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DETERMINATION OF HEROIN AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the simultaneous determination of heroin (3,6-diacetylmorphine, DAM) and its two active metabolites 6-acetylmorphine and morphine in blood by high-performance liquid chromatography using a normal-phase column and a UV detector at 218 nm. The compounds are stabilized in blood by rapid freezing and recovered by a multistep liquid—liquid extraction. The mobile phase is acetonitrile—methanol (75:25, v/v) buffered to apparent pH 7 with ammonium hydroxide and acetic acid. Using *l*-α-acetylmethadol as an internal standard, UV detection and a 1-ml biofluid sample, the lower limit of sensitivity is 12.5 ng/ml. Commonly used narcotic analgesics including codeine, propoxyphene, meperidine, methadone and levorphanol do not interfere with the analysis. The method has been applied to blood samples from humans and rats. Extracts of blood from a patient who had received an intravenous dose of 14 mg of DAM contained DAM and both of its active metabolites.

INTRODUCTION

Heroin (3,6-diacetylmorphine, DAM) is a commonly abused narcotic agonist which has been studied as an analgesic for the management of pain due to advanced cancer [1]. DAM is enzymatically deacylated to 6-acetylmorphine (AM) and subsequently to morphine (M) (Fig. 1) by a variety of tissues from man, rat, rabbit, mouse, dog [3, 4] and other species.

The clinical evaluation of DAM [4] has stimulated pharmacokinetic studies in man [5] and laboratory animals. DAM disposition has previously been studied using a number of analytical techniques including counter current distribution followed by spectrophotometric quantitation of methyl orange complexes or folin determination of free phenol content [2, 6], paper chromatography [6, 7], thin-layer chromatography [8—10], combined gas chro-

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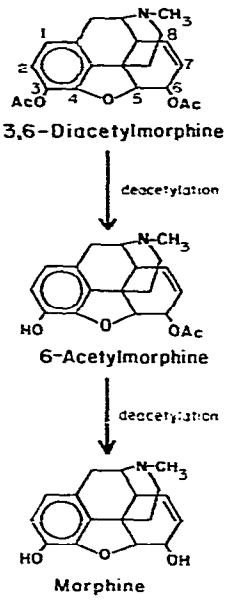


Fig. 1. Biotransformation pathway for heroin (diacetylmorphine, DAM), and structural formulae for DAM, acetylmorphine (AM) and morphine (M).

matography—mass spectrometry [11] and differential UV spectrophotometry [12]. High-performance liquid chromatography (HPLC) has been used in the analysis of DAM from pharmaceutical, illicit and other non-biological sample preparations [10, 13—15]. The lability of DAM's 3-ester at alkaline pH and to hydrolases in serum and red cells, coupled with the wide range in lipophilicity of the three compounds of interest have, however, hindered the development of a generally applicable analytical method with the requisite sensitivity for the routine simultaneous determination of DAM, AM and M in biofluid samples. Furthermore, Garrett and Gurkan's [10] method for stabilizing DAM in dog blood prior to extraction does not stabilize DAM in human or rat blood (Umans and Inturrisi, unpublished observations).

We describe here a method using rapid freezing of whole blood or biofluid samples, followed by solvent—solvent extraction and HPLC to resolve the compounds of interest; and demonstrate the utility of this method for measuring DAM and its metabolites in human and animal biological samples.

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·HCl, 3,6-diacetylmorphine·HCl and 6-acetylmorphine base. Morphine sulfate was obtained from Mallinckrodt (St. Louis, MO, U.S.A.).

Methanol, acetonitrile, *n*-butanol, chloroform, isopropanol, acetone and *n*-hexane (all distilled in glass) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Toluene (scintillation grade), glacial acetic acid and concentrated (29.2%) aqueous ammonia were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). All solvents and reagents were used as received. Reagent grade sulfuric acid, hydroxylamine·HCl and both 10% and 50% aqueous sodium hydroxide (w/v) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Glycylglycine buffer (1 *M*, pH 8.55) was prepared by titrating a solution of the free base (Sigma, St. Louis, MO, U.S.A.) in glass distilled water with 50% (w/v) sodium hydroxide.

