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A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE DETERMINATION OF ACETYLMETHADOL AND ITS METABOLITES IN HUMAN URINE

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SUMMARY

A method is described for the simultaneous determination of acetylmethadol, noracetylmethadol, methadol and normethadol in a sample of human urine. The compounds are recovered by the use of solvent extraction and separated by the use of a gas–liquid chromatographic system employing 3% XE-60 as the stationary phase.

The method has been used to identify acetylmethadol, noracetylmethadol, methadol and normethadol in the urine of patients receiving acetylmethadol for the treatment of heroin dependence. The use of an internal standard permits a determination of the concentrations of acetylmethadol and its metabolites in the urine of these patients.

INTRODUCTION

Alpha-*l*-acetylmethadol (AM), a derivative of *d*-methadone with analgesic activity[1], is currently under evaluation for the treatment of heroin dependence[2,3]. AM has the ability to suppress the narcotic withdrawal syndrome for a longer length of time than comparable doses of methadone[2,3]. Studies in laboratory animals have suggested that the biotransformation of AM to active metabolites is responsible for the time-action characteristics of certain of the pharmacologic effects of AM[4–6]. McMahon *et al.*[5] demonstrated that AM is biotransformed in the rat to noracetyl methadol (NAM). This report[5] also suggested that methadol (MOL) and normethadol (NMOL) might be metabolites of AM (Fig. 1).

There is no information available on the biotransformation of AM in man. The purpose of this report is to describe a specific and sensitive method for the identification and quantitation of AM, NAM, MOL and NMOL in the urine of patients receiving AM.

MATERIALS AND METHODS

Chemicals and reagents

The *l*-isomer of alpha-acetylmethadol was provided by Dr. M. Fink of New

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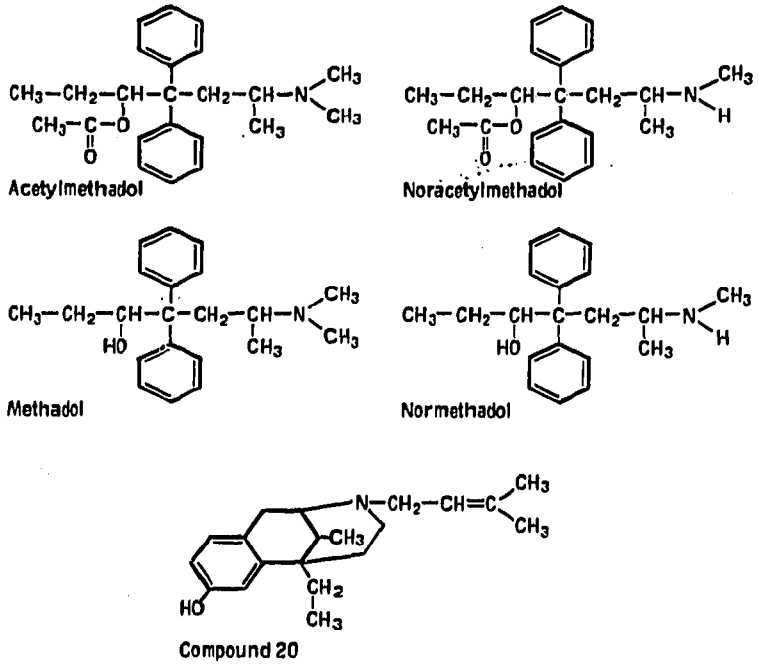

Fig. 1. Structural formulas of acetylmethadol, noracetylmethadol, methadol, normethadol and compound 20, the internal standard.

York Medical College. Alpha-*d,l*-noracetylmethadol hydrochloride, alpha-*l*-methadol hydrochloride and alpha-*l*-normethadol hydrochloride were complimentary research samples from Dr. A. Pohland of the Lilly Research Laboratories (Indianapolis, Ind.). Dr. E. L. May of the National Institutes of Health (Bethesda, Md.) also provided samples of alpha-*l*-methadol hydrochloride and alpha-*l*-noracetylmethadol hydrochloride. Compound 20 (2-allyl-5-ethyl-2′-hydroxy-9-methyl-6,7-benzomorphan) (Fig. 1) was a gift of Dr. S. Archer of the Sterling-Winthrop Research Institute (Rensselaer, N.Y.).

