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# SIMULTANEOUS DETERMINATION OF ACETYLMETHADOL AND ITS ACTIVE BIOTRANSFORMATION PRODUCTS IN HUMAN BIOFLUIDS*

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## SUMMARY

A method employing solvent extraction and gas–liquid chromatography has been developed for the quantitative determination of acetylmethadol simultaneously with its two major biotransformation products, noracetylmethadol and dinoracetylmethadol. Noracetylmethadol and dinoracetylmethadol are analyzed following their conversion to the corresponding amides. The amide structure is confirmed by the use of chemical ionization mass spectrometry and infrared spectroscopy.

The method can be used to determine the concentration of acetylmethadol and these compounds in plasma samples from acetylmethadol maintenance subjects. Methadol and normethadol do not attain measurable plasma levels.

Urine contains predominantly noracetylmethadol and dinoracetylmethadol. Evidence was also obtained for the urinary excretion of acetylmethadol, methadol and normethadol. A mean quantity equal to 28% of the administered dose was excreted in the urine of a 48-h dosing interval as acetylmethadol and metabolites.

## INTRODUCTION

In 1952 Fraser and Isbell[1] reported that a single dose of acetylmethadol (AM) can suppress narcotic withdrawal symptoms for at least three days. Recent clinical studies[2–5] have found that the administration of three oral doses per week of AM is as effective as daily methadone in the treatment of opiate dependence. Studies in laboratory animals[6–8] 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.

In a previous report[9] we identified AM, noracetylmethadol (NAM), methadol (MOL) and normethadol (NMOL) in the urine of AM maintenance subjects (Fig. 1). Billings *et al.*[10] initially identified dinoracetylmethadol (NNAM) in the biofluids of

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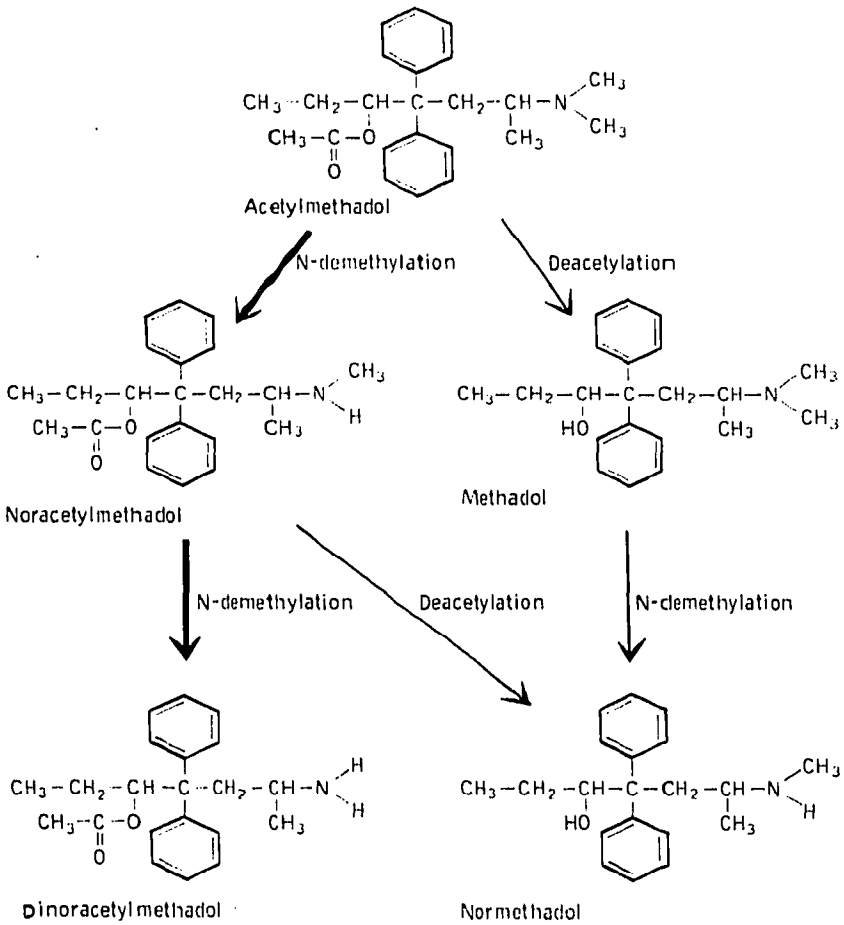


Fig. 1. Structural formulae of acetylmethadol, noracetylmethadol, dinoracetylmethadol, methadol and normethadol.

rats given AM. NNAM is found in the plasma and urine of AM maintenance subjects[11,12].

