Journal of Chromatography, 129 (1976) 329–338

CHROM. 9328

COMPARATIVE STUDY ON THE DERIVATIZATION OF *l*-α-ACETYLMETHADOL METABOLITES FOR ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

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(Received April 12th, 1976)

SUMMARY

Six reagents —trichloroacetyl chloride, trichloroacetic anhydride, pentafluorobenzoyl chloride, heptafluorobutyryl chloride, heptafluorobutyric anhydride, and trifluoroacetic anhydride— were evaluated as potential derivatizing reagents for quantitating the metabolites of *l*-α-acetylmethadol (LAAM) —noracetylmethadol, dinoracetylmethadol, methadol, and normethadol— by electron capture gas–liquid chromatography. All of the reagents studied reacted quantitatively with all of the metabolites except methadol; however, trichloroacetyl chloride was found to be the most satisfactory general reagent for analyzing these metabolites in biological fluids. A gas–liquid chromatographic method is presented which combines both flame ionization and electron capture detection for quantitating plasma and urine levels of methadone, *l*-α-acetylmethadol and its metabolites.

INTRODUCTION

The duration of the pharmacological activity of *l*-α-acetylmethadol (LAAM) is three times longer than that of methadone[1]. For this reason, LAAM is being investigated as a possible substitute for methadone in the maintenance treatment of heroin addicts[2]. LAAM is known to be metabolized in man to noracetylmethadol (N-LAAM), dinoracetylmethadol (DN-LAAM), methadol (MOL) and normethadol (N-MOL) (Fig. 1)[3,4]. Most of these metabolites have been found to be pharmacologically active, especially the N-demethylated compounds[5,6].

Therefore, the accurate determination of the levels of LAAM and its metabolites in biological fluids and tissues is of paramount importance in studying the pharmacokinetics of LAAM. Unfortunately, levels of the parent drug and its metabolites in plasma or urine are usually so low, especially following acute administration, that quantitation is difficult.

The conversion of compounds to their halogenated derivatives and subsequent detection by electron capture gas–liquid chromatography (GLC–ECD) is a sensitive

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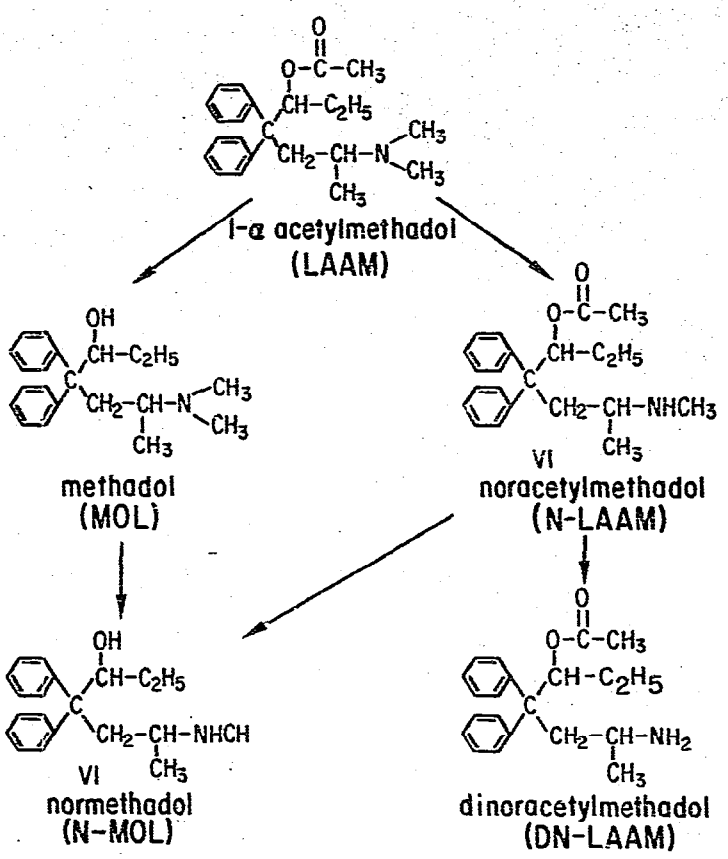


Fig. 1. Metabolic pathways for *l*-α-acetylmethadol.

