/e* 294 and *m/e* 223, caused by loss of $-CH_3$ and $-CH_2CH(NMe_2)CH_3$, respectively.

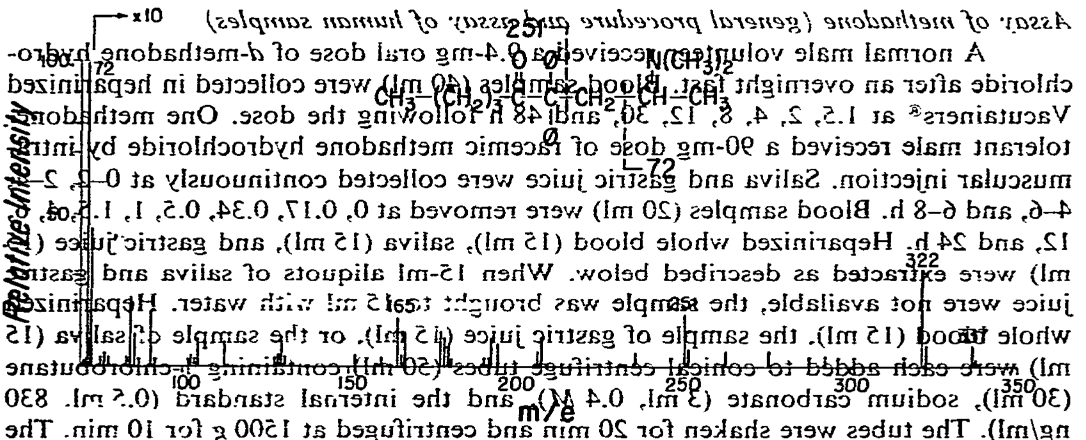

Fig. 1. Mass spectrum of 2-dimethylamino-4,4-diphenyl-5-nonanone as obtained by combined GC and mass spectrometry.

Partition coefficient of methadone

Countercurrent distribution was carried out according to the method of Bush and Densen[18] using manual countercurrent distribution machines obtained from Scientific Manufacturing Ind. (New York, U.S.A.). Methadone (*d*-, *l*- or *dl*) was added to the first tube of the countercurrent distribution apparatus to give an initial concentration of 0.216 mg/ml and four-tube countercurrent distribution was performed between 2,2,4-trimethylpentane (15 ml) and pH 6.8 phosphate buffer (15 ml, 0.2 *M*). At the end of the procedure the solvent layers were extracted into 15 ml of 1 *N* HCl and the concentration of methadone was measured in a Cary 15 recording spectrophotometer by scanning in the UV range from 360–220 nm. Methadone had an absorbance maximum at 295 nm with a molar extinction coefficient of 525. A

standard curve of methadone in 1 N HCl was prepared. The partition coefficient, C_0, of methadone in this system was determined from the relationship

$$C_0 = \frac{p}{q} \times \frac{V_y}{V_x}$$

where V_y is the volume of the lower (aqueous) layer and V_x is the volume of the upper (organic solvent) layer and p and q are the fractions of the drug which partition into the upper and lower layers, respectively, at each equilibration, *i.e.* $p + q = 1$ (Bush and Densen)[18]. In a similar manner the partition coefficients for *dl*-methadone were determined between 1-chlorobutane and aqueous phases at several different pH values using 4 × 4 countercurrent distribution. A curve of the partition coefficient (C_0) *versus* the pH was plotted. At pH values between 0–2, 3–4, and 5–9 volume ratios of 40:1 (organic:aqueous), 2:1, and 1:1, respectively, were used. The partition coefficient of methadone was also determined at different pH values using hydrochloric acid or sulphuric acid in the aqueous layer to determine whether the choice of acid influenced the partition coefficient and/or the recovery of the drug in the extraction procedure.

