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DETERMINATION OF ACETYLMETHADOL AND METABOLITES BY USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the simultaneous determination of *l*,α-acetylmethadol (LAAM) and five active metabolites — noracetylmethadol, dinoracetylmethadol, methadol, normethadol, and dinormethadol — in biofluids by high-performance liquid chromatography using a normal-phase column and a UV detector at 218 nm. The compounds are recovered from biofluids by a multistep liquid—liquid extraction. The mobile phase is methanol—acetonitrile (70:30, v/v) containing 0.015% ammonium hydroxide as the modifier. Retention times can be varied by adjusting the composition of the mobile phase to maximize peak height for quantitation using *l*-propranolol as the internal standard or peak separation for the collection of fractions. Using a UV detector the lower limit of sensitivity is 10 ng/ml of biofluid. Using fraction collection of radiolabeled drug and metabolites followed by liquid scintillation counting the lower limit of sensitivity is 1.0 ng/ml. Commonly used or abused narcotics including morphine, heroin, meperidine, methadone and propoxyphene do not interfere with the analysis. The method has been applied to plasma and urine samples from humans, sheep and rats. Extracts of urine from patients receiving maintenance treatment with LAAM contain LAAM and each of the five active metabolites.

INTRODUCTION

l,α-Acetylmethadol (LAAM) is a synthetic narcotic analgesic under development for the maintenance treatment of opiate dependence. LAAM has a relatively longer duration of action in the suppression of opiate withdrawal than methadone, the established maintenance drug [1]. Studies of the disposition of LAAM in maintenance patients indicate that this long duration of

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action is due predominantly to the formation of active and persistent metabolites [2]. These metabolites, all levo-alpha isomers, include noracetylmethadol (NAM), dinoracetylmethadol (DNAM), methadol (MOL), normethadol (NMOL) and dinormethadol (DNMOL) (Fig. 1). The clinical evaluation of LAAM has stimulated pharmacokinetic studies in man and laboratory animals. As might be expected for a drug with a complex metabolism, the disposition of LAAM has been studied using a number of analytical techniques including thin-layer chromatography [3—5], gas—liquid chromatography [6—8] and combined gas chromatography—mass spectrometry [9, 10]. None of these methods, as reported, fulfills both the selectivity and sensitivity requirements of a complete analytical method.

