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THE QUANTITATION OF CYCLAZOCINE AND ITS METABOLITES IN HUMAN URINE BY USE OF GAS–LIQUID CHROMATOGRAPHY*

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SUMMARY

A method is described for the quantitative determination of cyclazocine and its N-dealkylated biotransformation product, norcyclazocine, in human urine. The method can also be used to estimate the levels of conjugated cyclazocine and norcyclazocine by measurement of the amount of these compounds released by acid hydrolysis. The compounds are recovered from urine by the use of solvent extraction and separated as their trifluoroacetyl derivatives by the use of gas–liquid chromatography.

The levels of cyclazocine and metabolites were determined in the urine collected from two patients receiving 1.9 mg of cyclazocine every 12 h for the treatment of opiate dependence. Approximately 60% of the administered dose was recovered in patient urine as cyclazocine and metabolites. An average of 21% of the administered dose appeared in the urine as cyclazocine, 24% as conjugated cyclazocine, 4% as norcyclazocine and 11% as conjugated norcyclazocine.

INTRODUCTION

Cyclazocine, a member of the benzomorphan series, exhibits potent narcotic antagonist and agonist properties in the human. Currently, cyclazocine is under evaluation for treatment of opiate dependence. Martin *et al.*[1], Jaffe and Brill[2] and Freedman *et al.*[3] all have reported that cyclazocine can be an effective narcotic blocking agent in post-addicts.

At present there is no information available on the biotransformation of cyclazocine in the human. Mulé and Gorodetzky[4] and Mulé *et al.*[5] reported that following the subcutaneous administration of tritiated cyclazocine the drug is biotransformed in the dog to norcyclazocine and glucuronide conjugates of both cyclazocine and norcyclazocine. The purpose of this report is to describe a specific and sensitive method for the identification and quantitation of cyclazocine and norcyclazocine in the urine of two patients receiving cyclazocine for the treatment of opiate dependence.

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MATERIALS AND METHODS

Chemicals and reagents

d,l-Cyclazocine and *d,l*-norcyclazocine, each as the free base, were provided by Dr. S. Archer of The Sterling-Winthrop Research Institute (Rensselaer, N.Y., U.S.A.). Tritiated cyclazocine, as the cyclohexylsulfamate salt, was a gift of Dr. C. Gorodetzky of the Addiction Research Center (Lexington, Ky., U.S.A.). The internal standard, levallorphan as the tartrate salt, was provided by Hoffmann-LaRoche (Nutley, N.J., U.S.A.) (Fig. 1). Trifluoroacetylimidazole was obtained from Pierce (Rockford, Ill., U.S.A.). β-Glucuronidase, Type H-1, was obtained from Sigma (St. Louis, Mo., U.S.A.).

Hexane, *n*-butyl chloride and chloroform are Distilled in Glass® and obtained from Burdict and Jackson (Muskegon, Mich., U.S.A.). Isobutanol is reagent grade.

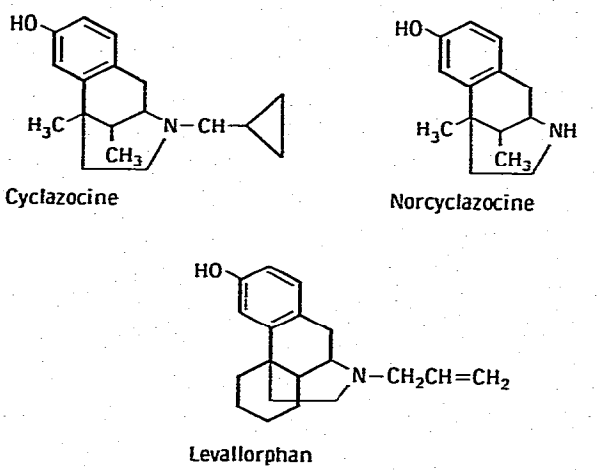

Fig. 1. Structural formulae of cyclazocine, norcyclazocine and levallorphan, the internal standard.

Stock solutions

Aqueous solutions of cyclazocine, norcyclazocine and levallorphan each at a concentration of 20 μg/ml are prepared and kept refrigerated.