The purpose of this report is to describe a specific and sensitive method for the quantitation of AM, NAM and NNAM in a sample of human plasma or urine.

## MATERIALS AND METHODS

### *Chemicals and reagents*

The *l*-isomer of α-acetylmethadol was provided by Dr. M. Fink of New York Medical College. α-*d,l*-Noracetylmethadol hydrochloride, α-*d,l*-dinoracetylmethadol maleate, α-*l*-methadol hydrochloride and α-l-normethadol hydrochloride were complimentary research samples from Dr. A. Pohland of the Lilly Research Laboratories (Indianapolis, Ind., U.S.A.). Dr. E. L. May of the National Institutes of Health (Bethesda, Md., U.S.A.) also provided α-*l*-noracetylmethadol hydrochloride and

α-*l*-methadol hydrochloride (Fig. 1). SKF 525-A (β-diethylaminoethyldiphenylpropylacetate hydrochloride) was provided by Smith, Kline and French Labs. (Philadelphia, Pa., U.S.A.). Trifluoroacetylimidazole was obtained from Pierce (Rockford, Ill., U.S.A.). β-Glucuronidase, Type H-1, was obtained from Sigma (St. Louis, Mo., U.S.A.).

Hexane, *n*-butyl chloride and chloroform are Distilled in Glass® and obtained from Burdict and Jackson (Muskegon, Mich., U.S.A.).

*Stock solutions*

Aqueous solutions of AM and its biotransformation products each at a concentration of 4 μg/ml and SKF 525-A at a concentration of 20 μg/ml are prepared and stored in opaque plastic containers in a refrigerator.

*Sample preparation from biofluids*

The extraction procedure is adapted from that described by Inturrisi and Verebely[13] for the extraction of methadone from plasma and urine. A flow sheet outlining this procedure is given in Fig. 2.

To plasma (1–4 ml) in a siliconized 15-ml centrifuge tube with a Teflon®-lined screw cap are added 50 μl of the aqueous solution of SKF 525-A, the internal standard, 1.0 ml of 1 *M* phosphate buffer, pH 7.4, and one drop of 1-octanol. After thorough mixing the sample is extracted with 9.0 ml of *n*-butyl chloride by shaking for 10 min followed by centrifugation at 1500 rpm (500 *g*) for 5 min. The upper, organic, phase is transferred to a second tube and the contents of the initial tube are discarded. The compounds are extracted into 5.0 ml of 0.2 *N* hydrochloric acid by

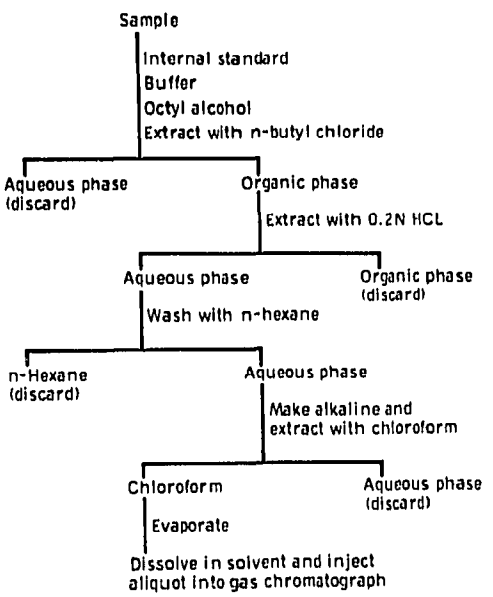


Fig. 2. A flow sheet describing the method for the isolation of acetylmethadol and metabolites from biofluids.