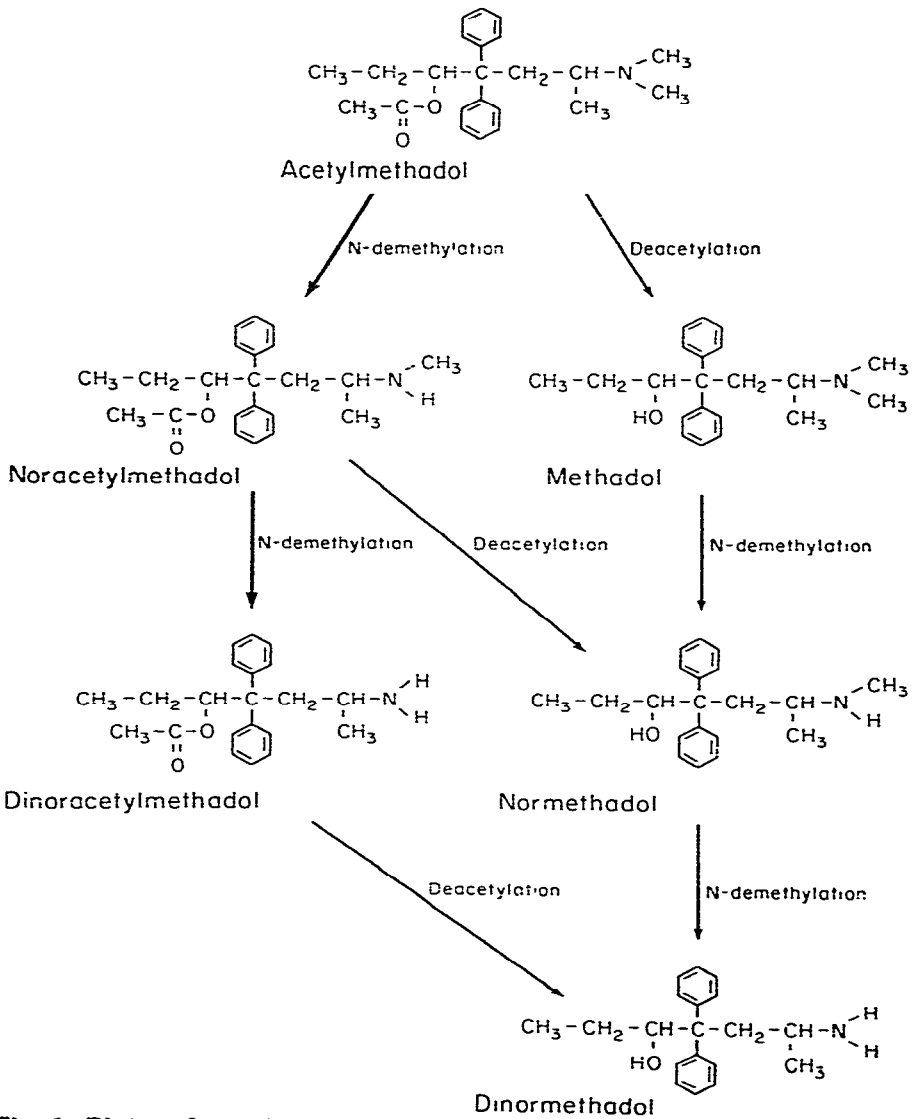

Fig. 1. Biotransformation pathways for acetylmethadol (LAAM), and structural formulae for LAAM, noracetylmethadol (NAM), dinoracetylmethadol (DNAM), methadol (MOL), normethadol (NMOL) and dinormethadol (DNMOL).

We will describe a method using high-performance liquid chromatography (HPLC) that can resolve the compounds of interest and demonstrate that this method has the requisite sensitivity to measure these compounds in biofluid samples from man and animals that have received single or multiple doses of LAAM.

MATERIALS AND METHODS

Chemicals and reagents

The following compounds were obtained from the Research Triangle Institute (Research Triangle Park, NC, U.S.A.) through the Medicinal Chemistry and Technology Section of the National Institute on Drug Abuse (NIDA), Rockville, MD, U.S.A.: *l*,α-acetylmethadol, *l*,α-noracetylmethadol, *l*,α-methadol and *l*,α-normethadol, all as the hydrochloride salt; *l*,α-dinoracetylmethadol and *l*,α-dinormethadol both as the maleate salt; and *l*,α-[O,O'-$^3H_2$]-acetylmethadol ([^{3}H] LAAM) at 1.4 Ci/mmole. The radiochemical purity of the [^{3}H] LAAM was found by HPLC to be greater than 98%. The *l*-propranolol was a gift of Dr. R. Levi, Cornell University Medical College (New York, NY, U.S.A.). Methanol, acetonitrile, *n*-butyl chloride, hexane and ethyl acetate were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Ethyl acetate was redistilled before use. Ammonium hydroxide [58% NH_4OH in water (w/w)] was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). The percentage of ammonium hydroxide in the mobile phase refers to the final concentration of NH_4OH expressed as a percentage (v/v).

Stock solutions

LAAM, MOL, NAM, DNAM, NMOL and DNMOL stock solutions at a concentration of 1 mg/ml and the internal standard, *l*-propranolol, at 0.1 mg/ml are prepared in methanol and stored at −5°C. Calibration standards are prepared by diluting the stock solutions with a mixture of methanol—acetonitrile (20:80, v/v).

Sample preparation from biofluids

Into a 13-ml ground-glass centrifuge tube with a Teflon stopper cap, add 1 ml of biofluid (plasma or urine), 0.10 ml of a 1.0 μg/ml solution of the internal standard and 0.5 ml of Delory and King's (D & K) carbonate—bicarbonate buffer (1 *M*, pH 9.6) [11]. The sample is extracted with 5 ml of *n*-butyl chloride for 7 min in an automatic shaker and centrifuged for 6 min at 500 *g*. The *n*-butyl chloride phase (upper) is transferred to a clean tube and evaporated to dryness (Vortex Evaporator Model 3-2200, Buchler Corp., Fort Lee, NJ, U.S.A.). This process is repeated again and the combined residue of the *n*-butyl chloride extract is reconstituted in 5 ml of *n*-hexane. The *n*-hexane phase is extracted with 2.5 ml of 0.2 *N* HCl by shaking for 7 min. After centrifugation for 6 min the *n*-hexane layer (upper) is discarded. The acid phase is washed with 5 ml of *n*-hexane and the pH adjusted to 9.6 by titration with 5 *N* ammonium hydroxide and the addition of 0.5 ml of the D & K buffer.

