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QUANTITATION OF *l*-α-ACETYLMETHADOL AND ITS METABOLITES IN HUMAN SERUM BY CAPILLARY GAS—LIQUID CHROMATOGRAPHY AND NITROGEN DETECTION

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SUMMARY

A procedure is described for the simultaneous measurement of *l*-α-acetylmethadol and its two pharmacologically active metabolites: noracetylmethadol and dinoracetylmethadol. In the method an intramolecular conversion reaction of the two metabolites to their amide configuration is utilized. The reaction is performed while the metabolites are still in the serum. Following solvent extraction the samples are analyzed by capillary gas—liquid chromatography coupled with nitrogen detection. Quantitation is achieved by internal standardization. The lower limit of sensitivity is 5 ng/ml in serum. Absolute sensitivity is 0.1 ng for all three compounds. The advantages over other procedures are: (1) speed due to the single extraction step; (2) increased recovery of noracetylmethadol and dinoracetylmethadol due to decreased polarity of the amides; (3) greater stability of the metabolites in the amide configuration; (4) better chromatographic quantitation and separation because detector response for the amides is greater than it is for the original configuration of the metabolites and the area of the chromatographic tracing is free of interfering substances.

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INTRODUCTION

Methadone maintenance of opiate-dependent subjects is now an accepted pharmacotherapeutic procedure. The rational for methadone use is based on the replacement of a short-acting, injectable opiate (heroin) with an orally effective long-acting one (methadone) and the production of a high-grade cross-tolerance to all opioids [1]. Methadone proved to be effective in the long-term stabilization of a large number of addicts. Programs provided a suitable environment for reaching the post-addicts by counseling, training and rehabilitation. Once the addicts are in an improved family environment with steady employment, daily clinic attendance for medication pick-up becomes a burden. Also, where take-home medication was instituted, methadone found its way into the illicit market becoming a drug of abuse. The long-acting congener of methadone, *l*-α-acetylmethadol (LAAM), became of interest since it provided up to 72 h protection against withdrawal symptoms. LAAM could be dispensed on alternate days, thus it eliminates take-home medication and diversion. The long-time action of LAAM is provided by the persistance of its active metabolites. These metabolites are also more potent than the parent compound [2]. Thus, accumulation of the metabolites may lead to toxicity [3]. These observations indicate that biotransformation characteristics of individual subjects may determine the dosing and safety of LAAM treatment. Therefore, it is necessary to monitor blood levels of LAAM, especially during induction and periodically thereafter during chronic treatment. Such monitoring detects under- and over-medication and aids in the attainment of correct drug administration. In this paper we present a method for the quantitative measurement

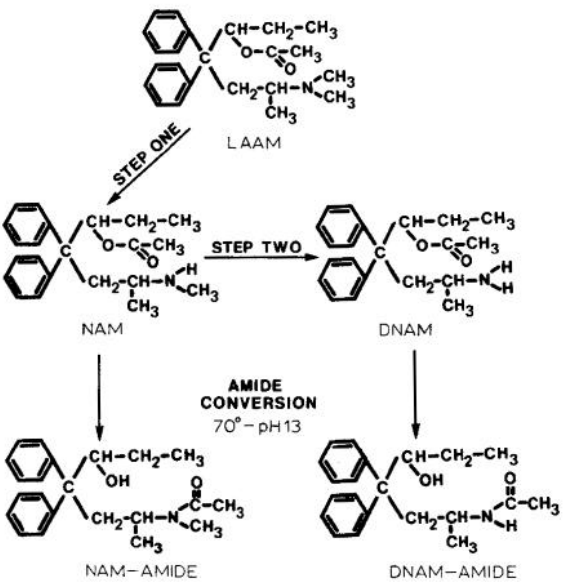

Fig. 1. The chemical structures of *l*-α-acetylmethadol (LAAM) and its active metabolites noracetylmethadol (NAM) and dinoracetylmethadol (DNAM). Following the exposure of the samples to 70° C temperature at pH 13 the metabolite structures convert to their amide configuration (NAM- and DNAM-amide).

of LAAM, noracetylmethadol (NAM) and dinoracetylmethadol (DNAM) (Fig. 1). The method provides a practical, simultaneous and rapid determination of the three compounds by capillary gas–liquid chromatography (GLC) and nitrogen detection.