Sample preparation from urine

The extraction procedure is adapted from that described by Inturrisi and Verebely[6] for the extraction of methadone from plasma and urine. A flow sheet outlining this procedure is given in Fig. 2. To urine (1–4 ml) in a siliconized 15-ml centrifuge tube with a Teflon®-lined screw cap are added 0.2 ml of the aqueous solution of internal standard, 0.5 ml of Delory and King's carbonate–hydrogen carbonate buffer[7], 1 *M*, pH 9.8, and one drop of 1-octanol. After thorough mixing the sample is extracted with 5.0 ml of *n*-butyl chloride–isobutanol (7:3) by shaking for 5 min followed by centrifugation at 1500 rpm (500 *g*) for 5 min. The upper, organic, phase is removed and saved. The extraction is then repeated with an additional 5.0 ml of the *n*-butyl chloride–isobutanol (7:3) mixture and this organic phase is added to the one

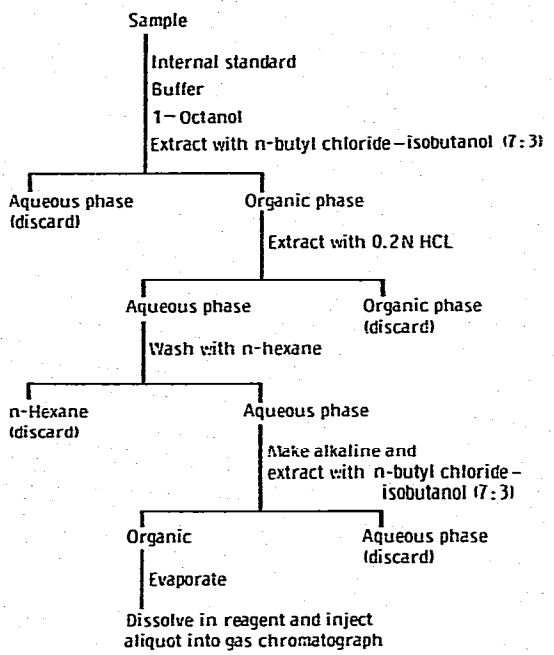

Fig. 2. A flow sheet outlining the procedure for the extraction of cyclazocine and norcyclazocine from urine.

resulting from the initial extraction. The compounds are extracted into acid by the addition of 5.0 ml of 0.2 *N* hydrochloric acid to the combined organic phases and shaking for 7 min followed by centrifugation at 500 *g* for 3 min. The upper, organic, phase is removed by aspiration and discarded. The acid phase is washed by the addition of 5.0 ml of *n*-hexane and shaking for 5 min followed by centrifugation at 500 *g* for 3 min. The *n*-hexane phase is removed by aspiration and discarded. The washed aqueous phase is made alkaline by the addition of 0.4 ml of concentrated ammonium hydroxide (pH adjusted to approx. 10). The compounds are extracted into 7.0 ml of the *n*-butyl chloride–isobutanol solvent mixture by shaking for 5 min followed by centrifugation for 3 min. The upper, organic, phase is transferred into a 12-ml siliconized centrifuge tube. The sample extract is concentrated by evaporating the organic phase to dryness with the use of a multiple flash evaporator with the bath at 65° (Evap-O-Mix, Buchler, Fort Lee, N.J., U.S.A.). The sample extract is concentrated in the lower tip of the tube by rinsing the lower sides of the tube with 50 μl of chloroform and allowing this to evaporate. The sample extract is dissolved in 10 to 20 μl of trifluoroacetylimidazole–chloroform (1:4) and between 1 and 4 μl are injected immediately into the gas chromatograph.

Gas–liquid chromatography (GLC)

The GLC analysis is performed on a Varian Model 1740 gas chromatograph equipped with a hydrogen flame ionization detector. The column is a 6-ft.-long spiral glass with a 2-mm I.D. The liquid phase is 3% SE-30 on Gas-Chrom Q, 80–100 mesh. The temperature of both the detector and injector port is 260°. Helium, at a flow-rate

of between 30 and 35 ml/min, is the carrier gas. Hydrogen and air flow-rates are between 32 and 40 ml/min and between 200 and 250 ml/min, respectively. The gas flows are adjusted to give maximal detector response. A column oven temperature of between 210 and 220° is used for the analysis. Detector sensitivity is varied from 8 to $32 \cdot 10^{-11}$ A/mV at full scale.