This aqueous phase is extracted twice with 5 ml of ethyl acetate by shaking for 7 min. After centrifugation for 6 min the ethyl acetate is transferred to a

12-ml siliconized conical centrifuge tube and evaporated to dryness as above. The sample extract is reconstituted in 100 μl of methanol—acetonitrile (20:80, v/v), and an appropriate volume of up to 100 μl is injected into the HPLC system.

Procedure for radiolabeled samples

To biofluid samples collected from animals that had received [^{3}H] LAAM are added 100 μl of a 15 μg/ml solution of unlabeled LAAM and metabolites as carriers. The internal standard is added and the sample extracted as above.

Chromatographic conditions

The analysis is performed on a Varian Model 8500 liquid chromatograph (Varian Instrument Group, Sunnyvale, CA, U.S.A.) equipped with a displacement syringe pump, a UV—Vis variable-wavelength detector (Varichrom Model VUV-10) and a Rheodyne Model 7125 syringe-loading sample injector (Rheodyne, Berkeley, CA, U.S.A.). The column is a 30 cm × 4 mm I.D. Varian Micropak containing 5-μm LiChrosorb Si-60. Chromatograms are recorded on a Model A-25 dual-channel chart recorder set at 1 mV. The mobile phase is a mixture of methanol—acetonitrile (70:30, v/v) containing 0.015% ammonium hydroxide. The flow-rate is 1.5 ml/min and the column and detector temperatures are maintained at 30°C. The column effluent is monitored at 218 nm at recorder scale that varies from 8 to 32 mA at full scale, and the chart speed is 10 in./h.

Following the injection of radiolabeled samples the column effluent corresponding to the peak area of each compound of interest is collected directly into a glass scintillation vial. The column effluent is evaporated to dryness and the residue reconstituted in 0.5 ml of methanol. Ten milliliters of a liquid scintillator containing 1% Liquifluor (New England Nuclear, Boston, MA, U.S.A.) in toluene are added and the sample is counted in a Model LS3100 liquid scintillation counter (Beckman Instruments, Irvine, CA, U.S.A.). Counting efficiency is determined by the internal standard method using a tritiated toluene standard. The extraction recovery is estimated by comparing the peak height of each compound of interest to an absolute standard curve.

Calibration curves and quantitation

Standard calibration curves are established by adding LAAM, MOL, NAM, DNAM, NMOL, DNMOL and *l*-propranolol to drug-free plasma or urine and proceeding as described above. Quantitation is performed by drawing the baseline and measuring the peak height of the compounds of interest. The ratio peak height standard/*l*-propranolol is calculated. A standard curve is constructed by plotting the peak height ratio against the amount added. Each calibration curve is constructed from at least triplicate determinations of five points.

Human and animal studies

Biofluid samples were collected from humans, sheep and rats following LAAM administration. Plasma and urine were collected from former heroin addicts receiving 40—60 mg of LAAM, orally, 3—5 times per week for 3—7

weeks as part of a LAAM maintenance treatment program. A Dorset ewe weighing 50 kg and prepared with polyethylene catheter inserted into the hindlimb artery and vein received a LAAM infusion of 0.02 mg/kg per min into the vein for 6 h. Blood samples were collected prior to, during and at 18 h after the cessation of drug administration. Male Sprague-Dawley rats weighing 300—350 g were prepared by cannulation of the right jugular vein. Blood and urine samples were collected prior to and from 5 min to 40 h after the rapid intravenous injection of 2.5 mg/kg unlabeled LAAM plus 130 μCi of [^{3}H] LAAM per rat. Blood samples were centrifuged at 500 *g* for 10 min and the plasma recovered. Plasma and urine are frozen at —20°C prior to analysis.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

