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EXTRACTION METHOD AND THIN-LAYER CHROMATOGRAPHIC SYSTEM FOR THE DETERMINATION OF α-*l*-ACETYLMETHADOL AND METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

An extraction method and thin-layer chromatographic (TLC) system for the determination of α-*l*-acetylmethadol and its known metabolites (methadol, noracetylmethadol, dinoracetylmethadol, normethadol, 6-acetamido-4,4-diphenyl-3-heptanol, and N-methyl-6-acetamido-4,4-diphenyl-3-heptanol) are described. The parent drug and metabolites are extracted from biological fluids with ethyl acetate and separated by TLC using silica gel plates and a developing system of ethyl acetate–methanol–water–ammonia (85:10:1:1). This system may be used to quantitatively determine levels of radiolabeled drug and metabolites by scraping the TLC plates into 3-mm zonal fractions and measuring the amount of radioactivity by scintillation counting. A representative radiochromatogram obtained from an extract of monkey urine is shown.

INTRODUCTION

The *l*-isomer of α-acetylmethadol (LAM) has been reported to be a long-acting, opiate-like analgesic in man[1,2]. LAM has a longer duration of action than methadone in suppressing narcotic withdrawal symptoms and is presently undergoing clinical studies as a substitute for methadone in the treatment of heroin addiction[3–6]. Recent data suggest that a significant portion of the activity of LAM is due to its metabolites[7–9]. LAM is N-demethylated first to α-*l*-noracetylmethadol (NAM) and then to α-*l*-dinoracetylmethadol (NNAM)[10]. Methadol (MOL), normethadol (NMOL)[11], N-methyl-6-acetamido-4,4-diphenyl-3-heptanol (N-acetylnormethadol) and 6-acetamido-4,4-diphenyl-3-heptanol (N-acetyldinormethadol)[7] have also been identified as metabolites of LAM (Fig. 1).

The purpose of this report is to describe an extraction method and a thin-layer chromatography (TLC) system for the separation of LAM and its metabolites NAM, NNAM, MOL, NMOL, N-acetylnormethadol and N-acetyldinormethadol with a view to their identification and quantitation in urine, plasma, bile and tissues.

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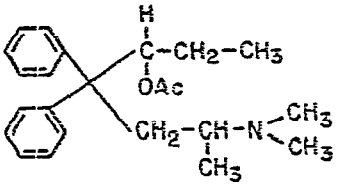
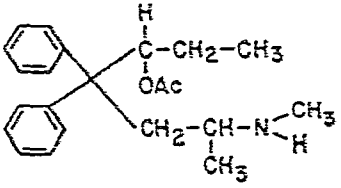
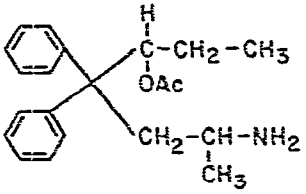


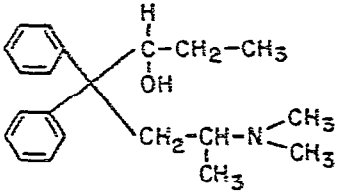
Acetylmethadol (LAM)



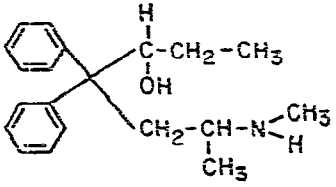
Noracetylmethadol (NAM)



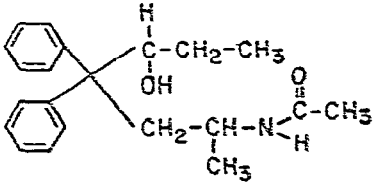
Dinoracetylmethadol (NNAM)



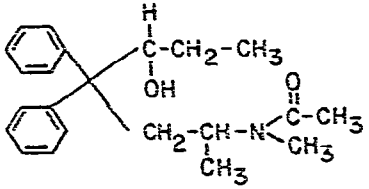
Methadol (MOL)



Normethadol (NMOL)



6-Acetamido-4,4-diphenyl-3-heptanol
(N-Acetyldinormethadol)



N-methyl-6-acetamido-4,4-diphenyl-3-heptanol
(N-Acetylnormethadol)

Fig. 1. Structural formulae of LAM, NAM, NNAM, MOL, NMOL, N-acetylnormethadol and N-acetyldinormethadol.

MATERIALS AND METHODS

Chemicals and reagents

LAM, NAM, NNAM, MOL, NMOL, N-acetylnormethadol and N-acetyldinormethadol were provided by the Research Triangle Institute (through the National Institute on Drug Abuse) and by Lilly Research Laboratories.

All solvents used in the procedure were reagent grade and were glass distilled in our laboratory.

Stock solutions. Methanolic solutions of LAM, NAM, NNAM, MOL and NMOL each at a concentration of 400 ng/μl were prepared and kept refrigerated.