Calibration curves and quantitation

Standard calibration curves are generated by the addition of cyclazocine and norcyclazocine in the selected amounts from 0.4 to 8.0 μg to a 4.0-ml sample of control urine and proceeding as described above. The peak height of the detector response to each compound is divided by the peak height of the internal standard to yield a ratio. Standard calibration curves are generated relating these peak height ratios to the amount of each compound added to the sample. Each calibration curve is constructed from triplicate determinations of five different points. The amount of each compound in an unknown urine sample is determined by converting the peak height ratio obtained into the absolute amount of compound present in the sample. The linearity of the standard calibration curves within the range indicated allows the use of calculated slopes for these conversions.

Recoveries

The recovery of cyclazocine and norcyclazocine from urine was determined by adding selected amounts from 0.4 to 8.0 μg of each compound to 2.0 ml of control urine samples and extracting as described above. The amount of each compound recovered was compared to the amount obtained when the same selected amounts were added to the extracts of a second set of control urine samples just prior to their evaporation to dryness. The single variable in this procedure for the determination of the percentage recovery from extraction is the time of the addition of compound to the urine, prior to or after the multi-step extraction procedure. That is, the only difference between the two sets of final extracts is that the former contains compound remaining after the losses incurred through the extraction and the latter set contains the total amount of each compound added. All other variables are controlled for. Quantitation was achieved in both sets of samples using GLC by the addition of internal standard to the extracts just prior to their evaporation to dryness. Samples from the two sets were alternately injected into the gas chromatograph in order to control for any changes in column or instrumentation conditions. After correcting for aliquot losses, the recovery from urine was 85.6% ± 3.7 S.D. for cyclazocine and 93.9% ± 12.0 S.D. for norcyclazocine. The mean recovery of each compound was based on triplicate determinations of four amounts. The recovery of cyclazocine was also determined using tritiated compound (approx. 18 mCi/mmole labeled at C-3). The mean recovery was 88.5% ± 3.1 S.D. for the same amounts as determined by GLC using the same controlled approach.

Hydrolysis of conjugation products

Acid-hydrolyzable conjugates of cyclazocine and norcyclazocine are released as free bases by adding a volume of concentrated hydrochloric acid equal to 1/10th of the urine sample volume and heating for 1 h at 121° under 18 p.s.i. in an autoclave according to the method of Mulé *et al.*[5].

To determine the extent to which glucuronide conjugates account for the acid-hydrolyzable conjugates of cyclazocine and norcyclazocine we compared the amount of free base of each compound released following acid-hydrolysis to that following hydrolysis by β-glucuronidase. Duplicate 2.0-ml urine samples (K.C., 0–4 h post-drug) are incubated for 48 h with 2.0 ml of β-glucuronidase, 5000 Fishman U/ml of 0.5 *M* acetate buffer, pH 5.0. Duplicate samples of the above urine are incubated with acetate buffer without enzyme added. A third set of urine samples is subjected to acid hydrolysis as described above. Prior to the extraction and GLC quantitation of the bases the pH of the urine samples is adjusted to approximately 10 by the addition of concentrated ammonium hydroxide.

Subjects

The excretion of cyclazocine and metabolites was determined in urine collected from two patients under treatment for opiate dependence during 4-h periods for the first 12 h after an oral dose of 1.9 mg of *d,l*-cyclazocine. The samples were frozen at −20 prior to analysis.

RESULTS AND DISCUSSION

In order to develop a method with sufficient sensitivity to measure the relatively low levels of cyclazocine and norcyclazocine that might be anticipated in the biofluids of these patients a comparison was made of the gas chromatographic detector response to cyclazocine, norcyclazocine and their trifluoroacetylated derivatives. When the peak height of the detector response to each compound is plotted as a function of the absolute quantity injected into the gas chromatograph (Fig. 3) it is clear that the linear dynamic range of the trifluoroacetyl derivatives extends through considerably lower nanogram quantities than that observed with the corresponding underivatized bases. We estimate that the lower limit of sensitivity (*i.e.* a 5% deflection above baseline) to cyclazocine was 50 ng and to norcyclazocine approximately 200 ng while the lower limit of sensitivity to the corresponding derivatives extends to approximately 10 ng. In addition, the relative S.E. for triplicate determinations was ±1.5% for trifluoroacetylated cyclazocine compared to ±15.5% for underivatized cyclazocine and ±1.0% for trifluoroacetylated norcyclazocine compared to ±34.5% for underivatized norcyclazocine.