LAAM and its metabolites are weak bases, which partially ionize in aqueous solution. This property makes it difficult to achieve a good separation using a reversed-phase column unless ionization of the compounds of interest can be suppressed by raising the pH of the mobile phase. Unfortunately, this approach often results in a degradation in column performance [12]. Paired-ion chromatographic techniques [13] may overcome the problem of column degradation, but the reagent used for ion-pairing has a UV cut-off at about 240 nm and therefore prevents use of the more sensitive short-wavelength absorption. Since LAAM and its metabolites have very similar structures but different functional groups (Fig. 1) they are amenable to separation by normal-phase chromatography. The polar nature of the compounds of interest suggests that methanol, a solvent with a low UV cut-off and rather good polarity, might serve as the mobile phase. All of the compounds except MOL and NAM were separated but with a very long analysis time and peak tailing. This is probably the result of an acid—base interaction, where the mass transfer is slow between a weakly basic drug and the acidic sites of silica gel [14]. A small amount of ammonium hydroxide was added as a modifier to compete at the acidic sites of silica gel, leaving the compounds of interest free to adsorb at other sites where mass transfer is faster. Ammonium hydroxide will also increase the pH of the mobile phase, resulting in suppression of the ionization of the compounds of interest. The addition of this modifier substantially decreased analysis time, but MOL and NAM were still not resolved.

The addition of acetonitrile can improve the resolution of MOL and NAM without compromising the resolution of the other compounds of interest. Fig. 2 shows the effect of the addition of acetonitrile to the mobile phase on column capacity factor (k') and analysis time. When the concentration of acetonitrile exceeds 50%, the resolution between DNMOL and NMOL and between DNAM and NAM begins to decrease (not shown in Fig. 2). Since acetonitrile has a lower polarity than methanol, the analysis time increases as the concentration of acetonitrile in the mobile phase is increased (Fig. 2). Fig. 3 shows the effect of relatively small changes in the concentration of ammonium hydroxide on k', analysis time and peak height. As the concentration of ammonium hydroxide is increased from 0 to 0.02%, k' and analysis

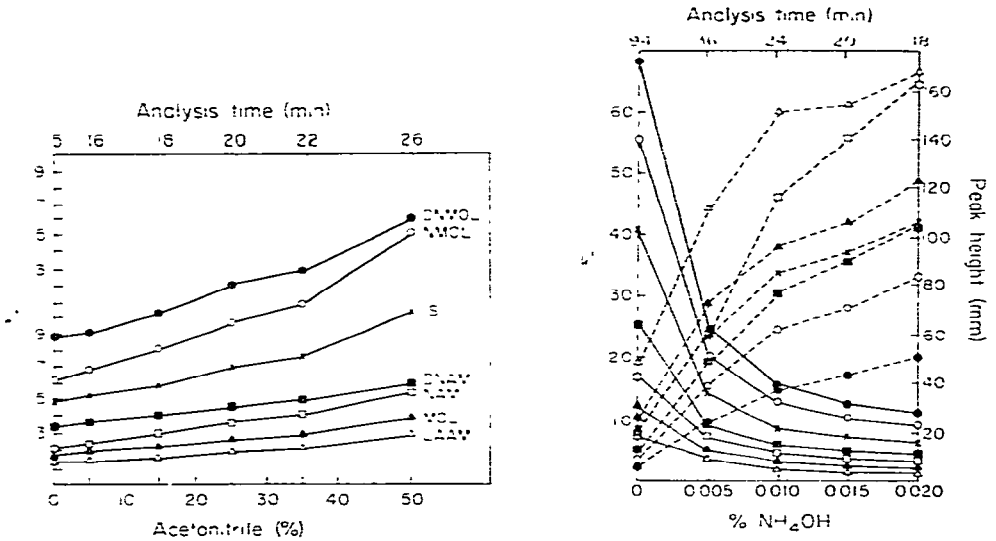

Fig. 2. Effect of increasing the percentage of acetonitrile in the mobile phase (methanol—acetonitrile) from 0% to 50% on the column capacity factor (k') of each compound of interest. The ammonium hydroxide modifier was kept constant at 0.015%. The chromatographic conditions are as described in Materials and methods. The abbreviations for LAAM and metabolites are as in Fig. 1 (IS = internal standard). The time required for a complete analysis is given on the upper abscissa.

