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Note

Sensitive method for the determination of methadone in small blood samples

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Quantitation of low levels of methadone in small size samples required a sensitive and specific method of analysis. Methods for clinical application have been described for blood and urine samples using immunoassay techniques[1–4], gas chromatography[5–11] and combined gas chromatography–mass spectrometry[12–14]. These methods are not sensitive enough for determination of plasma concentrations in small volume blood samples. The sensitivity of those methods was in the 40–50 ng/ml range; however, they required sample sizes of 4–15 ml[5,7]. These techniques were not appropriate to study the methadone–ethanol interaction in experimental animals. This report presents a simple and accurate method of quantification using the addition of an internal standard, extraction with an organic solvent and gas–liquid chromatography (GLC) determination. The internal standard selected, 4-dimethylamino-1,2-diphenyl-3-methyl-2-butanol (chirald), is commercially available.

METHODS

Reagents

All solvents and chemicals used were reagent grade. Chirald (99%) was purchased from Aldrich and used as the internal standard. Methadone hydrochloride was supplied by Burt Mayfield of Mallinckrodt.

Apparatus

The chromatographic determinations were performed using a Perkin-Elmer Sigma I analyzer equipped with a flame ionization detector. The inlet and detector temperatures were 275°C each. The column used was a 3% OV-7 on Gas-Chrom Q (100–120 mesh), with a flow of 35 ml/min of nitrogen. The column was heated to an initial temperature of 220°C for 7 min and then programmed at 20°C/min to 300°C and held constant for 7 min.

Sensitivity and linearity

A standard solution of methadone hydrochloride in water was prepared in concentrations ranging between 1 and 10 μg/ml and containing 6.5 μg/ml chirald in methanol (internal standard). Aliquots (1 μl) of these solutions were injected into the chromatograph.

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Recovery

Blood was withdrawn from naive mice. To each sample 6.5 μl of 0.1 mg/ml chirald in methanol and methadone solution to obtain concentrations of 0.1–1 μg/ml were added. The samples were then extracted as described below. The recovery rates were calculated by comparison with methanol standards containing both drugs.

Extraction procedure

To 50 or 100 μl of blood in a Teflon-lined, screw-capped testtube 6.5 μl of 1 mg/ml chirald in methanol (internal standard) and 75 or 150 μl of phosphate buffer (pH 8.0) were added. The extraction was carried out by the addition of 3 ml heptane containing 1.5% isoamyl alcohol. The mixture was vortexed for 1 min and centrifuged on a clinical bench centrifuge for 15 min. The organic mixture was removed to a second testtube and evaporated to dryness under a gentle stream of nitrogen. The dried extract was reconstituted with 10 μl of methanol and vortexed, and 1-μl aliquots were injected twice into the gas chromatograph. Other solvents (ethyl acetate) and buffers (Tris) were tried, but the recovery rates were lower and more interferences were observed.

Blood and methadone analyses

Male BL6J mice (28–30 g in weight), purchased from Jackson Labs. (Bar Harbor, ME, U.S.A.) were fasted overnight. A dose of 20 mg/kg methadone hydrochloride in saline was administered i.p. to 20 mice. Groups of 5 animals each were sacrificed after 1, 2, 3 and 4 h, and four blood samples (2 × 50 μl; 2 × 100 μl) were collected from each mouse with micropipettes. The blood samples (50 or 100 μl) were immediately poured into glass tubes containing 75 or 150 μl of phosphate buffer (pH = 8.0) and 6.5 μl of 1 mg/ml chirald in methanol and extracted as previously described. Multiple blood samples were withdrawn from the same animal to test the reproducibility between samples of different volume and to determine whether a 50-μl was sufficient and as accurate as a 100-μl sample.

RESULTS

Sensitivity and linearity

The detection limit for methadone was 1 ng injected into the chromatograph. The response curve for methadone (1-μl injection) was found to be linear ($y = 0.121 + 0.123x$) in the range of 1–10 μg/ml. The correlation coefficient calculated for the regression line was 0.961.

Recovery

The recovery rate, calculated on the basis of the amount of methadone measured after extraction compared with standard methanol solutions, was 95.4 ± 0.51%. Using a sample size of 50 μl of blood, methadone was readily quantitated to 0.1 μg/ml.

Methadone blood levels in mice

A typical chromatogram of a blood sample from a mouse receiving 20 mg/kg methadone i.p. is shown in Fig. 1. The retention times for methadone and chirald

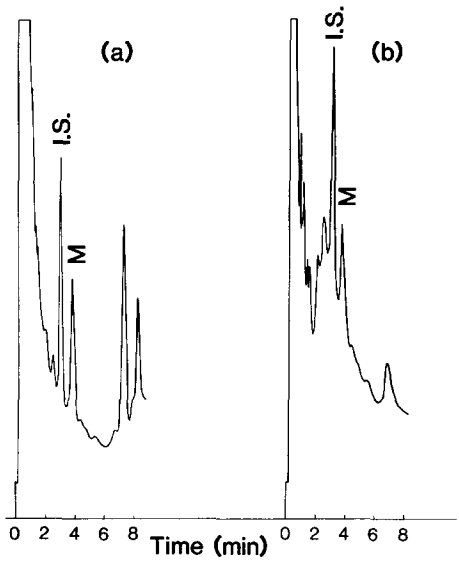

Fig. 1. Chromatogram of the extract of a blood sample taken from (a) naive mouse with added methadone (M) and (b) mouse injected with methadone. Chirald (I.S.) was added to both samples.

were 4.10 and 3.32 min, respectively. The chromatographic analysis was completed in 10 min, and although some peaks with shorter and longer retention times were present, there were no interferences with the analysis.

The concentration of methadone in the blood using 50- and 100-μl samples are shown in Table I. Comparison by Student's t-test between the two samples sizes for each time period did not show significant differences.

DISCUSSION

This report describes a rapid, sensitive and reproducible assay for methadone in blood samples. The small volume (50 μl) needed and the low limit of detection (0.1 μg/ml) allow for the determination of complete curves for methadone concentration in blood without significant risks to the animals.

In the present report, each mouse was sacrificed at each time period to ascertain the volume of blood needed and the reproducibility of the method. However, the

TABLE I

MEAN ± S.E.M. METHADONE CONCENTRATION (μg/ml)

Two samples from each mouse; five mice per time period.

Sample size (μl)	*Time (h)*			
	1	*2*	*3*	*4*
50	3.64 ± 0.09	3.48 ± 0.20	2.68 ± 0.22	2.25 ± 0.05
100	3.81 ± 0.13	3.56 ± 0.22	2.68 ± 0.23	2.32 ± 0.07

results show that it is possible to obtain serial blood samples (50 μl) from the tail tip of a mouse at hourly intervals to follow methadone concentration.

The peak blood levels of methadone occurred in 1 h or less and declined with time. The blood curve, although higher in values, follows the same pattern observed in rats receiving 5 mg/kg s.c. or 30 mg/kg p.o. for 4 weeks[15].

This method can also be used to quantitate methadone in clinical studies requiring high sensitivity and small sample size.

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