Assay of methadone (general procedure and assay of human samples)

A normal male volunteer received a 9.4-mg oral dose of *d*-methadone hydrochloride after an overnight fast. Blood samples (40 ml) were collected in heparinized Vacutainers® at 1.5, 2, 4, 8, 12, 30, and 48 h following the dose. One methadone-tolerant male received a 90-mg dose of racemic methadone hydrochloride by intramuscular injection. Saliva and gastric juice were collected continuously at 0–2, 2–4, 4–6, and 6–8 h. Blood samples (20 ml) were removed at 0, 0.17, 0.34, 0.5, 1, 1.5, 4, 6, 12, and 24 h. Heparinized whole blood (15 ml), saliva (15 ml), and gastric juice (15 ml) were extracted as described below. When 15-ml aliquots of saliva and gastric juice were not available, the sample was brought to 15 ml with water. Heparinized whole blood (15 ml), the sample of gastric juice (15 ml), or the sample of saliva (15 ml) were each added to conical centrifuge tubes (50 ml) containing 1-chlorobutane (30 ml), sodium carbonate (3 ml, 0.4 M), and the internal standard (0.5 ml, 830 ng/ml). The tubes were shaken for 20 min and centrifuged at 1500 g for 10 min. The organic phase (25 ml) was equilibrated with sulphuric acid (2 ml, 0.5 M) in a conical tube (50 ml) and centrifuged at 1500 g for 10 min. The 1-chlorobutane was discarded and the aqueous phase equilibrated with 2,2,4-trimethylpentane (15 ml) and centrifuged. In some samples the 2,2,4-trimethylpentane step was required to destroy the emulsion formed after equilibration of the sulphuric acid and 1-chlorobutane layers. The 2,2,4-trimethylpentane layer was aspirated off and discarded and the aqueous layer (1.8 ml) transferred to a Reacti-vial (Pierce, 5 ml), made alkaline (pH 9.6) by the addition of ammonium hydroxide (1 ml, 13.5%), and extracted with chloroform (50 μl). After centrifugation, 1–5 μl of the chloroform layer were examined by GC. The ratio of the methadone peak area to the internal standard peak area was determined by electronic integration and the concentration of methadone read directly from the standard curve. When low levels of methadone were anticipated duplicate 15-ml samples of blood were extracted as described above. The sulphuric acid extracts from each extraction were combined in a single vial, alkalinized, and extracted with chloroform (50 μl). In studies involving samples collected during a 72-h period each

sample was extracted immediately following collection and stored in the sulphuric acid phase at 4° until all samples had been collected. The group of samples was then analyzed on one day using a standard curve determined on that day. To prevent prolonged exposure to chloroform at the final stage of the extraction, samples in the sulphuric acid phase were extracted in groups of four and analyzed immediately by GC before extracting a second group of four samples.

Sample extracts were analyzed on a Hewlett-Packard Model 5830 A gas chromatograph using flame ionization detection and the calculating integrator for determination of peak areas and calculation of their ratios. The column was 6 ft. × 2 mm I.D. glass packed with 1.5% OV-101 on Gas-Chrom Q 100–120 mesh (supplied by Varian, Sunnyvale, Calif., U.S.A.). The oven temperature was programmed from 170–250° at 10°/min. The injector and detector temperatures were 250° and 275°, respectively. The hydrogen, air, and nitrogen flow-rates were 30, 250, and 30 ml/min, respectively.

A standard curve was prepared in each tissue from which methadone was quantitated. For example, for blood analysis outdated whole blood from the Red Cross Blood Bank was added in 15-ml aliquots to tubes containing 1-chlorobutane (30 ml), sodium carbonate (3 ml, 0.4 *M*), and the internal standard (0.5 ml, 830 ng/ml). Aliquots of a methadone stock solution were added to the tubes to give whole blood methadone concentrations of 1–50 ng/ml for non-tolerant volunteers and 1–600 ng/ml for methadone addicts. The samples were extracted as described above and analyzed by GC. The ratio of the methadone peak area to the internal standard peak area was determined by electronic integration. A plot of the experimental ratio *versus* the known concentration per milliliter in whole blood gave a linear standard curve within the range of concentrations employed. Using the experimental ratio of methadone to internal standard the concentration of methadone in the unknown samples was read directly from the standard curve provided the sample volume remained at 15 ml. A similar standard curve was constructed for each tissue studied using methadone and internal standard concentrations appropriate for that tissue. The standard curve samples were then analyzed by GC on the same day as the group of unknown samples to avoid possible errors due to slight instrument and column variations from day to day.