The solvents used in the procedure are spectral-grade chloroform and hexane and reagent-grade *n*-butyl chloride. All are glass-distilled in our laboratory.

Stock solutions

Aqueous solutions of AM, NAM and MOL each at a concentration of 4 μg/ml, NMOL at a concentration of 50 μg/ml and compound 20 at a concentration of 20 μg/ml are prepared and kept refrigerated.

Sample preparation from urine

The extraction procedure is adapted from that described by Inturrisi and Verebely[7] for the extraction of methadone from plasma and urine. A flow sheet outlining the procedure is given in Fig. 2.

To urine (1–4 ml) in a siliconized 15-ml centrifuge tube with a Teflon®-lined

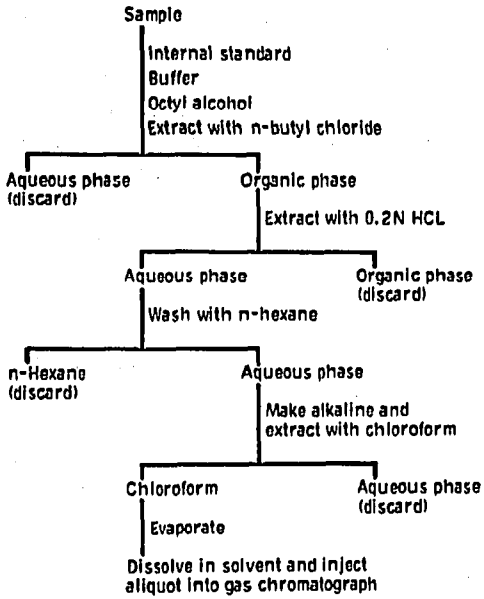

Fig. 2. A flow sheet outlining the procedure for the extraction of AM and its metabolites from urine.

screw cap is added 0.3 ml of the aqueous solution of compound 20, the internal standard, 0.5 ml of Delory and King's carbonate–bicarbonate buffer[8], 1 *M*, pH 9.8, and one drop of octyl alcohol. After thorough mixing, the sample is extracted with 5.0 ml of *n*-butyl chloride by shaking for 5 min, followed by centrifugation at 1500 r.p.m. for 5 min. The upper (organic) phase is removed and saved. The extraction is then repeated with an additional 5.0 ml of *n*-butyl chloride and this organic phase added to the one resulting from the initial extraction. The compounds are extracted into acid by the addition of 5.0 ml of 0.2 *N* HCl to the combined organic phases and shaking for 7 min, followed by centrifugation for 3 min. The upper (*n*-butyl chloride) phase is removed by aspiration and discarded. The acid phase is then washed by the addition of 5.0 ml of *n*-hexane and shaking for 5 min, followed by centrifugation for 3 min. The hexane phase is removed by aspiration and discarded. The washed acid phase is made alkaline by the addition of 0.4 ml of concentrated ammonium hydroxide (pH is approx. 10). The compounds are extracted into 7.0 ml of chloroform by shaking for 5 min, followed by centrifugation for 3 min. The upper aqueous phase is removed by aspiration and discarded. The organic phase is transferred into a 12-ml siliconized centrifuge tube. The sample extract is concentrated by evaporating the chloroform to dryness by the use of a multiple flash evaporator with the bath at 50° (Evap-O-Mix, Buchler Corp., Fort Lee, N.J.). The sample extract is concentrated in the lower tip of the tube by rinsing the sides of the tube with 0.15 ml of chloroform followed by evaporation to dryness. The sample extract is dissolved in 20 μl of chloroform and between 1 and 4 μl are injected into the gas chromatograph.

Gas–liquid chromatography

The gas–liquid chromatographic (GLC) 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 stationary phase is 3% XE-60 on Gas-Chrom Q, 80–100 mesh. The temperature of both the detector and the injector port is 260°. Helium, at a flow-rate of between 30 and 35 ml/min, is the carrier gas. Hydrogen and air flow are between 32 and 40 ml/min and between 200 and 250 ml/min, respectively. The gas flows are adjusted to give maximal detector response. A column oven temperature between 210 and 215° is used for the urine extracts. Detector sensitivity is varied from $8 \cdot 10^{-11}$ to $32 \cdot 10^{-11}$ A/mV at full scale deflection as required.