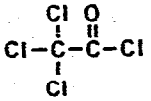
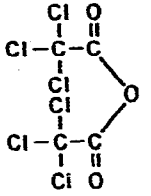
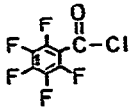
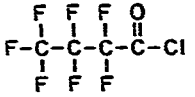
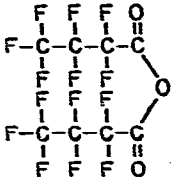
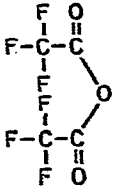
and selective method for quantitating drugs and metabolites at nanogram or even picogram levels[7]. Wallace *et al.*[8] used trifluoroacetic anhydride to derivatize morphine for subsequent analysis using GLC–ECD. Similarly, Hartvig *et al.*[9] determined terodiline in serum by GLC-ECD after heptafluorobutyrylation. Matin and Rowland[10] were able to measure as little as 5 pg of amphetamine as its pentafluorobenzoyl derivative.

Billings *et al.*[11] determined the LAAM metabolites, N-LAAM and DN-LAAM, in human plasma by transforming them into trichloroacetyl derivatives, and subsequently analyzing the derivatives by GLC–ECD. However, they did not report the effectiveness of using the trichloroacetyl chloride (TCAC) reagent to derivatize MOL or N-MOL. Kaiko *et al.*[12] used trifluoroacetylimidazole to derivatize N-LAAM and DN-LAAM but found that the standard calibration curves for these derivatives were nonlinear and inconsistent.

In the present study, the LAAM metabolites, N-LAAM, DN-LAAM, MOL and N-MOL, were derivatized with six halogenated reagents (Table I), *viz.* trichloroacetyl chloride (TCAC), trichloroacetic anhydride (TCAA), pentafluorobenzoyl chloride (PFBC), heptafluorobutyryl chloride (HFBC), heptafluorobutyric anhydride

TABLE I

CHEMICAL FORMULAE OF THE HALOGENATED DERIVATIZING REAGENTS

Chemical name	*Abbreviation*	*Structural formula*
Trichloroacetyl chloride	TCAC	
Trichloroacetic anhydride	TCAA	
Pentafluorobenzoyl chloride	PFBC	
Heptafluorobutyryl chloride	HFBC	
Heptafluorobutyric anhydride	HFBA	
Trifluoroacetyl anhydride	TFAA	

(HFBA), and trifluoroacetic anhydride (TFAA) with a view towards determining the most suitable reagent for quantifying these metabolites in biological fluids and tissues. Also, a GLC procedure is described which combines the use of both flame ionization and electron capture detection for the determination of methadone, LAAM, N-LAAM and DN-LAAM in biological fluids.

MATERIALS AND METHODS

Chemicals and reagents

LAAM·HCl, N-LAAM·HCl, DN-LAAM·HCl, MOL·HCl, N-MOL·HCl and methadone·HCl were provided by Lilly Research Labs. (Indianapolis, Ind.,

U.S.A.) and the National Institute on Drug Abuse (Bethesda, Md., U.S.A.). TCAC and triethylamine were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A), TCAA from K & K Labs. (Plainview, N.Y., U.S.A.), HFBC, HFBA, PFBC and TFAA from PCR (Gainesville, Fla., U.S.A.), and silylation grade pyridine from Pierce (Rockford, Ill., U.S.A.). Nanograde solvents —1-chlorobutane, acetone, toluene and hexane— were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). TFAA, 1-chlorobutane, and acetone were redistilled in glass in our laboratory.

Preparation of derivatives

Range finding studies were carried out by reacting 0.01–1.00 μg of the LAAM metabolites (as free bases) with 0.5 ml of 0.0005–50 m*M* solutions of the various derivatizing reagents in toluene.

All LAAM metabolite salts were first converted to their free bases to allow nucleophilic reactions to take place.

1–2 mg of each LAAM metabolite salt was dissolved in 4.0 ml of distilled water and the pH was adjusted to 9.5 with 0.1 *N* NaOH. The aqueous solution was extracted two times with 10.0 ml each of freshly distilled 1-chlorobutane. The organic layers were pooled and the concentration of the free base was then determined by flame ionization gas–liquid chromatography (GLC–FID).