Recovery of methadone

To determine the percentage of methadone recovered in the whole blood extraction procedure, *dl*-[^{14}C]methadone hydrochloride (2.7 μg, 2.8 mCi/mmole) was added to whole blood (15 ml) in quadruplicate, to give a concentration of 180 ng per ml. The samples were extracted as described above for whole blood. The distribution of [^{14}C]methadone was monitored throughout the extraction by counting small aliquots of the 1-chlorobutane after equilibration with blood and after equilibration with 0.5 *M* sulphuric acid. The sulphuric acid, 2,2,4-trimethylpentane, chloroform, and ammonium hydroxide layers were also analyzed by liquid scintillation counting. Organic samples were counted with 5 ml of a dioxane scintillator (600 g naphthalene and 5 g of diphenyloxazole per 1 of dioxane) and aqueous samples were counted with 15 ml of Aquasol-1® (New England Nuclear, Boston, Mass., U.S.A.). The percentage recovery of methadone from whole blood was 93 ± 2% S.D. in the 1-chlorobutane extract. In each of the following steps of the extraction procedure the recovery was greater than 99%.

Mouse liver microsomes. dl-Methadone hydrochloride at an initial concentration of 0.124 mg/ml was incubated in quadruplicate with the mouse liver 9000 *g* supernatant prepared according to Gerber and Arnold[19]. The protein concentration in the incubation mixture was approximately 18 mg/ml. Samples were removed at 0, 15, and 30 min and added to centrifuge tubes containing 1-chlorobutane (20 ml), water (3 ml), sodium carbonate (1 ml, 0.2 *M*), and the internal standard (70 μg/tube). The extraction was performed as for whole blood, omitting the 2,2,4-trimethylpentane wash. A standard curve was prepared by adding 1-ml aliquots of a microsome incubation mixture containing no drug to each of six centrifuge tubes containing 1-chlorobutane, sodium carbonate, water, methadone (22–177 μg), and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (10–120 μg).

Isolated perfused rat liver. dl-Methadone hydrochloride was added to the recirculating (10 ml/min) perfusate (100 ml) of the isolated perfused rat liver at an initial concentration of 200 μg/ml of perfusate. The procedure for isolation and perfusion of the rat liver was that of Gerber *et al.*[20]. Timed samples of perfusate were removed over a period of 3 h and extracted by the procedure described above for mouse liver microsomes.

RESULTS AND DISCUSSION

Assay of methadone

The new GC assay for the analysis of methadone, using 2-dimethylamino-4,4-diphenyl-5-nonanone as the internal standard, has been used to obtain kinetic data on methadone disappearance in whole blood from human volunteers and in *in vitro* preparations from rats and mice. The lower limit of reproducible quantification of methadone using 15 ml of whole blood was 5 ng/ml. In replicate samples (eight) from microsome incubations of methadone the standard deviation was 3%.

Fig. 2 is a typical gas chromatogram used for the quantification of methadone and its primary N-demethylated metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine. The sample was obtained from an incubation of methadone with the 9000 *g* supernatant of mouse liver. The pyrrolidine metabolite, methadone, and the internal standard have hydrocarbon numbers of 20.1, 21.3, and 22.8, respectively. Fig. 3 is a standard curve used for the determination of methadone in whole blood samples. Similar standard curves were constructed for the measurement of methadone in saliva, gastric juice, mouse 9000 *g* supernatant and the perfusate of the isolated perfused rat liver. Each standard curve was linear throughout the concentration range of methadone under study. A plot of the calculated molar ratio of methadone to the internal standard *versus* the experimental peak area ratio of methadone to the internal standard gave a straight line with a slope of 0.94 ± 0.06 S.D., indicating that the internal standard was recovered to a slightly greater extent than methadone in the extraction.

Partition coefficient of methadone

Fig. 4 illustrates a four-tube countercurrent distribution of *d*-methadone between 2,2,4-trimethylpentane (15 ml) and pH 6.8 phosphate buffer (15 ml, 0.2 *M*). The theoretical distribution which best fitted the experimental distribution was plotted and the partition coefficient calculated. The C_0 values of *d*-, *l*-, and *dl*-methadone were determined in an identical manner. *d*-, *l*-, and *dl*-methadone accumulate in the