[^{3}H]DAM (diacetyl[1(*n*)-^{3}H]morphine, specific activity = 26.5 Ci/mmol) and [^{3}H]M ([1(*n*)-^{3}H]morphine, specific activity = 22 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, U.S.A.). [^{3}H]AM was synthesized by one of two methods, as follows:

(1) In a modification of the methods of Small (cf. Wright [16]) and of Lerner and Mills [17], 25 μl of a saturated aqueous solution of hydroxylamine hydrochloride was added to [^{3}H]DAM, dissolved in 100 μl of ethanol (US Industrial Chemicals, New York, NY, U.S.A.) in a siliconized glass centrifuge tube. The solution was incubated at 60°C for 0.5 h and then neutralized by the dropwise addition of concentrated ammonium hydroxide. The solution was buffered to pH 8.6 (ammonia—ammonium chloride buffer) and extracted with chloroform—isopropanol (3:1, v/v).

(2) [^{3}H]DAM was added to 10 units of butyrylcholinesterase (equine serum acylcholine acylhydrolase, E.C.3.1.1.8, Sigma), dissolved in 100 μl of normal saline and incubated at 37°C for 1 h. Protein was precipitated by addition of 1 ml of acetone. The purity of both products was assessed by HPLC, followed by fractional collection of the column effluent and liquid scintillation counting using a 1% Liquifluor (New England Nuclear, Boston, MA, U.S.A.) in toluene cocktail in a Model LS3100 counter (Beckman Instruments, Irvine, CA, U.S.A.). Counting efficiency was determined using [^{3}H]toluene as an internal standard. Either method provides [^{3}H]AM of > 96% radiochemical purity in > 80% yield.

Stock solutions

LAAM, DAM, AM and M stock solutions at a concentration of 1 mg/ml were prepared in methanol and stored at −20°C. Calibration standards were prepared by diluting the stock solution with methanol—acetonitrile (20:80, v/v).

Sample preparation from biofluids

A flow sheet outlining the procedure is given in Fig. 2. Whole blood samples (up to 1.5 ml) are collected in 12-ml siliconized (Prosil 28, PCR, Gainesville, FL, U.S.A.) centrifuge tubes, fitted with PTFE-lined screw caps. The samples are rapidly frozen in a dry-ice—acetone bath and stored at −20°C. To each tube, while maintained at dry-ice temperature, is added 0.10 ml of a 4.0 μg/ml solution of the internal standard and 1.0 ml of the glycylglycine buffer. The sample is extracted with 5 ml of toluene—*n*-butanol (7:3, v/v) for 20 min on an automatic reciprocating shaker and centrifuged for 10 min

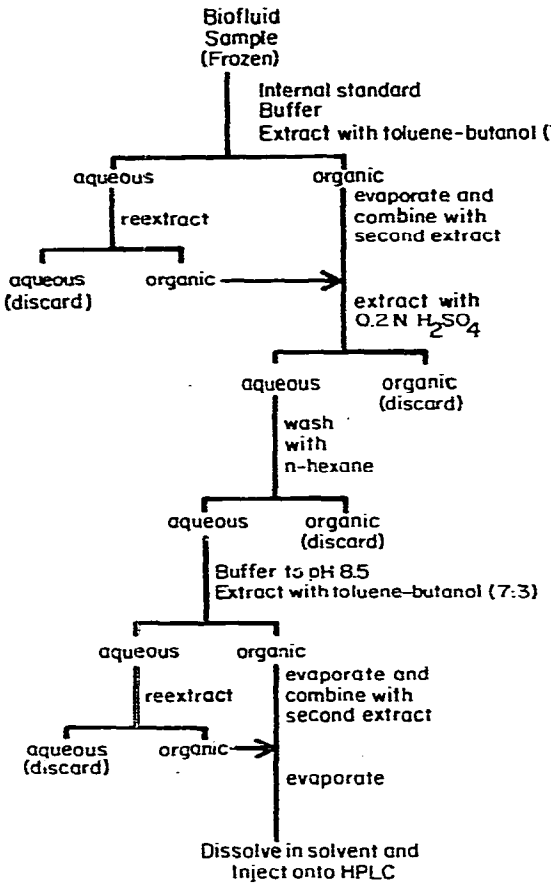


Fig. 2. Flow sheet outlining the stabilization and extraction procedures for DAM and its metabolites from blood.