Optimum derivatizing conditions were determined by varying the concentration of the derivatizing reagent, reaction time, and incubation temperature. The reaction mixture was evaporated to dryness under nitrogen and the residue was reconstituted with an appropriate amount of hexane. Aliquots of 2 μl were then analyzed by GLC–ECD.

Gas–liquid chromatography

GLC–FID parameters. A Tracor Model MT-220 gas chromatograph equipped with a FID and a 4 ft. × 1/8 in. I.D. glass column packed with 3% OV-25 on 100–120 mesh Gas-Chrom Q, was used for quantitating LAAM and methadone. The column temperature was set at 170°, the injector temperature at 240°, and the detector temperature at 270°. Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min.

GLC–ECD parameters. A Varian Model 2000 gas chromatograph equipped with a ^{63}Ni ECD and a 3 ft. × 1/8 in. I.D. glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q, was used for quantitating the metabolites as their halogenated derivatives. The column temperature was set at 235°, the injector temperature at 280°, and the detector temperature at 300°. Nitrogen was used as the carrier gas at a flow-rate of 60 ml/min.

Analysis of human plasma

Two milliliters of plasma was aliquoted into a 50-ml centrifuge tube, diluted with 2 ml of distilled water, and the pH was carefully adjusted to 9.5 with 0.1 *N* NaOH. The plasma was extracted twice with 10 ml each of freshly distilled 1-chlorobutane. The organic and aqueous layers were separated by centrifugation at 350 × *g* for 10 min. The organic layers were pooled and evaporated to dryness under nitrogen. The plasma extract was reconstituted with an appropriate amount of acetone and

2-μl aliquots were injected into a gas chromatograph equipped with a FID for quantitation of LAAM and methadone.

The extract was then evaporated to dryness under nitrogen and the residue reacted with 0.5 ml of freshly prepared 0.25 m*M* TCAC solution in toluene. The reaction mixture was incubated in a 13-mm tube fitted with a PTFE-lined screw cap in a water bath for 2 h at 70°. Excess reagent and toluene were evaporated to dryness under nitrogen and the reaction product was dissolved in an appropriate amount of hexane. A 2-μl aliquot was injected into a gas chromatograph equipped with an ECD for quantitation of the LAAM metabolites. Calibration curves were constructed by analysis of plasma fortified with 0, 50, 100, 250, and 500 ng each of LAAM and methadone and 0, 10, 25, 50, 100, and 200 ng each of the LAAM metabolites: N-LAAM, DN-LAAM and N-MOL.

RESULTS

Reaction conditions

Range finding experiments were conducted using 0.5 ml of the various derivatizing reagents at concentrations which ranged from 0.0005–50 m*M*. A concentration of 0.25 m*M* was found to be optimum for the six derivatizing reagents studied. This represents a 50- to 100-fold excess of derivatizing reagent. In some cases, increasing the reagent concentration decreased yields. Fig. 2 shows the yields (expressed as detector response) following the reaction of 200 ng N-LAAM, DN-LAAM, or N-MOL with 0.5 ml of increasing concentrations of TCAC in toluene at 70° for 2 h. TCAC reagent concentrations above 0.25 m*M* decreased the yield of N-LAAM and N-MOL derivatives and produced no increase in the yield of the DN-LAAM derivatives. Similarly, increasing the concentrations of the other derivatizing reagents above 0.25 m*M* produced no increase in yield.

A reaction temperature of 70° and a reaction time of 2 h were found to be optimum for the reagents studied. Generally, reaction temperatures higher than 70°

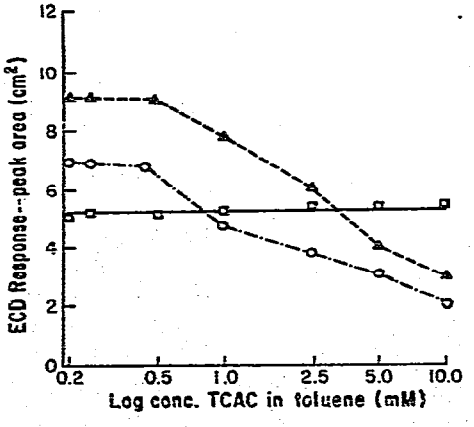


Fig. 2. Effect of derivatizing reagent concentration on yield. Shown are yields, expressed as detector response, following the reaction of 200 ng N-LAAM (▲--▲), DN-LAAM (□—□), or N-MOL (○--○) with increasing concentrations of TCAC in toluene at 70° for 2 h.

gave poor reproducibility probably due to rapid evaporation of reagents and solvents.