EXPERIMENTAL

Materials

LAAM, NAM and DNAM were obtained from the National Institute on Drug Abuse (Rockville, MD, U.S.A., Dr. R. Hawks) and β-diethylaminoethyldiphenylpropyl acetate (internal standard) was a gift of Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.). Methanol, *n*-butyl chloride and ethyl acetate were glass-distilled reagents from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). The 50% (w/w) sodium hydroxide solution was purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Water used during the extraction procedure was purified using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Standards and sample stability

Accurate quantitative studies depend on stability and reliability of standard solutions. Observations in this laboratory indicate that LAAM and its metabolites break down in a couple of days in dilute methanolic solutions even when refrigerated. Despite the standards instability, none of the early method papers cite this problem. If standards decompose 50%, for example, the results of the unknown would be 50% over estimated.

Quantitative determination of standards was performed by diluting a stock solution of 1 mg/ml LAAM, NAM and DNAM. The dilutions were 20 ng/ml and 1 ng/ml, respectively. Analyses were performed immediately, one week and one month later. The results indicate greater breakdown at greater dilutions and with longer standing time. We found that methanolic solutions of 1 mg/ml of the three substances were stable indefinitely when kept in a freezer at −12°C (for at least one year). Also, standards prepared in water were stable longer (used for three to four months) than those in methanol (used for one to two days). Because of these observations we became concerned about the stability of LAAM and its metabolites in our unknown serum samples. A pooled serum sample containing LAAM and its metabolites was analyzed under different conditions. (a) Immediately after mixing the pool; (b) left at room temperature for 8 h; (c) left at room temperature for one week; and (d) left for one month refrigerated. The quantitative results were identical in all samples. This indicates that the breakdown process was not occurring in serum for at least one month. Thus transportation of samples in a non-frozen state does not effect drug and metabolite levels.

Extraction

To siliconized 15-ml conical centrifuge tubes the following was added: (1) the internal standard, 150 ng (in 25 μl of methanol); (2) 0.5 ml of water; (3) 0.5 ml of serum; and (4) 100 μl of 50% sodium hydroxide. The contents were mixed on a vortex GENIE mixer (Scientific Industries, Springfield, MA,

U.S.A.), for 5 sec (pH > 12). The tubes, covered with PTFE-lined screw caps, were placed in a heating block at 70°C for 15 min. After removal from the heating block the samples were allowed to cool and 5.0 ml of *n*-butyl chloride were added. The samples were shaken for 10 min at high speed on an automatic shaker (Eberbach, Ann Arbor, MI, U.S.A.) and centrifuged for 5 min at 750 *g*. The upper layer (*n*-butyl chloride) was transferred into clean test-tubes, avoiding closeness of the pipet tip to the organic aqueous interphase and the organic solvent was evaporated to dryness on a Buchler vortex evaporator (Fort Lee, NJ, U.S.A.) for 15 min. The sample tubes were capped and stored in the refrigerator until reconstituted and injected into the gas chromatograph. Sampes are stable for at least two weeks in the dry state. The dried residues were reconstituted with 25 μl of ethyl acetate and 5-μl aliquots were injected on to the capillary column.

Instrumentation

The instrument used was a Hewlett-Packard 5880A gas chromatograph equipped with a nitrogen—phosphorus detector and adopted for capillary column capability (Hewlett-Packard, Valley Forge, PA, U.S.A.). The column was used as a 25 m × 0.2 mm I.D., wall-coated OV-1 open tubular capillary column (WCOT) made of fused flexible silica (Hewlett-Packard). The mode of operation was splitless injection and cold trapping with the split flow-rate being 20 ml/min. The injector and detector temperatures were 280°C and 300°C, respectively. The initial column temperature was 190°C, which was maintained for 35 sec. The temperature was programmed to reach 220°C at 30°C/min and held for 5.8 min. Then the final temperature of 240°C was reached at 18°C/min and held for 2 min. The carrier gas was helium, flow-rate 0.5 ml/min through the column, the hydrogen flow-rate at the detector was 3 ml/min and the air 90 ml/min. A complete run can be performed in 10 min. The retention times of LAAM, NAM-amide and DNAM-amide were 5.59, 10.10 and 9.13 min, respectively, while that of the internal standard was 7.16 min. In Fig. 2 chromatographic tracings are presented. The tracings are the result of the extraction and chromatography of a blank serum sample and also a serum sample to which LAAM, NAM, DNAM and internal standard were added. The arrows on the tracing (blank serum) indicate the areas where LAAM, its metabolites and internal standard would appear if present. Thus no interfering peaks were identified at the zones of interest.