Fig. 3. Effect of increasing the ammonium hydroxide modifier from 0 to 0.020% on the column capacity factor (k') of each compound of interest and on the peak height response. The mobile phase is methanol—acetonitrile (70:30, v/v). The chromatographic conditions are as described in Materials and methods. The abbreviations for LAAM and metabolites are as in Fig. 1. The time required for a complete analysis is given on the upper abscissa. (○) NMOL; (●) DNMOL; (□) NAM; (■) DNAM; (△) LAAM; (▲) MOL; (X) IS (internal standard).

time decrease while peak height is increased. The results shown in Figs. 2 and 3 demonstrate that by adjustment of the proportions of methanol, acetonitrile and ammonium hydroxide the system can be made optimal for quantitation by peak height measurement using the UV detector or for fractional collection of each compound of interest without cross-contamination.

A sample chromatogram demonstrating the resolution of the compounds of interest is given in Fig. 4. The use of elevated pH and methanol in a mobile phase can result in rapid deterioration of the column performance of a silica gel based packing material due to solubilization of the silica matrix. We have minimized this problem by placing a 20 × 4 mm guard column containing 40-μm silica (Vydac A, Varian Instrument Group) before the analytical column. The guard column serves to filter some contaminants in the applied samples and will saturate the mobile phase with silica to reduce the rate of dissolution of silica from the analytical column. These columns can be used for at least three months with only a slight loss in efficiency.

Column efficiency

The effects of flow-rate and temperature were investigated to determine optimum column efficiency. Since the mass diffusion term is much less im-

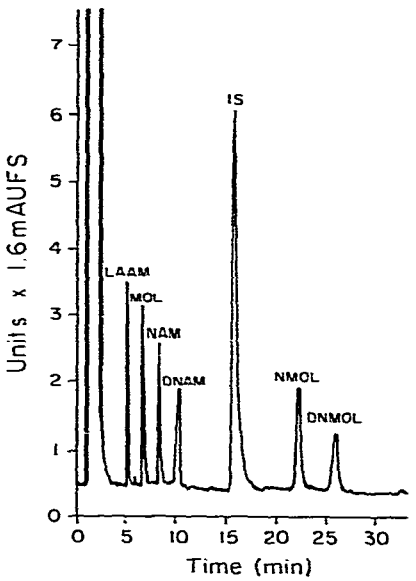

Fig. 4. Chromatogram of a calibration standard. To 1 ml of control human plasma were added LAAM and metabolites at 40 ng each and the internal standard (IS), propranolol, at 100 ng. The plasma extract was prepared as described in Materials and methods. The extract was reconstituted in 100 µl of methanol—acetonitrile (20:80, v/v) and 70 µl were injected. Chromatographic conditions are as described in Materials and methods. Abbreviations are as in Fig. 1.

portant in liquid chromatography than in gas chromatography, column efficiency is expected to be better at lower flow-rates due to good mass transfer. This is illustrated in Fig. 5 which shows the height equivalent to theoretical plates (HETP) obtained at different flow-rates. The HETP is quite low (< 0.09 mm) throughout the entire range and, except for MOL, the curves show a minimum at 50—70 ml/h. However, for a convenient analysis time we chose 80—90 ml/h. Increasing the column temperature from 20 to 50°C only slightly affects the column efficiency and analysis time. However, it is important to maintain the temperature of the column and detector constant when a wavelength in the far UV is used, since relatively small temperature fluctuations caused by heat generated from the pump or variations in room temperature will affect the precision of the retention time and the baseline. We chose to maintain the temperature of the column and detector cell at 30°C.

Reconstitution of the sample extract

The sample extract is dissolved in 100 µl of methanol—acetonitrile (20:80, v/v), a weaker solvent than the mobile phase, so that the sample will be concentrated at the column head as the reconstitution solution passes through the column.