Fig. 3 shows the yields obtained when 200 ng of N-LAAM, DN-LAAM and N-MOL were reacted with 0.25 m*M* TCAC in toluene at 70° with reaction times ranging from 30–180 min. A reaction time of 2 h seemed optimal since the per cent increase in yield with longer reaction times was low.

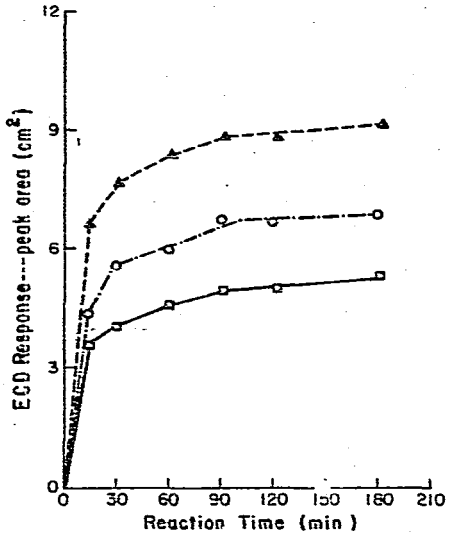


Fig. 3. Effect of reaction time on yield. Shown are yields, expressed as detector response, following the reaction of 200 ng N-LAAM (▲--▲), DN-LAAM (□—□), or N-MOL (○···○) with 0.5 ml of 0.25 m*M* TCAC in toluene at 70° for increasing lengths of time.

Pyridine and triethylamine have been commonly employed as catalysts in the derivatization of amines[9,13]. In this study, pyridine and triethylamine were found to be inadequate as catalysts. Pyridine did not evaporate under nitrogen, and subsequently interfered with detection, and triethylamine did not increase the yield of any of the reactions.

N-LAAM, DN-LAAM, and N-MOL could be quantitatively converted to their halogenated derivatives with any of the six reagents following reaction at 70° for 2 h. Quantitative conversion was assumed since none of the underivatized metabolites could be detected by GLC–FID after reaction.

Very little derivatized product of MOL was detected with any of the reagents or under any of the reaction conditions. Difficulty in derivatizing MOL might result from steric hindrance of the hydroxyl group and/or instability of the O-acyl derivative. After the derivatizing reaction, only about 50% of intact MOL was detected when the reaction residue was analyzed by GLC–FID. This implies that some of the MOL had been transformed to other products during the course of reaction. Cummins, in an attempt to derivatize pseudoephedrine, ephedrine, and norephedrine with heptafluorobutyric anhydride, also found that only the mono-N-acyl derivatives were formed and no O-acyl products could be detected[7]. Cummins concluded that the O-acyl products were very unstable and were hydrolyzed readily. Wallace *et al.*[8], on the other hand, were able to synthesize both mono- and di-O-acetylated morphine

by reacting morphine with TFAA. However, under our experimental conditions, TFAA produced no derivative with MOL that could be detected using our GLC–ECD system.

Gas chromatographic properties

The GLC properties of the various derivatives of N-LAAM, DN-LAAM and N-MOL are shown in Table II. The optimal column temperature was 235° for the TCAC, TCAA and PFBC derivatives, 185° for the HFBC and HFBA derivatives, and 165° for the TFAA derivatives (Table II, column 3). These oven temperatures gave short retention times, good resolution and reasonable sensitivity.

TABLE II

GLC PROPERTIES OF THE VARIOUS DERIVATIVES OF THE LAAM METABOLITES

The gas chromatograph was a Varian Model 2000 equipped with a ^{63}Ni ECD and a 3 ft. × 1/8 in. I.D. glass column packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q. The nitrogen carrier gas flow-rate was 60 ml/min.