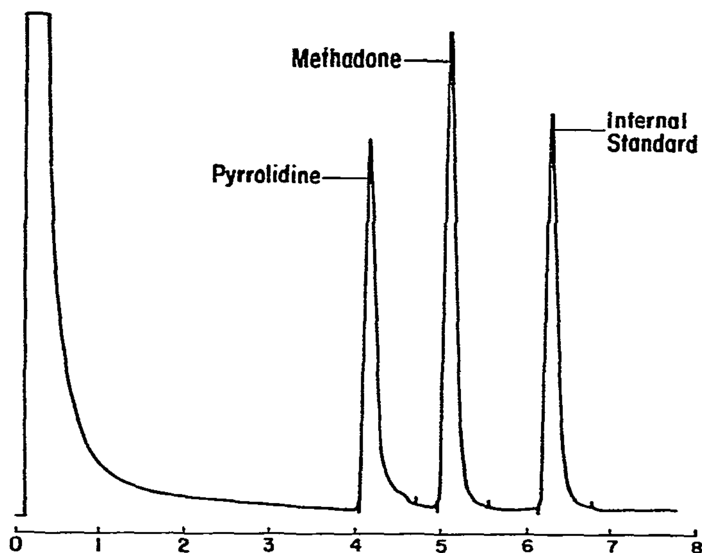

Fig. 2. Gas chromatogram illustrating the separation of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, methadone, and the internal standard.

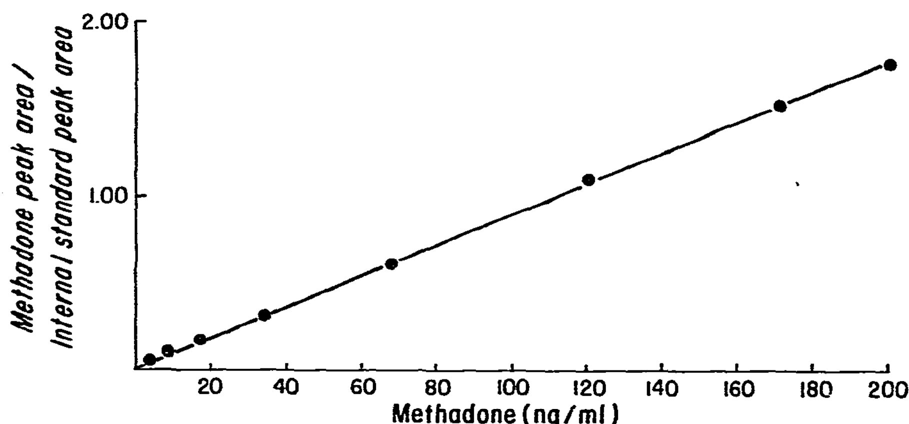

Fig. 3. Methadone standard curve from whole blood. Whole blood to which methadone and the internal standard had been added was extracted by the described procedure and analyzed by GC.

organic phase with partition coefficients of 4.88 ± 0.22 S.D., 4.60 ± 0.14 S.D., and 4.65 ± 0.09 S.D., respectively. In all 4 × 4 countercurrent distributions between 1-chlorobutane and buffer at various pH values, *dl*-methadone distributed as one compound (Fig. 4). No significant difference was detected between the partition coefficients of *d*-, *l*-, and racemic methadone using either 1-chlorobutane or 2,2,4-trimethylpentane as the organic solvent. This was in sharp contrast to the report of Misra and Mulé[16], who measured C_0 values of 28.3 and 57.3, respectively, for *d*- and *l*-methadone between 1-octanol and pH 7.4 buffer. The latter workers used radio-labelled drug and presumably performed a single equilibration so that the presence of a small quantity of radioactive impurity may have accounted for their anomalous results. Countercur-