Quantitation and sensitivity

LAAM, NAM and DNAM were prepared in concentrated stock solutions of 1 mg/ml in methanol, which were diluted to working solutions on the day of use. This procedure was adopted to avoid complications due to the instability of standards in dilute solutions even when stored in the refrigerator. Before each run 5, 10, 20, 50 and 100 ng of LAAM, NAM and DNAM were added to 0.5 ml of blank serum and 150 ng of internal standard were also added. The standards were extracted according to the procedure described above and injected into the gas chromatograph. The standard curves were linear up to 800 ng/ml, the highest concentration tried, although routinely the highest standard concentration used was 100 ng/ml. Quantitation is performed by programming the instrument to measure the peak heights of the unknown

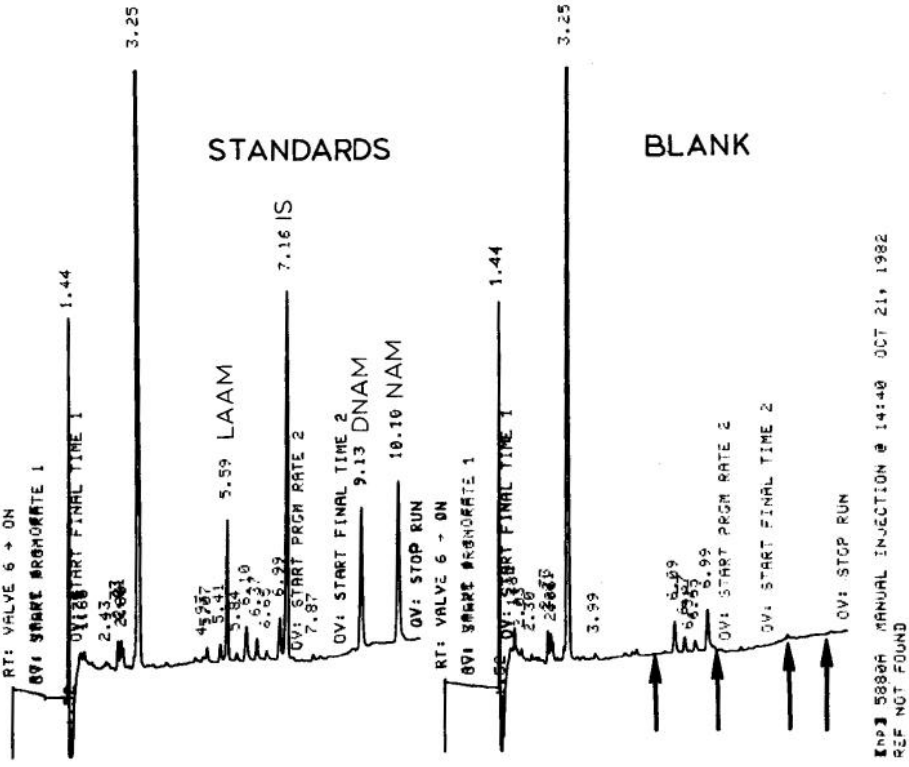

Fig. 2. Chromatograms of standards of LAAM, NAM and DNAM at 50 ng/ml and the internal standard (IS) at 150 ng/ml which were added to blank serum and extracted according to the method; and of a sample of blank serum which was also extracted. The arrows indicate the zones at which the peaks of interest are expected if present.