at 500 *g*. The frozen sample pellet thaws during this first room temperature extraction. The toluene—butanol phase (upper) is transferred to a clean, siliconized 15-ml screw-top centrifuge tube and evaporated to dryness at 35°C (Vortex Evaporator, Model 3-2200, Buchler, Fort Lee, NJ, U.S.A.). The sample is reextracted with 5 ml of toluene—butanol, which is then added to the dried residue of the first extract. The organic phase is extracted with 2.5 ml of 0.2 *N* sulfuric acid by shaking for 10 min. After centrifugation for 7 min the toluene—butanol layer (upper) is discarded. The acid phase is washed with 5 ml of *n*-hexane and the pH adjusted to 8.5 with the addition of 0.5 ml of glycylglycine buffer, followed by 0.195 ml of 10% sodium hydroxide. This aqueous phase is extracted twice with 5 ml of toluene—butanol by shaking for 10 min. After centrifugation for 7 min, each portion of toluene—butanol is transferred to a 12-ml siliconized conical centrifuge tube and evaporated to dryness as above. The sample extract is reconstituted in 260 μl of methanol, and 200 μl are injected into the HPLC system.

Chromatographic conditions

The analysis is performed on a Varian Model 8500 liquid chromatograph (Varian, Sunnyvale, CA, U.S.A.) equipped with a displacement syringe pump, a UV—visible 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 Varian Model A-25 dual-channel chart recorder set at 1 mV and 2 mV. The mobile phase is acetonitrile—methanol—solution A—solution B (75:25:0.040:0.216). Solution A is prepared by mixing concentrated aqueous ammonia and methanol (1:2, v/v) and solution B by mixing glacial acetic acid and methanol (1:1, v/v). The flow-rate is 80 ml/h and the column and detector are maintained at 30°C. The column effluent is monitored at 218 nm using a recorder scale that varies from 8—32 mA and a chart speed of 10 in./h.

Calibration curves and quantitation

Standard calibration curves are established by adding DAM, AM, M and LAAM to drug-free blood, plasma or normal saline and proceeding as described above. Quantitation is performed by drawing the baseline and measuring the peak heights of the compounds of interest. The peak height ratio (standard/LAAM) 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

Samples were taken from humans and rats following DAM administration and from in vitro incubations of DAM with rat blood. Blood was collected from three male cancer patients for 2—17 min following the intravenous administration of 4, 5 or 14 mg of DAM. Three male Sprague—Dawley rats weighing 300—350 g were prepared by cannulation of the right femoral artery and vein. Blood (0.5—1.0 ml) was collected just prior to and from 10 to 180 min after the intravenous infusion of DAM at 1.0 mg/kg/h for 3 h. Drug-free whole, heparinized blood was obtained by cardiac puncture from four rats and preincubated for 10—20 min in a 37°C water bath. DAM (1 μg/ml) was added to each sample, which was mixed thoroughly and returned to the water bath. Aliquots were taken at 0, 15, 30, 60 and 120 sec.