shaking for 7 min and centrifuging for 3 min under the said centrifugation conditions. The upper, organic, phase is removed by aspiration and discarded. The acid phase is washed by the addition of 5.0 ml of *n*-hexane and shaking for 5 min followed by centrifugation for 3 min. The hexane is removed by aspiration and discarded. The washed acid phase is adjusted to pH greater than 13 by the addition of four drops of 50% sodium hydroxide and incubated for 30 min in a heating block at 70° to effect the conversion of NAM and NNAM to their corresponding amides. The compounds are extracted into 8.0 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 organic phase to dryness with the use of a multiple flash evaporator with the bath at 50° (Evap-O-Mix®, Buchler, Fort Lee, N.J., U.S.A.). The sample extract is concentrated in the lower tip of the tube by rinsing the lower sides of the tube with 50 $\mu$l of chloroform and allowing this to evaporate. The sample extract is dissolved in 20 $\mu$l of chloroform and between 1 and 4 $\mu$l are injected into the gas chromatograph.

This extraction procedure is used for the isolation from plasma of AM as the free base and NAM and NNAM as their corresponding amides. NAM and NNAM are extracted from urine by the use of the above procedure. SKF 525-A is the internal standard at 250 $\mu$l per urine sample. AM is quantitated in urine according to the method of Kaiko and Inturrisi[9].

*Recovery*

The recovery of AM, NAM and NNAM from plasma was determined by adding 0.20 $\mu$g of each compound to 2.0 ml control human plasma and extracting as previously described. The amount of each compound recovered was compared to the amount obtained when 0.20 $\mu$g of each was added to 5.0 ml of 0.2 *N* hydrochloric acid and carried through the conversion and concentration procedures. Quantitation was achieved using gas–liquid chromatography (GLC) by the addition of internal standard to the acid phase in both cases.

The above procedure was also used to determine the recovery of 1.0 and 6.0 $\mu$g each of NAM and NNAM from urine.

*Gas–liquid chromatography*

The GLC analysis is performed on a Varian Model 1740 gas chromatograph equipped with a hydrogen flame ionization detector. The column is a 6-ft.-long spiral glass with an I.D. of 2 mm. The liquid phase is 3% SE-30 on Gas-Chrom Q, 80–100 mesh. The temperatures of the detector and injector port are 295 and 275°, respectively. Helium, at a flow-rate of 33 ml/min, is the carrier gas. The hydrogen and air flow-rates are adjusted to 17 and 273 ml/min, respectively, to give maximal detector response. A column oven temperature of 235° is used for the analysis. Detector sensitivity is varied from $4–64 \cdot 10^{-11}$ A/mV at full scale.

*Calibration curves and quantitation*

Standard calibration curves are generated by the addition of AM, NAM and NNAM in the selected amounts from 0.040–0.400 $\mu$g for AM and NNAM and from 0.100–0.400 $\mu$g for NAM to a 4.0-ml sample of control plasma and proceeding as de-

scribed above. Standard calibration curves for NAM and NNAM recovered from urine were established in the range from 1.00–6.00 μg per 4.0 ml urine. 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 generated relating these peak height ratios to the amount of each compound added to the sample. Each calibration curve is constructed from duplicate or triplicate determinations of four to five different points. The amount of each compound in an unknown plasma or 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.

*Identification of compounds in biofluids*

In addition to the identification of AM and its biotransformation products by the chromatographic systems on which they are separated for the purpose of quantitation, the presence of the compounds is confirmed by the use of other methods. A second GLC system is used for the identification of compounds present in plasma. Isobutane chemical ionization mass spectrometry is used similarly for urine.