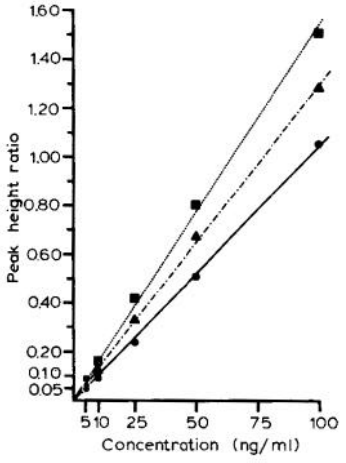

Fig. 3. Standard curves for LAAM (●), NAM (■) and DNAM (▲) at concentrations of 5, 10, 25, 50 and 100 ng/ml. The standards were added to blank serum, extracted and injected into the gas chromatograph. The points on the graph are each the ratio of the standard peak height divided by the internal standard peak height. Each point represents the mean of six determinations.

samples of LAAM, NAM and DNAM and divide them by the peak height of the internal standard. The instrument computer calculates the standard curve slope and multiplies the unknown sample peak height ratio with the slope factor. The results are obtained on the printout in ng/ml. A graphic representation of the standard curve for the three compounds of interest is shown in Fig. 3. The peak height ratio ± S.D. in six standard curves at concentrations of 5, 10, 25, 50, 75 and 100 ng/ml points were for LAAM 0.04 ± 0.004, 0.09 ± 0.088, 0.23 ± 0.02, 0.50 ± 0.04 and 1.05 ± 0.06, respectively; for NAM 0.08 ± 0.008, 0.16 ± 0.02, 0.42 ± 0.03, 0.80 ± 0.05 and 1.51 ± 0.09; and for DNAM 0.06 ± 0.005, 0.12 ± 0.01, 0.32 ± 0.02, 0.67 ± 0.05 and 1.27 ± 0.08 (see Fig. 3).

The detection sensitivity of absolute standards by nitrogen—phosphorus detection (NPD) is 0.1 ng for all three substances. After extraction of LAAM and its metabolites from serum and analysis by GLC 5 ng/ml can be accurately determined.

Reproducibility

To 20 ml of calf serum 1 μg of LAAM, 2 μg of NAM and DNAM and 1 μg of internal standard were added. Thus each ml contained 50 ng of LAAM, 100 ng of NAM and DNAM and 50 ng of internal standard. To each of twelve test tubes a 0.5-ml aliquot from the above described serum pool was added. The samples were processed through the procedure and analyzed. The mean ± S.D. values for LAAM, NAM and DNAM were 51.8 ± 2.1, 95.5 ± 3.5 and 100.6 ± 4.2 ng/ml, respectively. The coefficients of variation for LAAM, NAM and DNAM were 4.1, 4.7 and 6.2%, respectively.

Recovery studies

Recovery of LAAM, NAM and DNAM were performed using ^{3}H-labeled substances. A 10-ng amount of each ^{3}H-labeled substance was added in quadruplicate to 0.5 ml drug-free sheep serum and processed according to the method. Aliquots of *n*-butyl chloride were counted in a scintillation counter (Mark III, 6880, Searle Analytic, Des Plaines, IL, U.S.A.) adjusted for the total volume of extraction solvent and compared to counts of the absolute standards. The recoveries were: LAAM 63 ± 1.1%, NAM 94 ± 1.2% and DNAM 84 ± 2.0%.

RESULTS AND DISCUSSION

The paucity of metabolic data on LAAM in human subjects is probably due to the lack of simple and sensitive methodology. The analysis of LAAM in previous studies was performed by thin-layer chromatography [4–6], GLC [7–10], combined gas chromatography—mass spectrometry [11, 12], and high-performance liquid chromatography (HPLC) [13]. GLC and HPLC methods are suitable for the quantitative analysis of LAAM and its metabolites because of sufficient sensitivity and selectivity. The first detailed method was published by Kaiko et al. [7] in 1975. They utilized the intramolecular conversion of NAM and DNAM to their corrresponding amides at highly alkaline pH. A similar conversion of norpropoxyphene to norpropoxyphene amide was described by Verebey and Inturrisi [14]. Conversion was neces-

sary because the LAAM metabolites do not separate well on GLC columns and are not stable during extraction. The structural changes of NAM and DNAM to their amide configuration are shown in Fig. 1. The method of Lau and Henderson [10] provided greater sensitivity by derivatization of LAAM and its metabolites with halogens for electron-capture detection (ECD). This method measured only the metabolites. LAAM was determined by flame-ionization detection (FID). Quantitative analysis of LAAM and its known metabolites was carried out by Kiang et al. using HPLC [13].