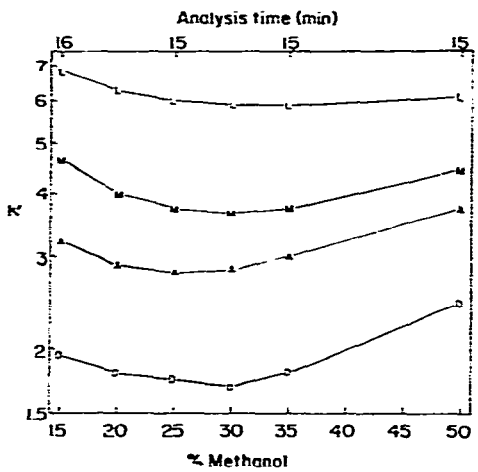
RESULTS AND DISCUSSION

Determination of chromatographic conditions

DAM and its metabolites are weak bases, which are partially ionized in aqueous solution. Separation by reversed-phase HPLC requires that ionization be suppressed by raising the mobile phase pH, which would unfortunately result in both DAM hydrolysis and column degradation [18]. The UV cut-off of paired-ion chromatographic reagents [19] would not allow detection at short wavelengths and would result in a less sensitive assay. By analogy to other aromatic, weakly basic alcohols and their esters, DAM and its metabolites are amenable to separation by normal-phase chromatography [20]. Ace-

tonitrile—methanol, buffered to apparent pH 7 with acetic acid and ammonium hydroxide provides a mobile phase with a low UV cut-off that fixes the extent of ionization of the compounds of interest.

Fig. 3 shows the effect of changing the concentrations of acetonitrile and methanol in the mobile phase on the column capacity factor (k') and analysis time. The four compounds are adequately resolved with a mobile phase containing up to 30% methanol, while at 50% methanol the resolution between AM and M begins to decrease. Since the k' value of LAAM is relatively insensitive to changes in percent methanol, the analysis time changes little over the range of 15—30% methanol.



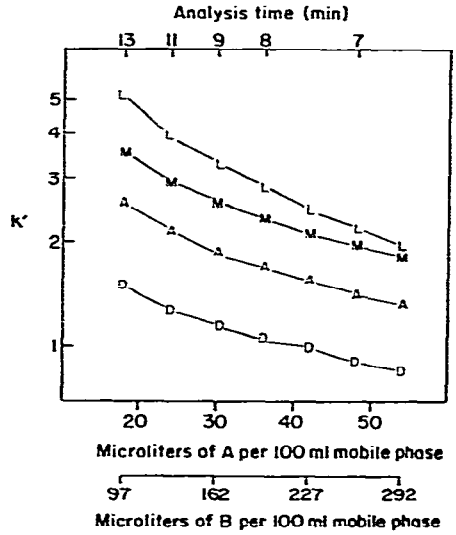


Fig. 3. Effect of increasing the percentage of methanol in the mobile phase (acetonitrile—methanol) from 15 to 50% on the column capacity factor (k') of each compound of interest. The buffer strength was kept constant at 20 μl solution A and 108 μl solution B per 100 ml of mobile phase. The time required for a complete analysis is given on the upper abscissa. Traces: L = LAAM; M = M; A = AM; D = DAM.

Fig. 4. Effect of increasing the buffer strength [μl of A and B per 100 ml of mobile phase, acetonitrile—methanol (70:30, v/v)], while maintaining the apparent pH constant at 7, on the column capacity factor (k') of each compound of interest. The time required for a complete analysis is given on the upper abscissa. Traces: L = LAAM; M = M; A = AM; D = DAM.

The variation of k' and analysis time with buffer strength is shown in Fig. 4. With the apparent pH maintained at neutrality, the analysis time decreases with increasing buffer strength. At high buffer strengths, the resolution between M and LAAM is lost. To provide adequate resolution and a short analysis time, we chose a mobile phase containing 25% methanol and 40 μl of solution A per 100 ml for routine use.