Stationary phases

A number of stationary phases were tested for their ability to separate the compounds of interest. They included: (all at 80–100 mesh) 3% SE-30 on Gas-Chrom Q, 3.8% W-98 on Diatoport S, 5% OV-17 on Gas-Chrom Q, 4% XF-1112 on Chromosorb W, 3% QF-1 on Chromosorb W, 3% XE-60 on Gas-Chrom Q and 3% 8-BP on Gas-Chrom Q. In addition 3% OV-225 on Gas-Chrom Q, 100–120 mesh, and Tenax-GC, 60–80 mesh, were evaluated.

The column oven temperature was varied within the ranges of temperature: for SE-30, OV-17 and XE-60 from 160 to 245°; for W-98, OV-225, QF-1, XF-1112 and 8-BP from 200 to 235°; and for Tenax-GC from 275 to 300°.

Calibration curves and quantitation

Standard calibration curves are generated by the addition of AM in the range of 0.1–2.0 μg, NAM and MOL in the range of 0.4–6.0 μg and NMOL in a range of 2.5–25 μg to a 1.0-ml sample of control urine and proceeding as described above. The peak height of the detector response to each compound is divided by the peak height of the internal standard to yield a ratio. Standard calibration curves are constructed relating these peak height ratios to the amount of each compound added to the sample. Each calibration curve is constructed from duplicate determinations of five different points. The amount of each compound in an unknown urine sample is determined by converting the peak height ratio obtained into the absolute amount of compound present in the sample. The linearity of the standard calibration curves within the range indicated allows the use of calculated slopes for these conversions. The mean deviation of individual determinations from the mean of duplicate determinations was approx. 10% of that mean for both the standard calibration and patient samples.

To determine the recovery of the bases, 4.0 μg of AM, 2.4 μg of NAM, 4.0 μg of MOL, 5.0 μg of NMOL and 6.0 μg of compound 20 were added to 1.0 ml of control urine and the extraction carried out as described above. The same quantities, representing an absolute recovery, were extracted from the alkaline aqueous phase (last step in *Sample preparation from urine*). The bases were quantitated by GLC. After correcting for aliquot losses, the recovery from urine was 90% for AM, 95% for NAM, 87% for MOL and 100% for both NMOL and compound 20. The average S.D. for these determinations was $\pm 6.5\%$.

The method can be used to detect as little as 0.1 μg of any of the bases. This high degree of sensitivity was not required for urine samples from AM maintenance patients.

RESULTS AND DISCUSSION

The compounds of interest are all weak organic bases. A number of stationary phases were tested for their ability to separate these bases. For each stationary phase shown in Table I the retention time for AM was given a value of 1.00 and the retention time for each of the other bases was divided by the retention time for AM such that the data are presented as values that are relative to that of AM. The non-polar phases tested, SE-30 and W-98, did not significantly distinguish between AM and NAM nor did they separate MOL from NMOL. Of the phases intermediate in polarity, XF-1112 did not sufficiently separate AM and MOL, QF-1 did not sufficiently separate AM and NMOL and OV-17 failed to give baseline separation between AM and NAM. Excessive tailing or the failure to obtain a retention time when microgram amounts of the bases were introduced on to the column (Table I) eliminated some of the stationary phases of high relative polarity (8-BP and Tenax-GC) from consideration. A decrease in the column temperature did not significantly improve the separation of the bases. For example, reducing the column temperature of SE-30 from 210 to 160° did not provide baseline separation of AM from NAM or MOL from NMOL.

TABLE I

RELATIVE RETENTION DATA FOR AM, NAM, MOL, NMOL AND COMPOUND 20 ON SELECTED GLC STATIONARY PHASES

The GLC conditions are as described in Materials and Methods.