Most published methods are suitable for research purposes but not for routine clinical application because of too many extraction steps in the procedure and the analysis time is too long. A summary is prepared of the published methods regarding the number of extraction steps, evaporations, volume of sample required, method's selectivity for LAAM and/or all metabolites, derivatization, mode of detection, retention time and lower limit of sensitivity (Table I). The method reported in this paper is the simplest and quickest to perform of the available methods. It has a single extraction step, one evaporation step, no derivatization, it requires only 0.5 ml of serum and provides for the simultaneous quantitation of the three pharmacologically active components of LAAM.

There is a note in the literature by Tse and Welling [8] which describes the breakdown of LAAM to methadol when heated at 70°C for 30 min in an alkaline solution. We repeated this experiment and confirmed its validity. However, when the same reaction conditions were carried out using serum instead of a protein-free aqueous media, no breakdown of LAAM was observed. LAAM breakdown is measured by the analysis for methadol which appears on the chromatographic tracing. No peak was seen in the methadol area following the amine-to-amide conversion reaction performed in serum.

The method was tested by analyzing serum samples from subjects who were stabilized on various doses of LAAM. The most practical sample collection times were 48 and 72 h after the dose, when the patients came to the clinic for their next dose or for counselling. Table II shows the results. At 48 h after the dose the average levels of LAAM, NAM and DNAM were 13.8 ± 8.0, 64.7 ± 40.7 and 78.8 ± 41.8 ng/ml, respectively. At 72 h the same for LAAM, NAM and DNAM were 7.9 ± 2, 29.4 ± 27.2 and 40.1 ± 19.4 ng/ml, respectively. The large dose differences and presumably the individual variation in LAAM metabolism resulted in large standard deviations from the average values. Table III shows the comparison of the data with the findings of other investigators. The average data in this study are in close agreement with those of Kaiko et al. [7] and Kiang et al. [13], while significantly higher levels of LAAM and its metabolites were reported by Henderson et al. [15], Finkle et al. [16] and Blaine et al. [17]. The reason for these differences is not known. The lower values for LAAM in this study are definitely not caused by the relatively low recovery of LAAM (63%). Using an internal standard method, the curves were straight, the unknown samples were treated the same way as the standard and the area of methadol, the known decomposition product of LAAM, was clear on the chromatogram. At lower pH vlaues, i.e. 9.0, LAAM recovery can be increased over 95% but the metabolite recovery is very low. Due to the higher chromatographic response of LAAM compared to

TABLE I

SUMMARY OF PUBLISHED METHODS FOR THE QUANTITATION OF LAAM AND ITS METABOLITES IN BLOOD

Author	Ref. No.	Number of extraction steps	Number of evaporation steps	Sample size (ml)	Simultaneous extraction and detection	Derivatization	Chromatographic method and mode of detection	Retention time (min)	Sensitivity (ng/ml)
Verebey et al.	Present paper	1	1	0.5	Yes	No	Capillary GLC with NPD	5.6 LAAM	5
								9.1 NNAM*	5
								10.1 NAM*	5
Lau and Henderson	10	2	3	2.0	No	Yes (2 h)	GLC with FID and ECD	15.0 LAAM	50
								10.0 NNAM	50
								14.0 NAM	50
Kaiko et al.	7	4	1	1—4	Yes	No	GLC with FID	1.8 LAAM	10
								3.6 NNAM*	10
								4.2 NAM*	25
Tse and Welling	8	4	1	1—4	No	No	GLC with FID	3.2 LAAM	10
								NR** NNAM	10
								NR NAM	25
Kiang et al.	13	6	3	1	Yes	No	HPLC with UV	5.0 LAAM	6
								10.0 NNAM	10
								8.0 NAM	6

*Amide.
**NR = Not reported.