LAAM metabolites	*Derivatizing reagents*	*Column temperature (°C)*	*Retention time (min)*	*Minimum detectable amount* (ng)*	*ECD response*** / *FID response*
N-LAAM	TCAC	235	10.5	0.3	7.6
	TCAA	235	10.5	0.2	14.1
	PFBC	235	7.7	0.6	4.0
	HFBC	185	10.2	0.3	6.9
	HFBA	185	10.2	0.3	7.4
	TFAA	165	8.1	2.0	1.2
DN-LAAM	TCAC	235	7.7	0.2	43.0
	TCAA	235	7.7	0.2	53.0
	PFBC	235	6.6	0.2	55.0
	HFBC	185	8.0	0.6	12.0
	HFBA	185	8.0	0.6	12.0
	TFAA	165	7.9	2.0	4.3
N-MOL	TCAC	235	10.8	2.0	1.8
	TCAA	235	10.8	1.5	2.6
	PFBC	235	7.8	0.3	13.5
	HFBC	185	9.6	0.6	7.5
	HFBA	185	9.6	1.0	4.0
	TEAA	165	7.0	1.0	3.5

* The minimum detectable amount is that amount which produces a detector response three times background noise.

** Relative response of derivatized metabolite using an ECD with respect to non-derivatized metabolite using a FID.

Most of the reagents studied produced derivatives of N-LAAM and N-MOL which had very similar retention times in our GLC systems (Table II, column 4); however, N-LAAM and N-MOL could be resolved as their heptafluorobutyryl derivatives. The retention times for heptafluorobutyryl-N-LAAM and heptafluorobutyryl-N-MOL were 16.5 and 17.5 min, respectively, at 175°.

The TCAC and HFBC derivatizing reagents produced few side products while the TCAA, HFBA, PFBC, and TFAA reagents produced substantially more artifacts.

Electron capture detector response

There was no single reagent which gave the maximum detector response for all its LAAM metabolite derivatives. For example, the GLC–ECD system was most sensitive to the TCAA derivatives of N-LAAM and DN-LAAM (minimum detectable amount of 0.2 ng per 2 μl aliquot, Table II) but was relatively insensitive to the TCAA derivatives of N-MOL. The minimum detectable amount is defined as that amount producing a detector response of three times background noise. The overall electronic structure of the derivatized compound, no doubt, is important in determining its electron-affinity properties. This unpredictable responsiveness of the ECD to different derivatized amines has also been reported by other investigators[7,10].

The sensitivity of detection of the derivatives using GLC–ECD could be increased as much as fiftyfold as compared with that of the corresponding underivatized compounds using GLC–FID (Table II, column 6). Derivatization was most effective in the case of DN-LAAM, since the FID was very insensitive to this metabolite.

The GLC–ECD system was also very selective in that it was very insensitive to any underivatized LAAM metabolites.

The responses of the ^{63}Ni detector to the derivatives of the LAAM metabolites were linear over a range of 0–8 ng of derivative per 2 μl injection (Fig. 4). The sensitivity of the detector decreased when more than 8 ng were introduced into the gas chromatograph, probably as a result of saturation of the detector.

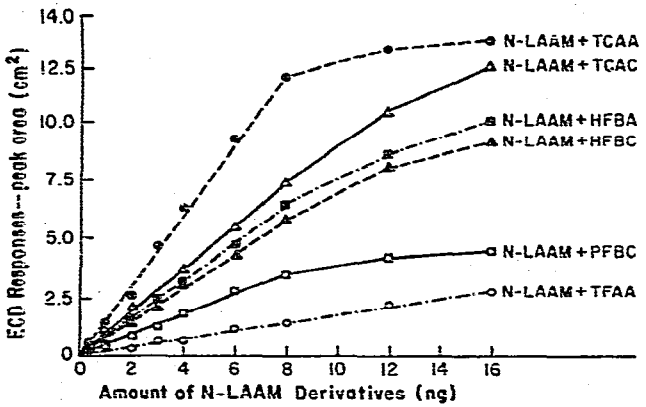


Fig. 4. ECD response to increasing amounts of the six halogenated derivatives of N-LAAM. The GLC conditions are those shown in Table II.