Plasma samples from AM maintenance subjects are screened for AM, NAM, NNAM, MOL and NMOL in concentrations above 0.020 μg/ml. Standard calibration samples of each compound in concentrations ranging from 0.020–0.200 μg/ml are extracted through the plasma extraction procedure. The procedure is modified to prevent the conversion of NAM and NNAM to their corresponding amides. The final extraction is from 0.2 *N* hydrochloric acid adjusted to pH 7.4 by the addition of 1.0 ml of 10% ammonium hydroxide. The final extract is dissolved in 20 μl of trifluoroacetylimidazole in chloroform (1:4) to effect trifluoroacetyl derivatization of the biotransformation products of AM. The derivatized extract is analyzed on 3% SE-30. Each compound is separated from the others. The temperature of the column oven is increased 0.75 min after each injection at a rate of 10°/min from 210–260°. Other conditions are as in the "amide" method. The retention times are: MOL, 1.9 min; NMOL, 2.5 min; AM, 2.8 min; NNAM, 3.2 min; NAM, 3.5 min.

The identification of AM and its biotransformation products in urine from AM subjects is accomplished by use of chemical ionization mass spectrometry with isobutane as the reactant gas. Aliquots of 40 ml of urine are collected from a subject receiving 100 mg of AM three times per week. The urine is extracted and concentrated to 10-μl extracts.

These urine extracts are introduced directly into the mass spectrometer after removal of solvent. The spectra obtained from the urine extract are compared to that of a synthetic mixture of AM, NAM, MOL and NMOL. A characteristic of isobutane chemical ionization mass spectra is that relatively little fragmentation of compounds is observed and the spectra of most compounds consist of a protonated molecule [an ion at $m/e^* = (M + 1)^+$] and one or two fragment ions[14]. With spectra of this degree of simplicity the components of a mixture can oftentimes be identified from a simple inspection of the mass spectrum of the mixture. The presence or absence

* $m/e$ is the mass to charge ratio. Most ions have unit charge and thus, the value of $m/e$ will be equal to the mass of the ion.

of the several peaks which are associated with each of the components may be taken as evidence for the presence or absence of the components.

*Hydrolysis of conjugation products*

For the determination of the presence of acid hydrolyzable conjugates of NAM and NNAM in urine from AM maintenance subjects, 0.2 ml of concentrated hydrochloric acid is added to 2.0 ml of urine and heated for 1 h at 121° under 18 p.s.i. in an autoclave according to the method of Mulé *et al.*[15]. Prior to the extraction of the bases, the pH is adjusted to 7.4 by the addition of ammonium hydroxide. Other samples are prepared as described above except that immediately after the addition of the acid the pH is readjusted to 7.4 and, thus, the samples are not hydrolyzed. A third set of samples is extracted according to the usual procedure and the concentrations of free NAM and NNAM obtained are compared to the concentrations obtained in the previous two sets of samples. Determinations are done in triplicate.

For the determination of glucuronide conjugates of NAM and NNAM according to the method of Mulé *et al.*[15], duplicate 1.0-ml urine samples are incubated with 1.0 ml of 0.5 *M* acetate buffer, pH 5.0, and 1.0 ml of a solution of $\beta$-glucuronidase (5000 Fishman Units per ml of acetate buffer) for 48 h at 37° with continuous gentle shaking. Duplicate 1.0-samples of the same urine are treated as above without enzyme added. After incubation and just prior to extraction, the pH is adjusted to 7.4 by the addition of 0.2 ml of 10% ammonium hydroxide.

*Further analytical procedure*

Infrared spectra in carbon tetrachloride solution were obtained on a Perkin-Elmer 257 grating infrared spectrophotometer.

*Sample handling*

Blood samples from an AM maintenance subject were drawn 4 and 48 h after an oral dose of 50 mg of AM. The heparinized blood was centrifuged and the plasma recovered. The excretion of AM and active biotransformation products was determined in urine collected from four subjects under treatment for opiate dependence during the 48-h period after an oral dose of AM. Plasma and urine samples were frozen at −20° until the day of analysis.