Detection in the ultraviolet

The absorbance of LAAM and its metabolites increases significantly in the far UV. A wavelength of 218 nm was selected as a compromise between the

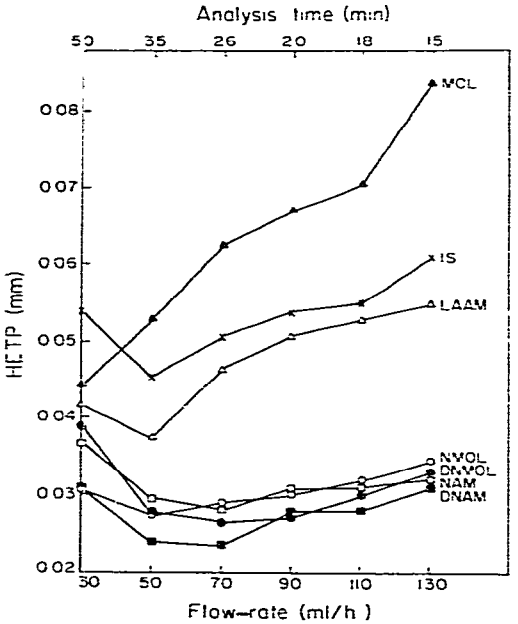

Fig. 5. Effect of the flow-rate of the mobile phase, methanol—acetonitrile (70:30, v/v) with 0.015% ammonium hydroxide on column efficiency as measured by height equivalent to theoretical plates (HETP). The chromatographic conditions are as described in Materials and methods. Abbreviations are as in Fig. 1 (IS = internal standard). The time required for a complete analysis is given on the upper abscissa.

maximum sensitivity and acceptable noise. At a cell temperature of 30°C (see above) the retention time of each compound is quite stable. The coefficient of variation for retention time was determined from eight consecutive injections of a mixture of the compounds of interest and was found to vary from 0.26% for LAAM to 0.73% for DNMOL. This reproducibility of retention time can permit the "blind" collection of fractions that correspond to the established retention times of the compounds of interest when the concentration of a sample falls below the lower limit of sensitivity of the UV detector. Samples collected in this manner can be analyzed using a sensitive radioimmunoassay procedure (studies in progress).

Extraction recovery and calibration curves

The optimal conditions for the simultaneous extraction of LAAM, metabolites and the internal standard, *l*-propranolol, require careful attention to pH and partition conditions. DNAM, a major metabolite is rapidly converted to the corresponding amide at pH 10.0 or greater [6]. The back-extraction of DNAM and DNMOL from *n*-butyl chloride into acid is incomplete and variable, therefore we found it necessary to evaporate the *n*-butyl chloride to dryness and reconstitute with a less polar solvent, *n*-hexane. The back-extraction from *n*-hexane into acid is nearly complete. The recovery of the compounds of interest is independent of concentration from 40 to 2000 ng/ml. After a correction for aliquot losses the mean recovery and coefficient of variation (C.V.) as

percentages are: LAAM = 95 (C.V. = 1.1), MOL = 88 (C.V. = 1.7), NAM = 89 (C.V. = 1.1), DNAM = 67 (C.V. = 3.7), NMOL = 85 (C.V. = 2.1), DNMOL = 77 (C.V. = 2.0).

The extraction procedure yields an extract that is free of peaks that interfere with the quantitation of the compounds of interest (Fig. 4).

Fig. 6 shows that, using the UV detector, standard calibration curves can be constructed that are linear from 10 to 100 ng. Although not shown in Fig. 6, the standard calibration curves are linear up to 2000 ng. The precision of triplicate determinations of 20 and 100 ng calibration standards is given in Table I. Using an initial volume of 1 ml, this system has a lower limit of sensitivity (signal-to-noise ratio of 5) of 10 ng/ml for DNAM, NMOL and DNAM and 6 ng/ml for LAAM, MOL and NAM.

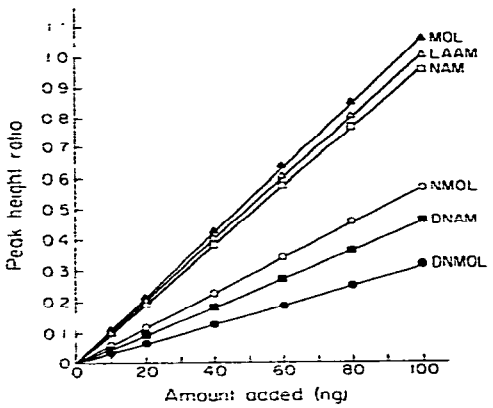

Fig. 6. Standard calibration curves for LAAM and metabolites recovered from control human plasma. Each point represents the mean of triplicate determinations. Abbreviations are as in Fig. 1.