The trifluoroacetylation of cyclazocine and norcyclazocine favors their GLC quantitation using the flame ionization detector by producing more volatile compounds resulting in less adsorption onto column packing materials. This is especially true for norcyclazocine. The method of Ahmad and Medzihradsky[8] for the determination of benzomorphans using underivatized compounds included neither norcyclazocine nor a demonstration of the quantitation of cyclazocine in the biofluids of the human. Pittman and Williams[9] have reported a radio-immunoassay method for cyclazocine but as yet it has not been used to quantitate levels of the drug in human biofluids following its administration. The method reported here is sufficiently sensitive for the quantitation of both cyclazocine and norcyclazocine in the urine of human subjects receiving cyclazocine for the treatment of opiate dependence. However, if flame ionization detection of these compounds proves inadequate for plasma-level determinations, these halogenated derivatives permit the utilization of an electron

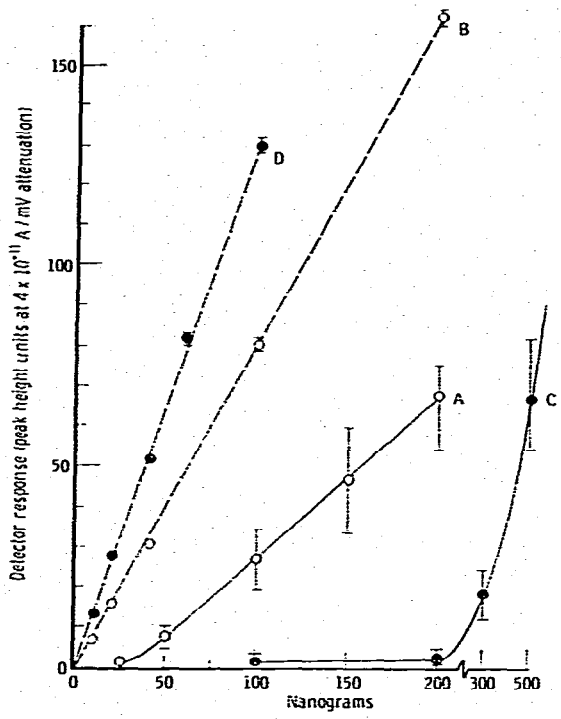

Fig. 3. Effect of trifluoroacetylation of cyclazocine and norcyclazocine on linear dynamic range. Each point represents the mean of triplicate determinations; the brackets represent the range. GLC conditions are as described in Materials and methods. A = Cyclazocine; B = trifluoroacetylated cyclazocine; C = norcyclazocine; D = trifluoroacetylated norcyclazocine.

capture detector which would further increase the limits of sensitivity of the method.

The method can be used to quantitate as little as 40 ng of cyclazocine and norcyclazocine per 4-ml aliquot of urine. A relative S.E. of $\pm 3.0\%$ was obtained for triplicate determinations of points representing known amounts of drug and metabolite added to urine and used as the standard calibration curves. A relative S.E. of $\pm 7.5\%$ was obtained for triplicate determinations of cyclazocine and norcyclazocine in patient urine samples.

Examples of chromatograms obtained under the conditions described in Materials and methods are given in Fig. 4. The multi-step extraction procedure results in an extract that is free of interfering peaks. In most cases it was possible to introduce samples into the gas chromatograph every 12 min. Fig. 4a shows a chromatogram of an extract of the urine collected during the 8–12-h period after an oral dose of 1.9 mg of cyclazocine. The extract contains peaks that correspond in retention time to the trifluoroacetyl derivatives of norcyclazocine (NC), cyclazocine (C) and the added internal standard, levallorphan (L). Fig. 4b shows the chromatogram of an extract of an equivalent aliquot of this sample urine. This urine sample, however, was subjected to acid hydrolysis prior to extraction. It is clear, even by visual inspection, that the ratio of the peak height of either NC or C to that of the internal standard, L, is greater in Fig. 4b than in Fig. 4a. Thus, samples exposed to acid hydrolysis have