Column efficiency

The effect of flow-rate on column efficiency, as determined by the height equivalent to a theoretical plate (HETP), was investigated. As expected, Fig. 5

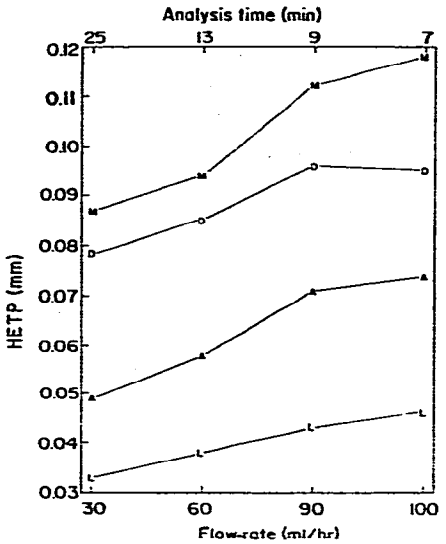


Fig. 5. Effect of the flow-rate of the mobile phase, acetonitrile—methanol (80:20, v/v) with 36 μl A and 194 μl B per 100 ml of mobile phase, on column efficiency as measured by the height equivalent to a theoretical plate (HETP). The time required for a complete analysis is given on the upper abscissa. Traces: M = M; D = DAM; A = AM; L = LAAM.

shows that the HETP is smallest at the lowest flow-rates. However, while the HETP is largest for M, it does not exceed 0.12 mm over the range of flow-rates tested. For a convenient analysis time we chose a flow-rate of 80 ml/h.

UV detection

The absorbance of DAM and its metabolites increases significantly in the far UV. A wavelength of 218 nm was selected as a compromise between maximum sensitivity and acceptable noise. At a column and detector temperature of 30°C, the retention time of each compound is quite stable. The coefficients of variation (C.V.) for retention times from eleven consecutive extracted samples are shown in Table I. This reproducibility can permit the blind collection of peak fractions when the sample concentration falls below the limits of sensitivity for UV detection. These fractions may be subsequently quantitated using a sensitive morphine radioimmunoassay [21] which also recognizes AM and DAM (studies in progress).

TABLE I

PRECISION VALUES FOR DAM AND ITS METABOLITES

Compound	Mean retention time (min)	C.V. (%)	Recovery		C.V. (%) for determination of	
			Mean	C.V. (%)	25 ng	300 ng
DAM	5.7	0.2	87.8	6.3	3.9	2.0
AM	8.1	0.3	94.3	5.8	5.1	3.4
M	10.0	0.5	92.8	8.7	3.5	2.9
LAAM	13.8	0.2	28.5	7.3	—	—

Determination of radiolabeled samples

The availability of tritiated forms of DAM and its metabolites led us to adapt the HPLC system we have described so as to be able to collect fractions containing the radiolabeled compounds of interest for analysis by liquid scintillation counting. The location and recovery of the compounds of interest are facilitated by adding 1 μg of each as unlabeled carrier such that the expected contribution of the radioisotope to the detector response will be less than 5%. The recovery of radiolabeled drug from the column is 93% (C.V. = 5%) for all three compounds and is independent of radioisotope amount from 0.0006 to 0.02 μCi. The sensitivity of this approach is therefore a function of the specific activity of the radiolabeled drug and the average background. We can easily determine 0.01 ng of [^{3}H] DAM, [^{3}H] AM or [^{3}H] M using this method with extraction recovery assessed by comparison of the cold carrier detector response to absolute standard curves for the three compounds of interest.

Extraction recovery and calibration curves

The optimal conditions for the simultaneous extraction of DAM and its metabolites are particularly dependent on pH and partition conditions. DAM is rapidly converted to AM at pH 9 or greater. The uniform recovery of M depends on forming the sulfate rather than the hydrochloride salt in the second step of the extraction. The recovery of the compounds of interest is independent of concentration from 12.5 to 500 ng/ml. After correction for aliquot losses, the mean recoveries and their coefficients of variation are shown in Table I.

While extraction from blood yields a small and variable unidentified peak eluting between the solvent front and DAM, the extraction yields a sample which is free of peaks that might interfere with the quantitation of the compounds of interest (Fig. 6).