## RESULTS AND DISCUSSION

During the course of the development of an extraction procedure it was observed that when NNAM is extracted from an aqueous solution at pH 9.8 or greater, less compound is recovered as measured by GLC analysis. At a pH of 13 (using 50% sodium hydroxide) the recovery of NNAM is negligible. The decreased recovery concurrent with an increased extraction pH coincided with an increased recovery of an unknown compound. This observation led to the suggestion that NNAM was being converted to another compound under highly alkaline conditions. Subsequently, NAM was also extracted under highly alkaline conditions and found to react similarly. At a pH of 13 with heating at 70° for 30 min NAM is quantitatively converted to another unknown compound. The alkaline conversion products of NAM and NNAM are of interest because of their potential use as alternative forms for the

separation and quantitation of NAM and NNAM. The chromatographic liquid phase, SE-30, does not separate NAM and NNAM from AM. However, AM and the alkaline conversion products of NAM and NNAM are all separable on SE-30.

Isobutane chemical ionization mass spectral analysis of NAM and its conversion product demonstrates that they are of equal mass ($m/e$ = 340) but have slightly different fragmentation patterns. Infrared spectroscopic analysis of the alkaline conversion product of NAM indicates the absence of an ester and the presence of an alcohol and an amide function as would be expected of the proposed structure. There is no absorbance corresponding to that of the ester function of NAM, which exhibits characteristic bands at 1730 and 1240 $cm^{-1}$. A broad peak, attributable to a hydroxyl function, appears at 3400 $cm^{-1}$. An intense band at 1630 $cm^{-1}$ provides evidence for the presence of an amide function. The conversion products of both NAM and NNAM are not extractable into 0.2 *N* hydrochloric acid from *n*-butyl chloride. The chemical nature of the newly formed compounds and the spectral features enumerated lead to the assignment of the amide structure. The amides would result from an intramolecular acyl shift as shown in Fig. 3. Two compounds of similar structure to NAM and NNAM, norpropoxyphene and dinorpropoxyphene, undergo such a rearrangement under highly alkaline conditions. McMahon *et al.*[16] showed that at pH 11 or above norpropoxyphene undergoes an intramolecular acyl shift.

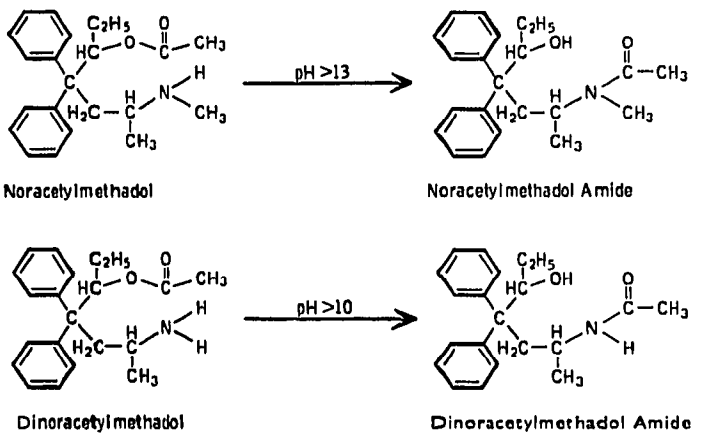


Fig. 3. The alkaline conversion of noracetylmethadol and dinoracetylmethadol to their corresponding amides.

The conditions of the extraction procedure developed allow the amines to be recovered from plasma or urine and then converted to amides by the addition of strong base at the last step in the extraction procedure. This is similar to the method for the determination of norpropoxyphene as described by Verebely and Inturrisi[17]. AM itself is unaffected by this procedure.

After correcting for aliquot losses, the mean recovery from control plasma based on the determinations was 105.0% ± 6.0 S.D. for AM, 86.0% ± 8.2 S.D. for NAM and 91.3% ± 4.6 S.D. for NNAM. After correcting for aliquot losses, the

mean recovery from control urine based on six determinations was 88.2% ± 6.8 S.D. for NAM and 93.0% ± 3.9 S.D. for NNAM. The recovery of the compounds was independent of concentration in the above range.

The method can be used to quantitate as little as 0.010 μg of AM and NNAM and 0.025 μg of NAM per ml of biofluid. The mean precision is 5% relative S.D. for triplicate determinations for each concentration of compound added to plasma and used as the standard calibration curves. The mean linear correlation coefficient for the standard calibration curves is 0.997. The mean precision is 1.7% relative S.D. for duplicate determinations for each concentration of NAM and NNAM added to urine. The mean linear correlation coefficient for the standard calibration curves is 0.995.