TABLE I

PRECISION VALUES FOR THE CALIBRATION CURVE OF LAAM AND METABOLITES

Compound	Coefficient of variation (%)	
	20 ng	100 ng
LAAM	3.3	1.6
MOL	4.1	1.7
NAM	3.1	3.2
DNAM	5.2	4.8
NMOL	3.3	2.0
DNMOL	5.0	4.5

Potential interference

As shown in Table II, LAAM and metabolites are resolved from commonly used or abused narcotics and their metabolites. Thus, this method can be used to analyze samples from subjects taking or receiving the drugs given in Table II.

TABLE II

RESOLUTION OF LAAM AND METABOLITES FROM SELECTED NARCOTICS AND THEIR METABOLITES

Drug*	Column capacity factor (k')
Heroin	1.18
6-Monoacetylmorphine	1.37
Propoxyphene	1.52
Codeine	1.52
Morphine	1.59
Meperidine	1.71
LAAM	2.03
MOL	2.72
NAM	3.70
DNAM	4.70
Pentazocine	6.82
NMOL	10.57
Normeperidine	11.17
DNMOL	12.88
Methadone	13.14
Levorphanol	14.88
Norpropoxyphene	>15
Methadone metabolite 1	>15

*Abbreviations as in Fig. 1.

HPLC and liquid scintillation counting of radiolabeled samples

The availability of a tritiated form of LAAM with high specific activity led us to adapt the HPLC system we have described for the collection of fractions containing the radiolabeled compounds of interest so that they could be analyzed by liquid scintillation counting. The location and recovery of the compounds of interest are determined by adding unlabeled "carrier" in amounts such that any contribution to the UV detector response by the radioisotope is less than 5%. The limit of detection is a function of the specific activity of the radiolabeled drug and the average background. We can easily determine 1.0 ng/ml of [^{3}H]LAAM. The chromatographic conditions are adjusted to prevent cross-contamination of the peaks. We have found a satisfactory mobile phase to be methanol—acetonitrile (65:35, v/v) with 0.01% ammonium hydroxide (see also Figs. 2 and 3).

Human and animal studies

Examples of the application of the method using the UV detector for the quantitation of LAAM and metabolites are presented in Tables III and IV. LAAM and each of the five metabolites are found in the urine of maintenance patients (Table III). Fig. 7 shows the chromatogram of an extract of a sample of urine obtained from patient 4. At the low attenuation used to obtain Fig. 7 the peaks corresponding to the retention times of LAAM, NAM and DNAM are "off scale". However, peaks corresponding to MOL, NMOL and DNMOL are present and can be quantitated (Table III). These results confirm an earlier

TABLE III

LAAM AND METABOLITES IN MAINTENANCE-PATIENT BIOFLUIDS AT 24 HOURS AFTER A DOSE

Patient	Biofluid	Concentration (ng/ml)					
		LAAM	NAM	DNAM	MOL	NMOL	DNMOL
1	Plasma	41	140	80	nd*	nd	nd
1	Urine	1000	3050	4200	31	23	29
2	Plasma	12	28	26	nd	nd	nd
2	Urine	450	1500	2800	23	13	22
3	Plasma	24	68	57	nd	nd	nd
3	Urine	1450	2800	4000	46	26	40
4	Plasma	27	44	42	nd	nd	nd
4	Urine	600	1575	3700	14	22	46
5	Plasma	8	38	24	nd	nd	nd
5	Urine	50	575	1150	8	6	14

*nd = not detected.

TABLE IV

LAAM AND METABOLITES IN SHEEP PLASMA

The dose was an intravenous infusion of LAAM, 0.02 mg/kg per min for 6 h.

Time (h)	Plasma concentration (ng/ml)		
	LAAM	NAM	DNAM
0	0	0	0
0.25	97	13	0
0.5	116	19	0
1	228	54	6
2.5	253	97	23
4	340	159	50
6	423	211	78
24	36	72	168

qualitative study [6] which used mass spectrometry to identify LAAM, NAM, DNAM, MOL and NMOL in patients' urine. At the time of this report by Kaiko et al. [6] an analytical standard of DNMOL was not available. The discovery of DNMOL in patients' urine using HPLC will require confirmation using mass spectrometry (studies in progress). As we have reported earlier [6, 15], only LAAM, NAM and DNAM are present in detectable amounts in the plasma of LAAM maintenance patients (Table III). LAAM, NAM and DNAM are found in sheep plasma during and following the infusion of LAAM (Table IV). LAAM levels continued to rise during the 6-h infusion and were greater than those of NAM and DNAM. DNAM was not detected until 1 h after the start of the LAAM infusion. At 24 h (18 h after the cessation of the infusion) LAAM levels are lower than those of NAM and DNAM. These results suggest