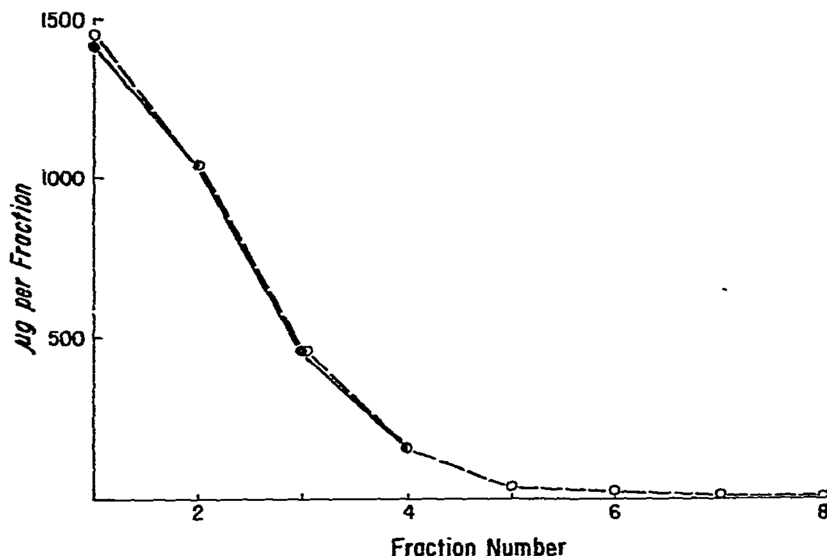

Fig. 4. Four-tube countercurrent distribution of *d*-methadone. The partition coefficients for *d*-, *l*-, and *dl*-methadone were 4.88 ± 0.22 S.D., 4.60 ± 0.14 S.D., and 4.65 ± 0.09 S.D., respectively. ●—●, Experimental; ○--○, theoretical ($p = 0.82$).

rent distribution techniques for the determination of C_0 values avoided this hazard because impurities of different polarity were separated from the drug under investigation and the C_0 could be determined when mathematical analysis indicated that the substance under investigation was distributing as a single compound[18]. The partition coefficient work of Misra and Mulé[16] was repeated in our laboratory using four-tube countercurrent distribution between 1-octanol and pH 7.4 phosphate buffer at a volume ratio of 5:40. Both *d*- and *l*-methadone accumulated in the organic phase with partition coefficients of 38.3 ± 1.2 S.D. and 38.4 ± 4.6 S.D., respectively.

Fig. 5 illustrates the change in partition coefficient (1-chlorobutane:buffer) of racemic methadone with changing pH of the aqueous layer. As was expected for a weak base such as methadone (pK_a 8.6)[21], the partition coefficient decreased with increasing hydrogen ion concentration in the aqueous layer between pH 7 and 2. However, between pH 2 and 0 an interesting anomaly occurred when hydrochloric acid was used for the aqueous layer. The partition coefficient increased as the normality of the hydrochloric acid was increased. When sulphuric acid of equal normality was substituted for the hydrochloric acid the partition coefficient decreased as expected. At pH 0.2 the partition coefficients of methadone were 0.0005 and 0.027, using sulphuric acid or hydrochloric acid as the aqueous layer, respectively. This suggests that the hydrochloride salt had a higher partition into 1-chlorobutane than the sulphate salt and for this reason sulphuric acid was selected for use in the methadone extraction procedure.

Assay of methadone in human samples

Fig. 6 illustrates the decline in the concentration of methadone in the blood of a volunteer who received a single oral dose of 9.4 mg of *d*-methadone hydrochloride.

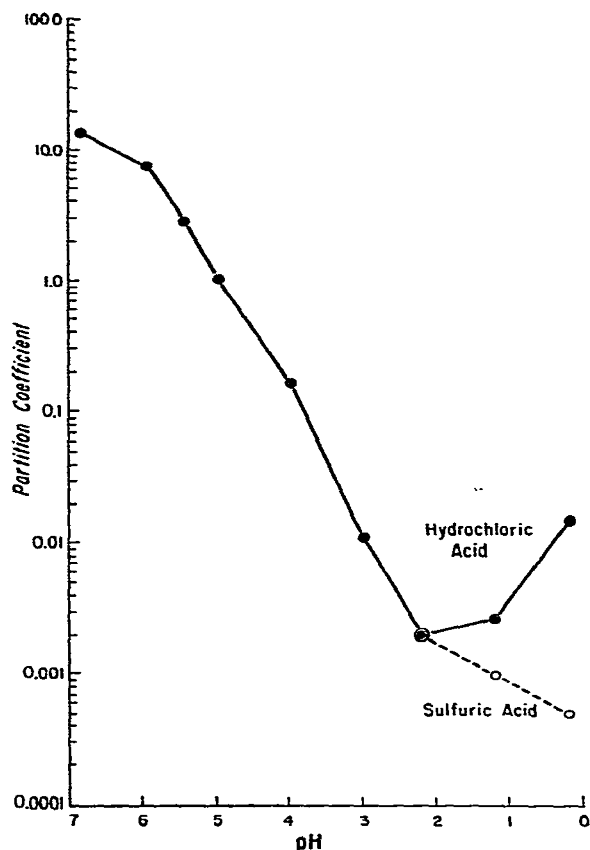

Fig. 5. Partition coefficient of racemic methadone *vs.* pH. Partition coefficients of racemic methadone were determined by four-tube countercurrent distribution between 1-chlorobutane and aqueous layers at several pH values. Between pH 2 and 0 the partition coefficient was dependent upon the choice of acid.