Sample preparation

Each 2-ml urine, plasma, or bile sample, in a 30-ml centrifuge tube with a PTFE lined screw cap, was diluted with 2 ml of distilled water and the pH carefully adjusted to 9.5 with 0.1 *N* NaOH solution. The sample was then extracted with 10 ml of distilled ethyl acetate by shaking for 1 min using a Vortex mixer followed by centrifugation at 362 *g* for 5 min. The upper organic phase was transferred to a second tube and the extraction was repeated with another 10 ml of ethyl acetate. The ethyl acetate was evaporated under nitrogen and the sample extract concentrated in the lower tip of the tube by rinsing the lower sides of the tube with approximately 100 μl of ethyl acetate and allowing this to evaporate. The free drug and metabolites in the extract were then determined by TLC.

Recoveries

To determine the per cent recovery of LAM, NAM, MOL and NMOL from urine, one set of ten urine samples was fortified with LAM and NAM, and another set with MOL and NMOL, each at a concentration of 1 μg/ml. The samples were then extracted with ethyl acetate following the same procedure described above for sample preparation. The extract was dissolved in about 100 μl of distilled acetone and between 2–3 μl were injected into the gas chromatograph fitted with a flame ionization detector.

The recovery of LAM was also determined by fortifying urine with tritiated LAM at a concentration of 1 μg/ml (about 9755 dpm) and extracting following the procedure described above. The extract was transferred to a glass scintillation vial, Searle Scintillation Cocktail (PCS) added, and the amount of radioactivity in the extract determined using a liquid scintillation counter (Searle Mark II). Tritiated toluene was used as an internal standard to correct for quenching.

The recovery of NNAM was determined using tritiated metabolite isolated from rat bile. The radioactive metabolite was purified by TLC, and its purity and structure were determined by gas chromatography–mass spectrometry (GC–MS). Ten urine samples were fortified with the radioactive metabolite (about 1 μg/ml, 12,000 dpm) and were extracted as described above. The recovered radioactivity was determined by liquid scintillation counting.

Gas–liquid chromatography

Gas–liquid chromatographic (GLC) analysis was performed on a Tracor MT-220 gas chromatograph equipped with a hydrogen flame ionization detector. The column was a 6 ft. × 1/8 in. I.D. U-shaped glass column. The packing consisted of 3% OV-25 on 100–120 mesh Gas-Chrom Q. The column oven temperature was 165°. The injector temperature and the detector temperature were 240° and 270°, respectively. The carrier gas was nitrogen at a flow-rate of 40 ml/min. Hydrogen and air flow were adjusted to give maximal detector response. The retention times of LAM, NAM, MOL and NMOL were 14.67, 16.06, 15.59, and 17.30 min, respectively.

Calibration curves and quantitation

The concentrations of the different compounds were determined by comparing the peak areas of the standards. Calibration curves were constructed using standards of different concentration. Each calibration curve was constructed from duplicate

determinations of at least four different points and has been found to be linear over the range of concentrations used.

Thin-layer chromatography

Precoated thin-layer plates (20 × 20 cm, silica gel (Merck) 0.25 mm) were marked into six lanes with a spatula. The extracts were reconstituted in about 50–75 μl of distilled methanol and streaked about 2 cm from the bottom of the plate using a 100-μl Hamilton syringe. Standards were spotted on the same plate to determine the position of the compounds under study. The plates were developed at room temperature in equilibrated tanks lined with Gelman adsorbent pads using the solvent system ethyl acetate–methanol–water–concentrated ammonia (85:10:1:1). Development time was about 50 min. The compounds were localized after development by spraying the standards with iodoplatinate spray reagent.

Quantitative thin-layer chromatography

The developed silica gel plate was scraped into 3-mm zones and the silica gel was transferred to glass scintillation vials, mixed with 3.5 ml of water and 10 ml of PCS and then counted in a liquid scintillation counter. Tritiated toluene was used as an internal standard to correct for quenching. A plot of the radioactivity found in each fraction (dpm) *versus* the distance of the fraction from the origin showed a distribution of the radioactivity (Fig. 2). In each case, radioactive peaks which corresponded to the R_F values of authentic standards were assumed to be identical to the standards.

RESULTS AND DISCUSSION

Solvent extraction

A number of solvents, including ethyl acetate, diethyl ether, *n*-butyl chloride, methylene chloride, chloroform and chloroform–isopropanol in different proportions were evaluated for their ability to extract LAM and its metabolites from urine. Urine samples obtained from dogs and monkeys which received ^{3}H-labeled LAM were extracted with the different solvents at pH 9.5 and the amount of radioactivity recovered was determined by liquid scintillation counting. On the basis of such determinations, ethyl acetate was chosen as the solvent for the extraction of the free drug and metabolites. Therefore, per cent recovery of the different metabolites from urine using ethyl acetate was determined by GC as described in Materials and methods. Mean recoveries for ten urine samples quantitated by GC were 91 ± 5.4% for LAM, 91 ± 6.0% for NAM, 88 ± 9.0% for MOL, and 89.6 ± 8.0% for NMOL. Recovery of LAM using tritiated drug was 93 ± 2.4% when analyzed by liquid scintillation counting.