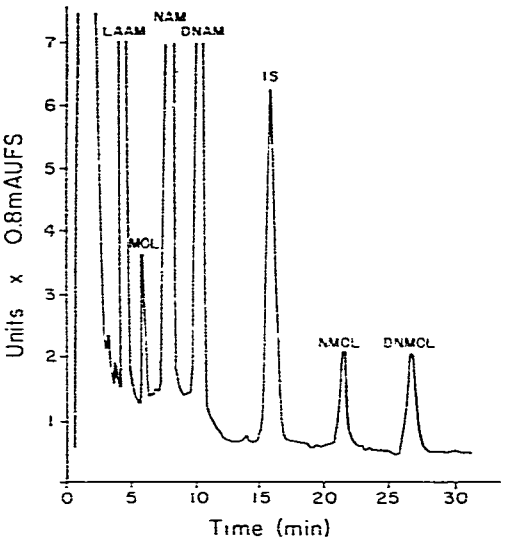

Fig. 7. Chromatogram of urine from a LAAM-maintenance patient. The extract was prepared from 2 ml of patient urine collected at 24 h after a dose, to which was added 50 ng of propranolol, the internal standard (IS). Extraction is as described in Materials and methods. Mobile phase is methanol—acetonitrile (75:25, v/v) with 0.008% ammonium hydroxide. Abbreviations are as in Fig. 1.

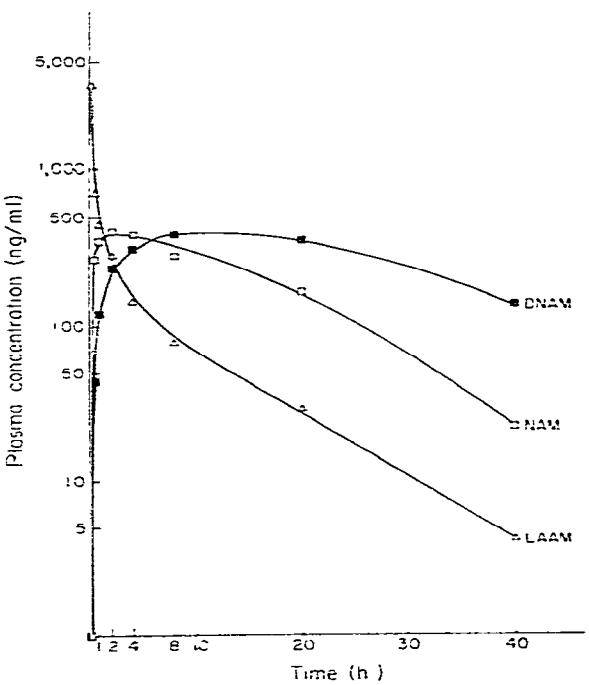

Fig. 8. Plasma levels of LAAM (△), NAM (□) and DNAM (■) following the intravenous injection of [^{3}H]LAAM (2.5 mg/kg, 130 μCi) to a rat.

that, in sheep, NAM and DNAM are persistent metabolites (see also ref. 15).

Fig. 8 demonstrates the results obtained when the HPLC system is combined with fraction collection and liquid scintillation counting to measure the disposition of [^{3}H] LAAM. Radiolabeled LAAM, NAM and DNAM were found in rat plasma following the intravenous administration of [^{3}H] LAAM. We could not demonstrate significant (> 1.0 ng/ml) radioactivity in the fractions that correspond to the UV peaks for MOL, NMOL or DNMOL. As would be expected from their respective product—precursor relationships, as LAAM levels fall, NAM and then DNAM increase. In the rat, at 40 h after dosing with [^{3}H] LAAM, DNAM levels are the highest and LAAM levels the lowest.

We have developed an HPLC method for the quantitative determination of LAAM and metabolites in biofluids. This system can be used to characterize the pharmacokinetics of LAAM in man and laboratory animals. In addition, the method can be used to determine the disposition of any one of the metabolites.

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