The peak blood level, 29 ng/ml, was observed at 1 h and declined thereafter with an apparent half-life ($t_{\frac{1}{2}}$) of about 7.5 h. This was followed by a slower rate of decline ($t_{\frac{1}{2}}$ = 37 h) after 12 h. Forty-eight hours after the dose the whole blood concentration of methadone was approximately 5 ng/ml. The apparent change in the rate of decline of the drug in blood with time was not observed in all volunteer subjects.

Fig. 7 is a comparison of the whole blood concentration of methadone, and the salivary and gastric juice concentrations obtained concurrently from a methadone addict who received an intramuscular dose of 90 mg of *dl*-methadone hydrochloride. The whole blood concentration of methadone reached a peak within 1 h and declined steadily in subsequent samples with an apparent $t_{\frac{1}{2}}$ of 13.5 h. In gastric juice the peak concentration of methadone was attained at 1.5 h. One and a half hours after administration of the drug the concentrations of methadone in saliva and gastric juice, respectively, were 54 and 100 times those measured in whole blood. The total amount of methadone recovered in the 8-h gastric juice collection was 10.5 mg, accounting for 13.0% of the administered dose. Other workers[22] have shown that drugs

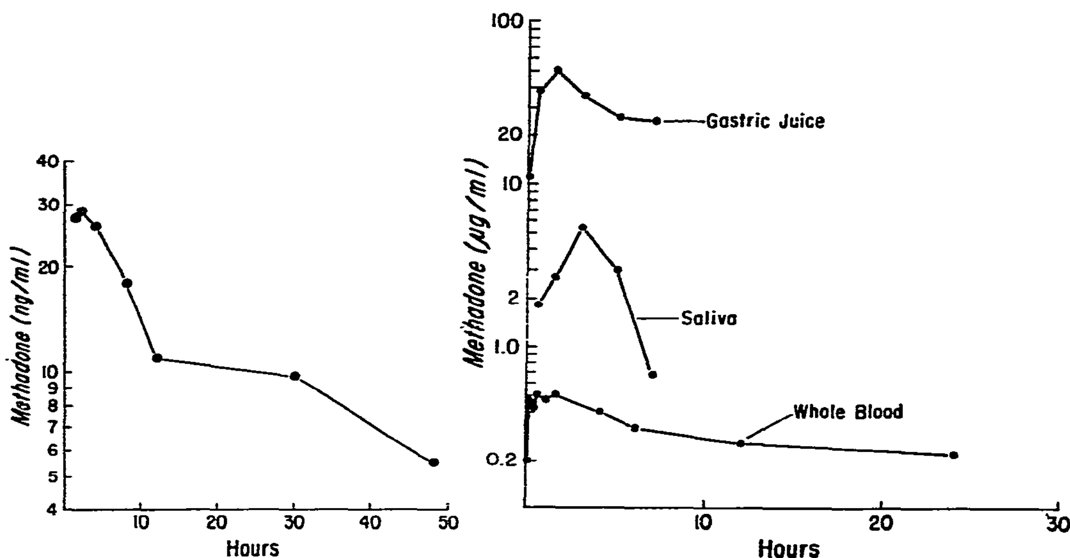

Fig. 6. *d*-Methadone in a normal volunteer. The concentration of methadone was measured in whole blood samples from a normal male volunteer following administration of a 9.4-mg oral dose of *d*-methadone hydrochloride.

Fig. 7. *dl*-Methadone in a former heroin addict. The concentration of methadone was measured in gastric juice, saliva, and whole blood following intramuscular administration of *dl*-methadone hydrochloride (90 mg).

such as aminopyrine and aniline are excreted in the gastric juice of dogs following parenteral administration. In humans meperidine administered iv was recovered in the gastric juice at a maximum gastric juice to blood concentration ratio of 430 (ref. 23). Robinson and Williams[14] found methadone in the gastric juice of man post mortem, following an acute intravenous overdose of methadone hydrochloride.