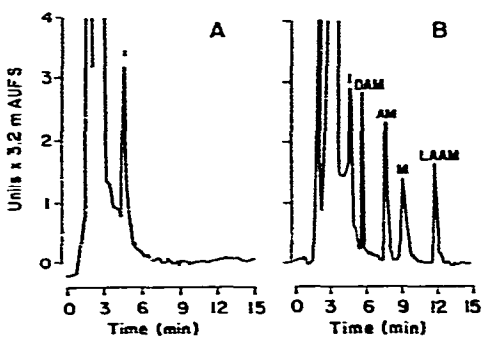


Fig. 6. Chromatograms of the extract of control human blood (A) and calibration standards recovered from human blood (B). To 0.5 ml of control human blood were added DAM (100 ng), its metabolites AM and M at 75 ng each and 400 ng of LAAM, the internal standard. The extract was reconstituted in 260 μl of methanol and 200 μl were injected. Peak 1 is unidentified. Chromatographic conditions are as described in Materials and methods.

Fig. 7 shows that, using the UV detector, linear standard calibration curves can be constructed from 12.5 to 200 ng. Although not shown in Fig. 7, linear curves may be constructed up to 4000 ng. The precision of determination of extracted 25 and 300 ng calibration standards is given in Table I. With a sample volume of 1 ml and this calibration curve the lower limit of sensitivity is 12.5 ng/ml. The effective sensitivity may be increased by using sample volumes up to 1.5 ml. The lower limit of detection (signal-to-noise ratio of 2) is 6 ng for all three compounds.

While the rapid freezing and mildly alkaline extraction procedures permit only minimal (11.2 ± 0.36% S.E.M., n = 12 at 150 ng DAM) DAM hydrolysis, the extent of hydrolysis is routinely monitored in each extraction and the DAM and AM standards are extracted separately to avoid the systematic underestimation of AM.

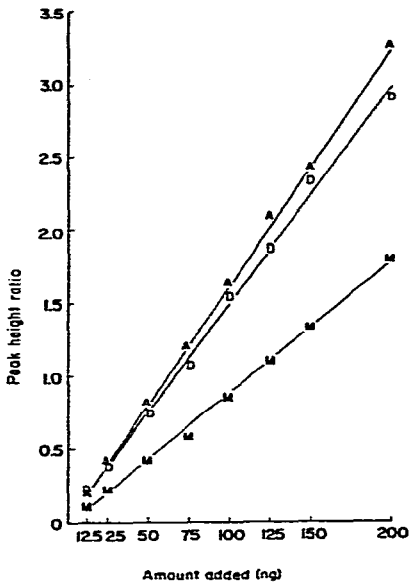


Fig. 7. Standard calibration curves for DAM (D) and metabolites M and AM (A) recovered from control human blood. Superimposible curves were derived from normal saline. Each point represents the mean of triplicate determinations.

Potential interference

As shown in Table II, DAM and its metabolites are resolved from commonly used or abused drugs and their metabolites. Only hydrocodone and quinine were not adequately resolved when injected together with morphine and analyzed with the routine system described in Materials and methods. However, if samples were suspected to contain either of these compounds, the mobile phase could be modified to resolve them from all three compounds of interest. The relatively low pH of the extracted sample also serves to selectively diminish the recovery of many of these compounds. Thus, this method may be used to analyze samples from subjects taking the drugs listed in Table II.

TABLE II

RESOLUTION OF HEROIN AND METABOLITES FROM SELECTED DRUGS AND THEIR METABOLITES

Drug	Column capacity factor (k')
Naloxone	0.57
Heroin (DAM)	0.97
Acetylcodeine	1.46
6-Acetylmorphine (AM)	1.66
Codeine	1.81
Naltrexone	1.89
Morphine (M)	2.17
Hydrocodone	2.24
Quinine	2.28
Oxycodone	2.38
Hydromorphone	2.48
Meperidine	2.61
Propoxyphene	2.85
Oxymorphone	2.85
Norpropoxyphenamide	2.85
l-α-Acetylmethadol (LAAM)	3.16
Dinoracetylmethadol	3.57
Cocaine	3.58
Dinormethadol	3.73
Methadol	4.04
Amphetamine	4.07
Normeperidine	4.13
Normethadol	4.14
Pentazocine	4.24
Methadone	5.07
Levorphanol	5.13
Acetaminophen	> 5
Caffeine	> 5