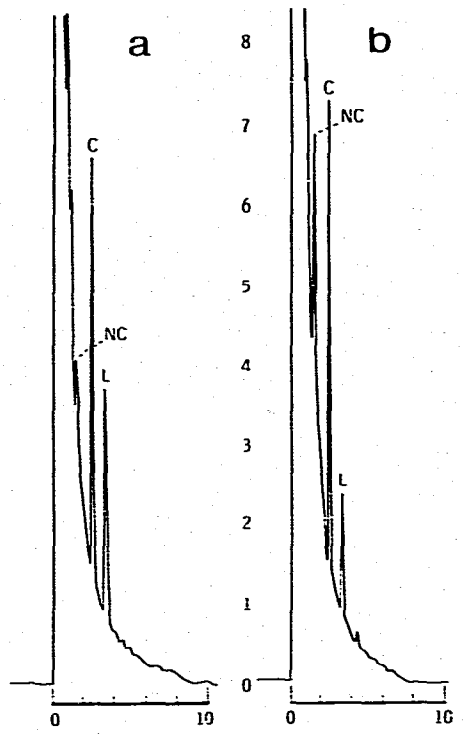

Fig. 4. Chromatograms of human urine extracts from a patient who received an oral dose of 1.9 mg cyclazocine (C). The internal standard, levallorphan (L), was added directly to the urine and the extract prepared. Norcyclazocine (NC), C and L present in the extract were trifluoroacetylated just prior to GLC analysis. (a) Free unconjugated C and NC. (b) Total free and conjugated C and NC resulting from acid hydrolysis of the urine prior to extraction. Retention times are NC = 1.8 min, C = 2.7 min and L = 3.6 min. GLC conditions are as described in Materials and methods.

a greater total amount of both compounds than the corresponding unhydrolyzed samples. The amount of cyclazocine and norcyclazocine present as a conjugate was calculated by subtracting the amount determined in Fig. 4a (free compound) from the amount determined in Fig. 4b (total compound). No significant difference was found between the amount of conjugated compounds computed by this method and the amounts obtained when samples were subjected to acid hydrolysis after the free bases had been removed by extraction.

Table I contains data on the total urinary excretion of cyclazocine and metabolites for the 12 h following a 1.9-mg oral dose of cyclazocine. During this period the total base recovered was predominantly as cyclazocine and its conjugate. In patient K.C. 24.4% of the administered dose (0.464 mg) was excreted as conjugated cyclazocine and 14.8% (0.281 mg) as free cyclazocine. For patient B.W. 23.4% of the administered dose (0.445 mg) was excreted as conjugated cyclazocine and 26.2% (0.498 mg) as free cyclazocine. In patient K.C. 14.0% of the administered dose (0.266 mg) was excreted as conjugated norcyclazocine and 5.7% (0.109 mg) as free norcyclazocine. For patient B.W. 8.8% of the administered dose (0.168 mg) was excreted as conjugated norcyclazocine and only 2.0% (0.037 mg) as free norcyclazocine.

TABLE I
TOTAL URINARY EXCRETION OF CYCLAZOCINE, NORCYCLAZOCINE AND THEIR CONJUGATES IN THE 12 h FOLLOWING A 1.9-mg ORAL DOSE OF CYCLAZOCINE

Subject	*Base (mg)*					*Percent of dose recovered*
	Cyclazocine		*Norcyclazocine*		*Total base recovered*	
	Free	*Conjugated*	*Free*	*Conjugated*		
K. C.	0.281	0.464	0.109	0.266	1.120	58.9
B. W.	0.498	0.445	0.037	0.168	1.148	60.4

Approximately 60% of the administered dose was recovered in the urine of both patients as cyclazocine and metabolites.

These data indicate that the biotransformation of cyclazocine in the human resembles that found by Mulé *et al.*[5] in the dog to the extent that cyclazocine was excreted principally as free and conjugated drug. In addition, free and conjugated norcyclazocine were excreted in appreciable amounts. In the urine excreted for 12 h after drug administration, an average amount equal to 60% of the orally administered dose could be accounted for as cyclazocine and biotransformation products. To explain the incomplete recovery of administered dose it may be suggested that the drug was not completely adsorbed from the gastrointestinal tract, that there are routes of elimination other than the kidney, that other biotransformation products are formed as suggested by Mulé *et al.*[5], and that the patients had not yet reached a balance between drug absorption and elimination. There are, as yet, insufficient data to support or reject any of these explanations. It is likely, however, that the excretion of a given dose of cyclazocine in these patients requires considerably more than 12 h. This is indicated from the relatively unattenuated rate of excretion of cyclazocine and metabolites illustrated in Fig. 5. This figure represents the cumulative urinary excre-