Trifluoroacetyl derivatization of extracts of plasma from AM maintenance subjects provided a preliminary screen for the compounds that achieve measurable plasma levels. Most plasma samples contained AM, NAM and NNAM in concentrations above 0.020 μg/ml. Concentrations of MOL and NMOL above this were not observed in any samples obtained from eight subjects drawn at 4, 8, 24, 48 and 72 h following a mean dose of 50 mg of AM. Combined gas chromatography–mass spectrometry analysis of extracts of plasma from AM subjects confirms the absence of methadol and any demethylated biotransformation products of methadol[18]. The trifluoroacetyl derivatization procedure is not used for the routine quantitation of any compound because of the nonlinearity of the standard calibration curves and the relative inconsistency between triplicate determinations compared to the system used. In addition, the need for an extra step, derivatization, is obviated.

TABLE I

PARTIAL CHEMICAL IONIZATION MASS SPECTRUM (ISOBUTANE) OF AN EXTRACT OF URINE FROM AN ACETYLMETHADOL MAINTENANCE SUBJECT

| *m/e** | *Associated compound* | *Absolute intensity*** |
|---|---|---|
| 354 | Acetylmethadol | 676 |
| 340 | Noracetylmethadol | 2925 |
| 326 | Dinoracetylmethadol | 4600 |
| 312 | Methadol | 384 |
| 298 | Normethadol | 134 |

* Molecular weight of protonated nolecule.
** Arbitrary intensity units.

Table I lists the absolute intensity of the protonated molecular ion for each compound in the isobutane chemical ionization mass spectrum of the urine extract. All five bases were identified. The presence of an intense ion at *m/e* 326 corresponds to NNAM*. This is 14 mass units lower than the ion at *m/e* 340 observed for NAM. Fourteen corresponds to the mass of a methylene group. The two demethylated products of AM are, apparently, present in the urine in a high concentration relative to the other compounds.

* At the time of the mass spectral analysis a synthetic chemical standard of NNAM was not available. Subsequently, a standard was obtained, the mass spectral analysis of which revealed the protonated molecular ion at *m/e* 326.

The acid hydrolysis of urine samples obtained from AM maintenance subjects results in a decrease in the measurable concentrations of free NAM and NNAM compared to the non-hydrolyzed control samples. One would expect an increase in these concentrations if NAM and NNAM are excreted in the urine as acid-hydrolyzable conjugates (*e.g.*, glucuronide or sulfate). No change in concentration would have been observed had the compounds not been present as conjugates. The decrease in free base of both compounds might be explained on the basis that they are acid-labile under the rigorous conditions employed for the hydrolysis. $\beta$-Glucuronidase hydrolysis results in no change in the concentrations of free NAM and NNAM. This preliminary observation suggests that glucuronidation is not a major route of biotransformation for NAM and NNAM in the human. An alternate explanation is that the conjugates are excreted principally via the gastrointestinal tract.

Examples of chromatograms obtained under the conditions described in Materials and methods are given in Fig. 4. The multi-step solvent extraction procedure