NNAM recovery was determined using the pure radioactive metabolite isolated from rat bile and purified by TLC. Structure and purity of the metabolite were confirmed by GC–MS. Recovery from urine was found to be 96 ± 2.0% using ethyl acetate (ten samples fortified with 12,000 dpm, 1 μg/ml). Careful adjustment of the pH of the urine sample to 9.5 was found to be important, since spontaneous conversion of NAM to N-acetylnormethadol and NNAM to N-acetyldinormethadol in appreciable amounts was observed when the pH was adjusted to 13. Such an intramolecular acyl shift under alkaline conditions has been reported by McMahon *et al.*[12]

in norpropoxyphene and dinorpropoxyphene, which are of similar structure to NAM and NNAM. Preliminary pH studies in our laboratory have shown that conversion of NAM to N-acetylnormethadol and NNAM to N-acetyldinormethadol increases when the pH is increased from 9.5 to 13. The amount of N-acetylnormethadol and N-acetyldinormethadol formed at pH 9.5 used in our recovery study has been found to be negligible as determined by the GC system described in Materials and methods. At a column temperature of 210° the retention times of the N-acetylnormethadol and N-acetyldinormethadol are 9.6 and 8.0 min, respectively. Billings *et al.*[7] have reported the presence of metabolically formed N-acetylnormethadol and N-acetyldinormethadol in human urine. N-Acetyldinormethadol has also been reported as a major urinary and biliary metabolite of methadol[13].

Thin-layer chromatography

A number of TLC solvent systems were evaluated for the separation of authentic standards of LAM, NAM, NNAM, MOL, NMOL, N-acetylnormethadol, and N-acetyldinormethadol. Both acidic and basic solvent systems were attempted in one- and two-dimensional chromatography on silica gel plates. Some of the solvent systems evaluated included butanol–ammonia, butanol–acetic acid, butanol–chloroform–ammonia, ethyl acetate–methanol–ammonia and others. The best separation of all the seven standards was achieved in one-dimensional chromatography using the solvent system ethyl acetate–methanol–water–concentrated ammonia (85:10:1:1). The R_F values of the different compounds are shown in Table I.

TABLE I

R_F VALUES OF LAM AND METABOLITES USING THE SOLVENT SYSTEM ETHYL ACETATE–METHANOL–WATER–CONC. AMMONIA (85:10:1:1)

Compound	*R_F value*	*Iodoplatinate*
N-Acetylnormethadol	0.76	yellow
LAM	0.70	purple
MOL	0.63	purple
N-Acetyldinormethadol	0.58	yellow
NNAM	0.53	purple
NAM	0.42	purple
NMOL	0.28	purple

Quantitative thin-layer chromatography

Fig. 2 is a representative radiochromatogram showing the spectrum of metabolites and their amounts in a monkey urine extract as determined by scraping the plate in 3-mm sections and counting the radioactivity. A great amount of radioactivity remains at the origin and represents conjugated and polar metabolites extracted by the ethyl acetate. Enzymatic and acid hydrolysis of the conjugates is being carried out and the samples analyzed using the same procedure. Direct counting of the silica gel by suspending it in a liquid scintillation gel (PCS and water) has been found to be a fast, sensitive and quantitative method. Elution of the silica gel fractions with methanol and removal of the methanol prior to scintillation counting was lengthy and tedious with no appreciable improvement in counting efficiency. Use of omnifluor scin-

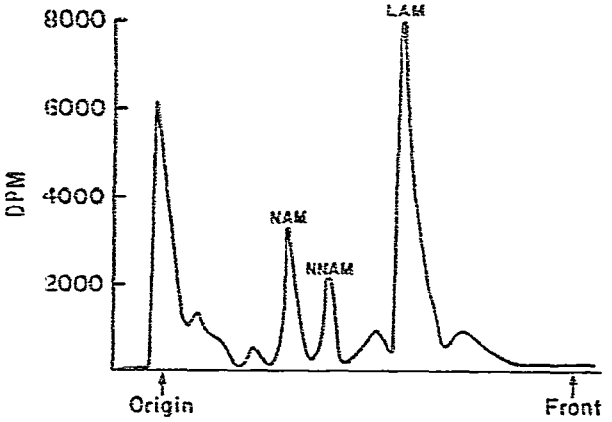


Fig. 2. Representative TLC radiochromatogram of a monkey urine extract collected 24 h after ^{3}H-labeled LAM administration.

tillation cocktail to elute directly the compounds from the silica gel particles in the scintillation vial showed poor efficiency. This could be due to incomplete elution of the various compounds and to reabsorption of the compounds on the silica gel particles in the bottom of the vial.

The method we have described should provide a means for quantitative determination of the different metabolites of LAM in different species administered radiolabeled LAM. It should also provide a means for the elucidation of the role of biotransformation in the pharmacological action of LAM.

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