Previous reports[9,10] noted that the metabolites of methadone could not be detected in whole blood or plasma, although authentic methadone metabolites, added to plasma, could be recovered and detected. In one of our studies of an addict, receiving daily oral doses of 70 mg of methadone, the primary N-demethylated metabolite of methadone (pyrrolidine) was detected in the whole blood and its structure confirmed by mass spectrometry using the authentic material as the standard. The ratio of methadone to the pyrrolidine metabolite was 0.05.

Analysis of methadone in vitro

Fig. 8 demonstrates the rate of decline of the concentration of methadone in the perfusate of the isolated perfused rat liver. There was an initial rapid decline in the concentration of the drug in the perfusate, caused by uptake of the unchanged compound into the liver. The $t_{\frac{1}{2}}$ at later times was 121 min and resulted primarily from the metabolism of methadone to its primary N-demethylated metabolite which accumulated in the liver and perfusate with the passage of time.

Fig. 9 illustrates the metabolism of methadone in the 9000 *g* supernatant from mouse liver. Each point represents the mean ± S.D. of four incubations. The major

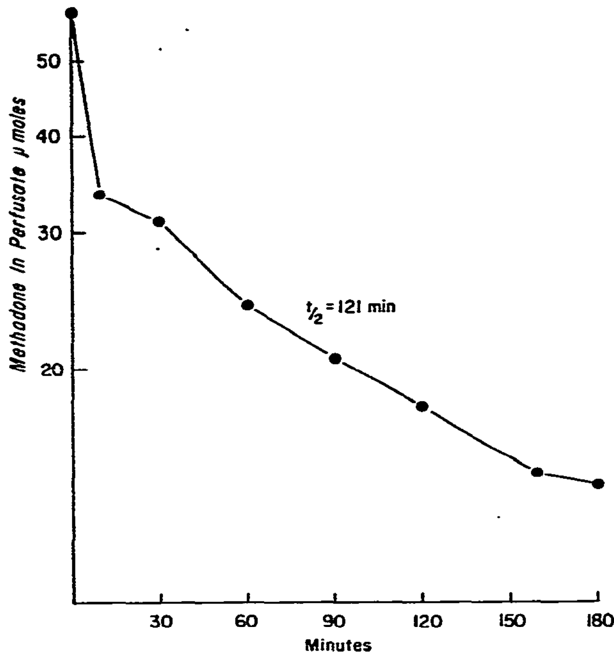

Fig. 8. Decline of methadone in the perfusate of the isolated perfused rat liver. Racemic methadone was added to the perfusate of the isolated perfused rat liver preparation and samples removed at timed intervals thereafter for determination of the methadone concentration by GC.

metabolite in these incubations was also the mono-N-demethylated metabolite (pyrrolidine) which was identified by comparison with the mass spectrum of the authentic synthetic sample. The appearance of pyrrolidine in the incubation with time accounted for over 90% of the methadone, which disappeared by 30 min. Other metabolites were produced in the incubation. These were probably hydroxylated demethylated compounds. Preliminary work in this laboratory demonstrated that the

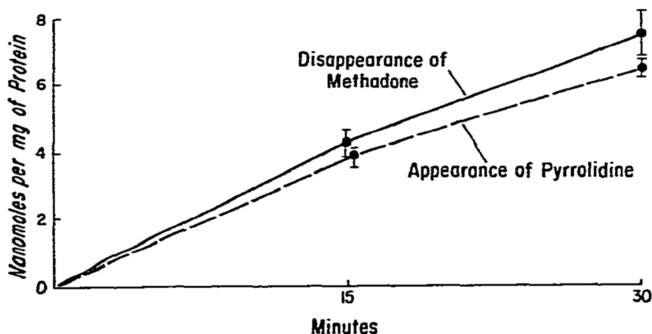

Fig. 9. Metabolism of methadone in the mouse liver 9000 *g* supernatant. Methadone was incubated with mouse liver 9000 *g* supernatant in quadruplicate and samples were removed at 0, 15, and 30 min. The concentration of methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine was determined by GC. Each point represents the mean ± S.D. of four incubations.

percentage of these metabolites increased in the incubations of methadone with microsomes from mice which have been pre-treated with phenobarbital.

The assay for methadone is currently being used for detailed tissue distribution studies of methadone in mice and rats.

ACKNOWLEDGEMENTS

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