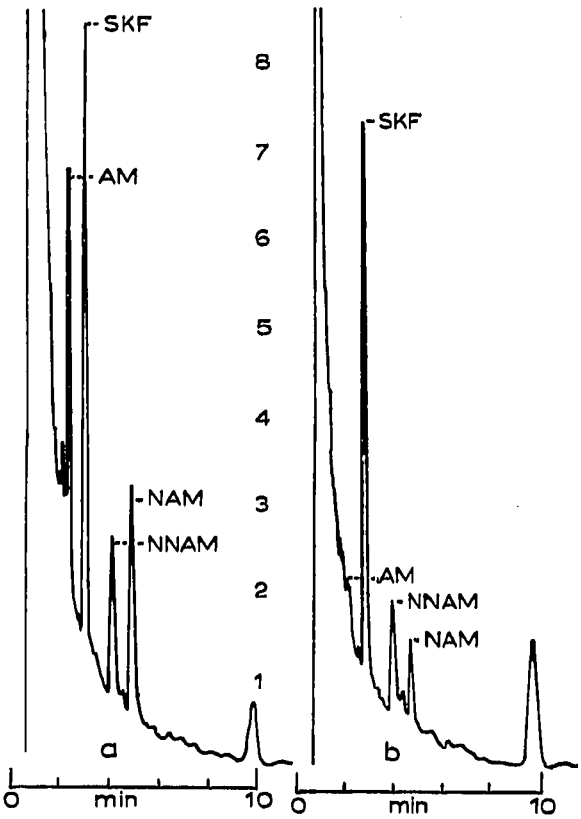


Fig. 4. Chromatograms of human plasma extracts from a patient who received an oral dose of 50 mg of acetylmethadol (AM). The internal standard, SKF 525-A, was added directly to the plasma and the extract prepared. Noracetylmethadol (NAM) and dinoracetylmethadol (NNAM) present in the extract were quantitatively converted to their corresponding amides just prior to the final step of the extraction procedure. (a) 4-h Post-drug plasma. (b) 48-h post-drug plasma. Retention times are: AM, 1.8 min; SKF, 2.5 min; NNAM, 3.6 min; NAM, 4.2 min.

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. Fig. 4a shows a chromatogram of an extract of plasma collected 4 h after an oral dose of 50 mg of AM. The extract contains peaks that correspond in retention time to AM, NAM amide and NNAM amide in addition to the added internal standard, SKF 525-A. Fig. 4b shows the chromatogram of an extract of plasma collected 48 h after AM administration from the same subject. Still, NAM and NNAM are present while AM is not. The principle advantage of this method over that of Billings *et al.*[10] is that it allows the simultaneous determination of the parent compound, AM, along with its two major active biotransformation products, NAM and NNAM, in plasma. The method of Billings *et al.* is not applicable to the quantitation of the parent compound. In addition, the method presented here obviates the need for derivatization. However, the method of Billings *et al.* is more sensitive and thus may be more appropriately used for the determination of NAM and NNAM in plasma following initial AM doses.

Table II shows the percentage of administered dose recovered in the total 48-h post-drug urine as AM and biotransformation products. A mean of 28% with a range of 16.7–37.7% was recovered for four subjects. This included approximately 2% as AM, 8% as NAM, 5% as MOL (using the method of Kaiko and Inturrisi[9] for the quantitation of AM and MOL in urine), and 13% as NNAM. The pattern of excretion for each of the subjects is nearly the same despite a fivefold difference in administered dose.

TABLE II

PERCENTAGE OF ADMINISTERED ACETYLMETHADOL DOSE EXCRETED IN TOTAL 48-h URINE AS ACETYLMETHADOL AND BIOTRANSFORMATION PRODUCTS

| *Subject* | *Dose (mg)* | *Acetyl-methadol* | *Noracetyl-methadol* | *Methadol* | *Dinoracetyl-methadol* | *Total* |
|---|---|---|---|---|---|---|
| L.M. | 20 | 1.5 | 2.9 | 6.0 | 6.3 | 16.7 |
| R.G. | 50 | 1.4 | 15.0 | 2.9 | 18.4 | 37.7 |
| G.E. | 100 | 1.3 | 5.0 | 3.5 | 12.5 | 22.3 |
| I.R. | 100 | 2.8 | 10.1 | 6.8 | 15.5 | 35.2 |
| Mean | | 1.8 | 8.2 | 4.8 | 13.2 | 28.0 |

Twenty-eight per cent of the administered dose of AM is excreted in the total 48-h dosing interval urine. It is likely that there are other routes of elimination for AM and that there are biotransformation products in addition to those discussed. Elimination via the gastrointestinal tract should be investigated. NAM and NNAM may be excreted as N-acetylated products[18]. These amides would not be extracted through the procedure described in this report. There is as yet no evidence that these amides or any possible biotransformation products in addition to those discussed here possess narcotic activity.