TABLE II

SERUM LEVELS OF LAAM, NAM AND DNAM IN PATIENTS RECEIVING VARIOUS DOSES OF LAAM AT 48 AND 72 h AFTER THE DOSE

Patient	Dose (mg)	Serum levels (ng/ml)		
		LAAM	NAM	DNAM
48-h Samples				
1	40	5.3	52.9	35.5
2	45	7.5	20.5	29.8
3	50	11.3	31.9	66.7
4	55	11.3	59.0	95.1
5	55	24.9	129.8	132.9
6	65	22.3	93.9	112.5
Mean ± S.D.	51.7 ± 8.8	13.8 ± 8.0	64.7 ± 40.7	78.8 ± 41.8
72-h Samples				
7	25	ND*	11.8	25.7
8	35	7.1	23.9	24.8
9	40	7.5	10.6	24.1
10	45	8.9	34.0	43.8
11	45	5.3	21.4	36.5
12	60	10.7	88.2	78.8
13	65	ND	15.7	46.8
Mean ± S.D.	45 ± 13.8	7.9 ± 2.0	29.4 ± 27.2	40.1 ± 19.4

*ND = Not detected.

TABLE III

COMPARATIVE RESULTS OF PLASMA LEVELS OF LAAM, NAM AND DNAM IN VARIOUS STUDIES

Author	Ref. No.	Number of subjects	Sample time (h)	Dose (mg)	Plasma levels (ng/ml)		
					LAAM	NAM	DNAM
Kaiko et al.	7	12	48	40–60	ND*	48	70
Kiang et al.	13	5	24	40–60	22	63	46
Verebey et al.	Present paper	6	48	40–65	14	65	78
Henderson et al.	15	3	48	85	80	70	175
Blaine et al.**	17	12	48	60–80	79	184	102

*ND = Not detected.
**Blood level data are from Finkle et al. [16].

its metabolites (unconverted) it was more useful to extract at a high pH which yielded cleaner chromatograms; higher metabolite recoveries and sufficient amount of LAAM to allow quantitation of LAAM and its metabolites simultaneously.

Further studies in our laboratory are in progress to investigate the serum levels expected in individuals following various doses of LAAM and various

conditions which may alter the blood levels of LAAM during maintenance treatment.

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REFERENCES

1 V.P. Dole and M.E. Nyswander, JAMA, 193 (1965) 646.
2 R.E. Billings, R.E. McMahon and D.A. Blake, Life Sci., 14 (1974) 1437.
3 G.L. Henderson, J.A. Weinberg, W.A. Hargreaves, D.H.M. Lau, J. Taylor and B. Baker, J. Anal. Toxicol., 1 (1977) 1.
4 G.L. Henderson, N. North-Roof and S.H. Kutterb, Drug Metab. Dispos., 5 (1977) 321.
5 A.L. Misra, R. Bloch and S.J. Mulé, J. Chromatogr., 106 (1975) 184.
6 S. Campos-Flor and C.E. Inturrisi, J. Anal. Toxicol., 1 (1977) 75.
7 R.F. Kaiko, N. Chatterjie and C.E. Inturrisi, J. Chromatogr., 109 (1975) 247.
8 F.S.L. Tse and P.G. Welling, J. Chromatogr., 135 (1977) 205.
9 R.E. Billings, R. Booher, S. Smits, A. Pohland and R.E. McMahon, J. Med. Chem., 16 (1973) 305.
10 D.H.M. Lau and G.L. Henderson, J. Chromatogr., 129 (1976) 329.
11 H.R. Sullivan, S.L. Due and R.E. McMahon, Res. Commun. Chem. Pathol. Pharmacol., 6 (1973) 1072.
12 T.A. Jennison, B.S. Finkle, D.M. Chinn and D.J. Crouch, J. Chromatogr. Sci., 17 (1979) 64.
13 C.-H. Kiang, S. Campos-Flor and C.E. Inturrisi, J. Chromatogr., 222 (1981) 81.
14 K. Verebey and C.E. Inturrisi, J. Chromatogr., 75 (1973) 195.
15 G.L. Henderson, B.K. Wilson and D.H.M. Lau, Clin. Pharmacol. Ther., 21 (1976) 16.
16 B.S. Finkle, T.A. Jennison, D.M. Chinn. W. Ling and E.D. Holmes, J. Anal. Toxicol., 6 (1982) 100.
17 J.D. Blaine, D.B. Thomas, G. Barnett, J.A. Whysner and P.F. Renault, in J.H. Lowinson and P. Ruiz (Editors), Substance Abuse: Clinical Problems and Perspectives, Williams and Wilkins, Baltimore, MD, London, 1981, Ch. 28, p. 379.