Human and animal studies

Following the rapid intravenous (i.v.) administration of DAM to two cancer patients, Fig. 8 shows the rapid ($t_{½}$ = 1.7, 2.2 min) exponential disappearance of DAM from blood. This disappearance is more rapid than that of DAM from blood in vitro (unpublished observations) and may be due to both distribution and biotransformation, in blood and extravascularly. While neither AM nor M was detected in these patients, both metabolites have been observed following the administration of 14 mg DAM (i.v.) to a highly narcotic-tolerant patient (Fig. 9). This is the first report of the detection of DAM in human blood and confirms the inadequacy of the analytical methods used in prior studies [22].

Following the termination of a 3-h infusion of DAM to three rats, no DAM was detected in any blood samples. Based on the lower limit of detection of 6 ng we can estimate the minimum steady state blood DAM clearance for the rat from the equation:

$$\text{Clearance} = \frac{k_0}{C_{ss}}$$

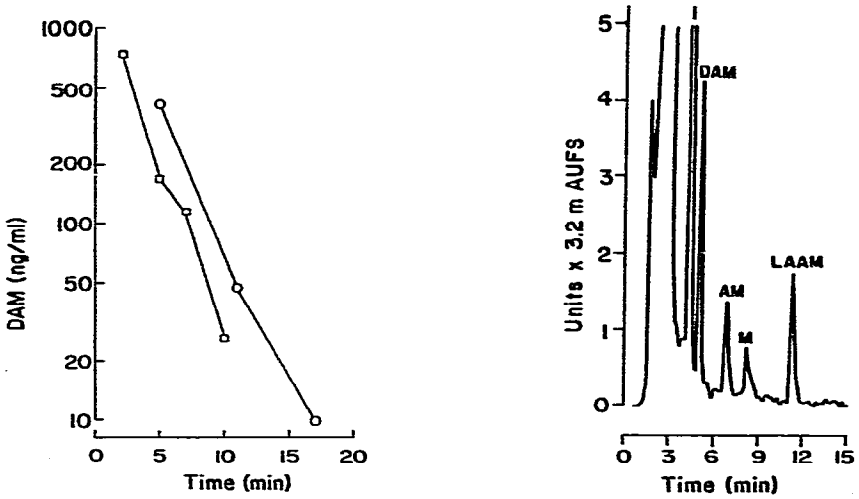


Fig. 8. Blood levels of DAM following the i.v. injection of DAM, 5 mg (○) and 4 mg (□), to two cancer patients.

Fig. 9. Chromatogram of a blood sample collected from a cancer patient 11.25 min after the i.v. administration of 14 mg DAM. Peak 1 is unidentified. Extraction and chromatographic conditions are as described in Materials and methods.

where k_0 is the zero order DAM infusion rate (1.0 mg/kg/min) and C_{ss} is the steady state blood DAM concentration (assumed to be 6 ng/ml). This calculation indicates that in the rat the blood DAM clearance must exceed 2800 ml/min/kg. The elimination of AM and M following these infusions is displayed in Fig. 10. The rapid clearance of i.v. DAM in the rat is substantiated by a study of DAM hydrolysis and reciprocal AM formation by rat blood, in vitro (Fig. 11). Under the conditions used, DAM was hydrolyzed with a mean half-life of 23 sec.

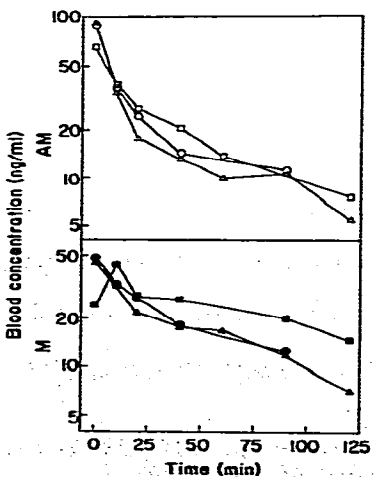


Fig. 10. Blood levels of AM (upper panel) and M (lower panel) following a continuous infusion of DAM at 1 mg/kg/h to three rats. Zero time represents the cessation of drug infusion. No DAM was detected in any samples. Extraction and chromatographic conditions are as described in Materials and methods.