An incomplete recovery of the administered dose within a single dosing interval would be consistent with the suggestion that an equilibrium had not yet been attained between drug absorption and elimination in these subjects. Clearly, one would expect that the recovery of initial maintenance doses within a single dosing interval would

necessarily be quite low if these doses are to protect the subjects from abstinence symptoms for the complete duration of the dosing interval. There is evidence for a very slow rate of attainment of an equilibrium between absorption and elimination for acetylmethadol in the human[18]. The concentration of basic drugs by extravascular tissue is well documented[19]. It is likely that these tissues sequester the compounds to an extent capable of dramatically reducing their excretion. Once the tissues become "saturated" excretion increases. Dole and Kreek[20] have suggested that narcotic drugs which have high tissue-binding affinities would become longer acting in the suppression of abstinence once the reservoir of drug in tissues had been established upon repeated drug administration. Likewise, one might speculate that such drugs could be administered in considerably lower doses once the tissue reservoir had been adequately "saturated".

The results of these preliminary studies indicate that biotransformation is a prerequisite for the elimination of AM and that N-demethylation is quantitatively more important than deacetylation for AM in the human. Plasma contains measurable levels of only AM, NAM and NNAM (Fig. 5). Studies in animals[6-8] strongly suggest that NAM and NNAM are important in determining the time-action characteristics of certain effects of AM.

The methods we have described should prove valuable in the elucidation of the pharmacokinetics of AM in the human. We are currently completing the analysis of pharmacokinetic data in relation to the long duration of action of AM in maintenance subjects.

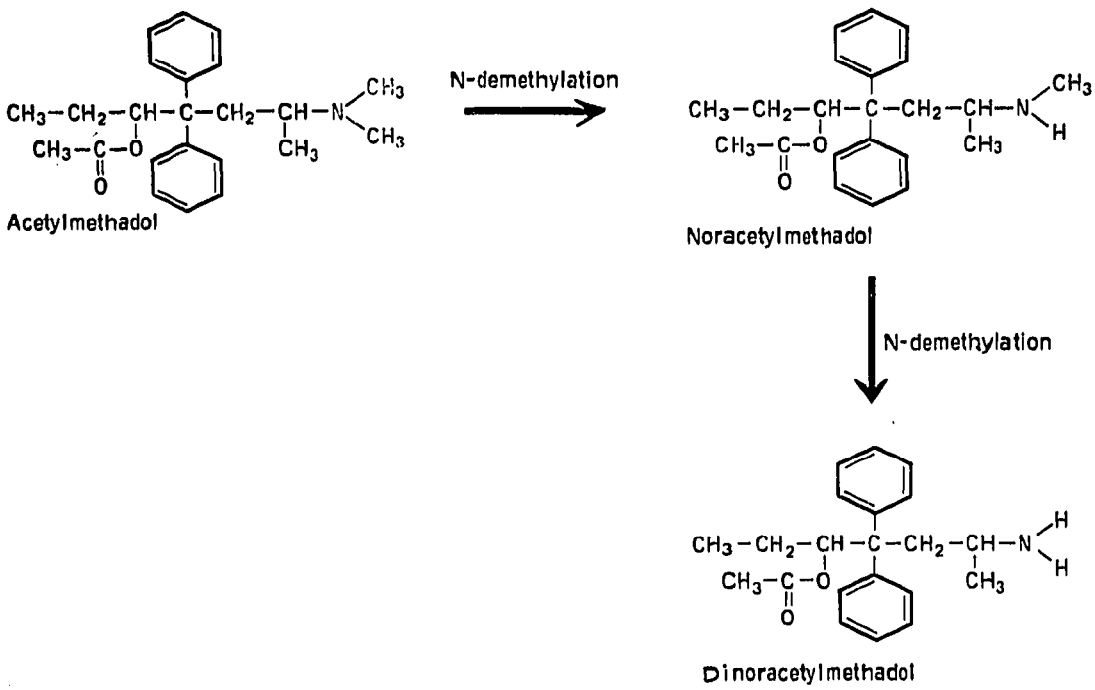


Fig. 5. Acetylmethadol and active biotransformation products, noracetylmethadol and dinoracetylmethadol, predominant in plasma from acetylmethadol maintenance subjects.

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