Quantitative applications

Under our conditions TCAC was found to be the reagent of choice for quantitating N-LAAM, DN-LAAM, and N-MOL in complex biological fluids such as plasma and urine. For most of the metabolites, the detector responses to the derivatives of this reagent were greater and more linear as compared with the other reagents. The minimum detectable amounts of the trichloroacetyl derivatives of N-LAAM, DN-LAAM and N-MOL were 0.3, 0.2, and 2.0 ng per 2-μl injections, respectively. Also, the TCAC reagent produced fewer interfering side products.

The disadvantage of the TCAC reagent is that the N-LAAM and N-MOL

derivatives had very similar retention times under our conditions. To date, N-MOL has not been reported as being present as a metabolite in plasma. Aliquots of plasma samples obtained from human subjects who had received 60 mg LAAM p.o. for up to 90 days were analyzed for the presence of N-MOL using the heptafluorobutyryl chloride reagent. No detectable amounts of N-MOL were observed in any of the samples.

Fig. 5 shows the gas chromatograms of a plasma sample obtained from a patient who had received a single 60-mg oral dose of LAAM 24 h after a 50-mg oral dose of methadone. This patient had been receiving 50 mg methadone per day for 90 days prior to this study. Methadone and LAAM could be quantitated in the plasma extract by GLC–FID while N-LAAM was barely detectable and DN-LAAM could not be detected. After the extract had been derivatized with the TCAC reagent and analyzed by GLC–ECD according to the methods described above, the N-LAAM and DN-LAAM derivatives gave distinct peaks which were easily quantitated. Thus the derivatizing reagent trichloroacetyl chloride may be usefully employed for quantitating the very low levels of N-LAAM and DN-LAAM such as might occur following an acute oral dose[14] or when small samples are available such as are obtained from experimental animals.

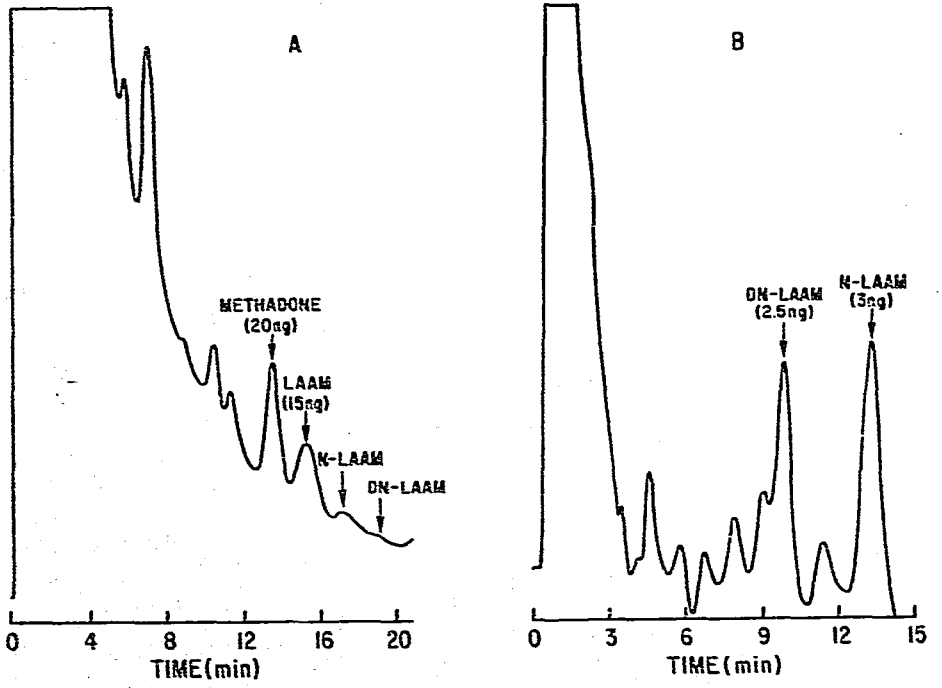


Fig. 5. Gas–liquid chromatograms of a plasma sample obtained from a methadone maintenance patient who had received a single 60-mg dose of LAAM. Chromatogram A shows the FID response to a 2-μl aliquot of the extracted sample and chromatogram B shows the ECD response following derivatization with TCAC.

ACKNOWLEDGEMENTS

Supported in part by grant DA 00284 and contract HSM-42-73-211 (ND).

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