Stationary phase	*AM*	*NAM*	*MOL*	*NMOL*	*Compound 20*
SE-30	1.00	1.00	0.94	0.94	0.94
W-98	1.00	1.00	0.91	0.90	—*
OV-17	1.00	1.00	—	—	—
XF-1112	1.00	1.12	1.02	neg**	—
QF-1	1.00	1.20	0.88	1.00	—
OV-225	1.00	1.25	1.15	1.55	—
XE-60	1.00	1.26	1.17	1.63	2.82
8-BP	1.00	neg	neg	—	—
Tenax-GC	1.00	neg	0.75	0.75	—

* —=Not determined.
** neg= retention time could not be obtained.

OV-225 and XE-60 furnished retention results more compatible with the objective of the simultaneous quantitation of AM, NAM, MOL and NMOL. Complete baseline separation of the bases was effected (Table I) and therefore XE-60 was chosen as the stationary phase for this method.

Examples of chromatograms obtained under the conditions described in Materials and Methods are given in Fig. 3. The multi-step extraction procedure results in an extract that is free of interfering peaks. In most cases it was possible to introduce samples into the gas chromatograph every 12 min.

Panel (a) of Fig. 3 shows the chromatogram obtained from an extract of control urine to which were added 0.25 μg of AM (1), 0.50 μg of MOL (2), 1.00 μg of NAM (3) and 6.00 μg of compound 20 (5).

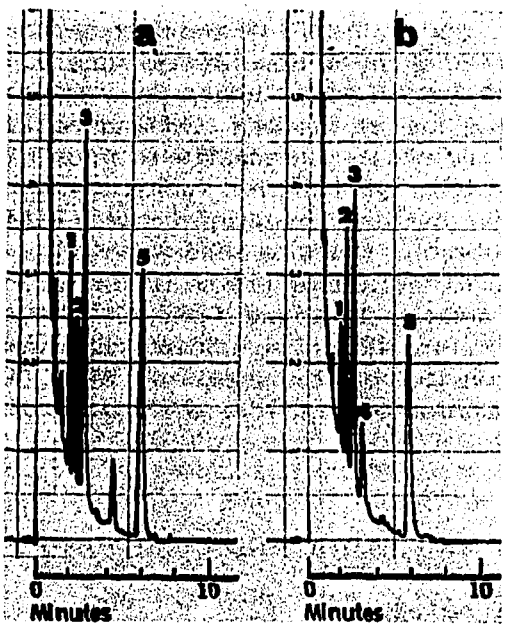

Fig. 3. Chromatograms of human urine extracts. Retention times: acetylmethadol (1), 2.0 min; methadol (2), 2.4 min; noracetylmethadol (3), 2.7 min; normethadol (4), 3.2 min; and compound 20 (5), 5.8 min. (a) Extract of control urine to which 0.25 μg of acetylmethadol (1), 0.50 μg of methadol (2), 1.00 μg of noracetylmethadol (3) and 6.0 μg of the internal standard, compound 20 (5), were added and the extract prepared. Detector sensitivity was $16 \cdot 10^{-11}$ A/mV at full scale. (b) Extract of urine from a patient who received an oral dose of acetylmethadol. The internal standard (5) was added directly to the urine and the extract prepared. Detector sensitivity was $32 \cdot 10^{-11}$ A/mV at full scale.

Panel (b) of Fig. 3 shows the chromatogram obtained from an extract of the urine of a patient who was receiving AM for the treatment of heroin dependence. The urine was collected during the 4–8 h period after an oral dose of AM of 100 mg. The internal standard was added to a 1.0-ml aliquot of the urine and the extract prepared as described in Materials and Methods. As can be seen from Fig. 3b the extract contained AM (1), MOL (2), NAM (3) and NMOL (4). Fig. 3 also shows that compound 20 (5) produces a single peak well separated from the other four bases, as required of a suitable internal standard.

This method has been used for the identification and quantitation of AM, MOL, NAM and NMOL in the urine of AM maintenance patients. In a preliminary report[9] we indicated that the urine collected for 48 h after AM administration contains an average of 2% of the dose as unchanged AM, 5% as MOL and 10% as NAM. In addition 2% of the dose appears as NMOL. A complete report on the biotransformation of AM in these patients is in preparation.

The method we have described should provide the means for the elucidation of the role of biotransformation in the pharmacologic action of AM in man.

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