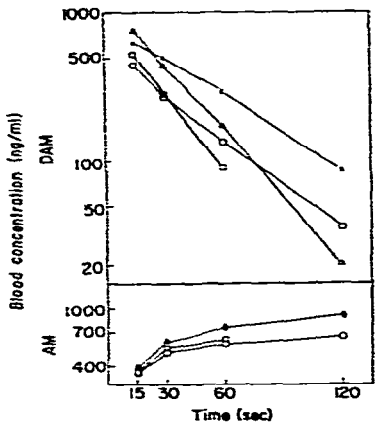


Fig. 11. Disappearance of DAM (upper panel) and the reciprocal formation of AM (lower panel) during the incubation of DAM (1 μg/ml) with rat blood (n = 4) at 37°C, in vitro. Extraction and chromatographic conditions are as described in Materials and methods.

We have developed an HPLC method for the quantitative determination of DAM and its metabolites, following their stabilization in, and extraction from whole blood. The system can be used to determine the pharmacokinetics of DAM or either of its metabolites in man and laboratory animals.

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REFERENCES

1 R.G. Twycross, Postgrad. Med. J., 49 (1973) 309.
2 E.L. Way, J.M. Young and J.W. Kemp, Bull. Narc., 17 (1965) 25.
3 C.I. Wright, J. Pharmacol. Exp. Ther., 75 (1942) 328.
4 R.F. Kaiko, S.L. Wailenstein, A.G. Rogers, P.Y. Grabinski and R.W. Houde, N. Engl. J. Med., 304 (1981) 1501.
5 C. Inturrisi, M. Max, J. Umans, M. Schultz, S. Shin, K. Foley and R. Houde, Clin. Pharmacol. Ther., 31 (1982) 235.
6 E.L. Way, J.W. Kemp, J.M. Young and D. Grassetti, J. Pharmacol. Exp. Ther., 129 (1960) 144.
7 G.R. Nakamura and T. Ukita, J. Pharm. Sci., 56 (1967) 294.
8 G.R. Nakamura, J.I. Thornton and T.T. Noguchi, J. Chromatogr., 110 (1975) 81.
9 S.Y. Yeh, R.L. McQuinn and C.W. Gorodetzky, J. Pharm. Sci., 66 (1977) 201.
10 E.R. Garrett and T. Gurkan, J. Pharm. Sci., 68 (1979) 26.
11 W.O.R. Ebbighausen, J.H. Mowat, M. Stearns and P. Vestergaard, Biomed. Mass Spectrom., 1 (1974) 305.

12 O. Lockridge, N. Mottershaw-Jackson, H.W. Eckerson and B. LaDu, J. Pharmacol. Exp. Ther., 215 (1980) 1.
13 J.H. Knox and J. Jurand, J. Chromatogr., 87 (1973) 95.
14 P.J. Twitchett, J. Chromatogr., 104 (1975) 205.
15 G.K. Poochikian and J.C. Cradock, J. Chromatogr., 171 (1979) 371.
16 C.I. Wright, J. Pharmacol. Exp. Ther., 71 (1941) 164.
17 M. Lerner and A. Mills, Bull. Narc., 15 (1963) 37.
18 F.L. Vandermark, R.F. Adams and G.J. Schmidt, Clin. Chem., 24 (1978) 87.
19 N.A. Parris, Instrumental Liquid Chromatography, Elsevier, Amsterdam, 1976, p. 187.
20 C.-H. Kiang, S. Campos-Flor and C.E. Inturrisi, J. Chromatogr., 222 (1981) 81.
21 S. Spector, J. Pharmacol. Exp. Ther., 178 (1971) 253.
22 H.W. Elliott, K.D. Parker, J.A. Wright and N. Nomof, Clin. Pharmacol. Ther., 12 (1971) 806.