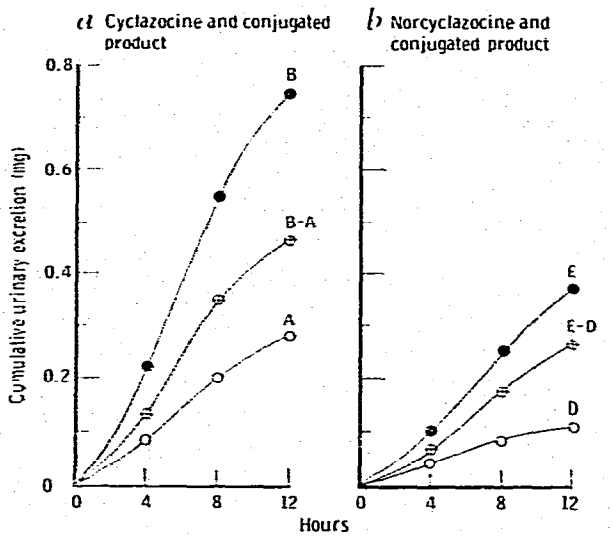

Fig. 5. Urinary excretion of cyclazocine (A), conjugated cyclazocine (B-A), cyclazocine plus conjugated cyclazocine (B), norcyclazocine (D), conjugated norcyclazocine (E-D) and norcyclazocine plus conjugated norcyclazocine (E) following an oral dose of 1.9 mg of cyclazocine to patient K.C.

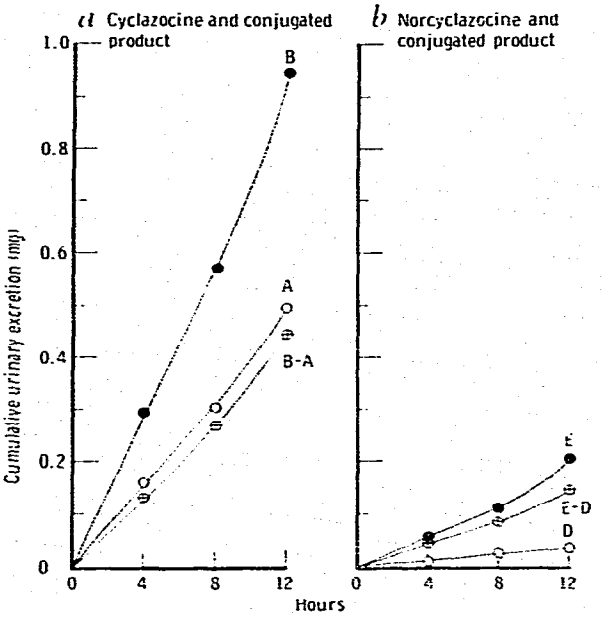

Fig. 6. Urinary excretion for patient B.W. See Fig. 5 for description.

tion of cyclazocine and metabolites for the 12 h following drug administration to patient K.C. The relative rate of excretion of each form of each base remained in the same order throughout the 12-h collection period. If a comparison of the amount of each compound excreted during each period is made, it reveals that the highest rates of excretion of cyclazocine and metabolites occurs during the 4–8 h period after drug administration. However, the considerable quantities excreted during the 8–12-h period suggests that there is continued excretion beyond 12 h. Indeed, for patient B.W. the highest rates of excretion of cyclazocine, cyclazocine conjugate and norcyclazocine conjugate occur during the 8–12 h period as illustrated in Fig. 6. The patients receive cyclazocine every 12 h for the maintenance of a narcotic blockade. If the blockade is to be effective throughout the 12-h dosing interval, one would not expect to recover the total administered dose within the interval.

The amount of free cyclazocine released by β-glucuronidase hydrolysis of the 0–4-h post-drug urine of patient K.C. was equivalent to the amount released by acid-hydrolysis. In contrast, β-glucuronide hydrolysis accounted for only 25% of the total acid-hydrolyzable norcyclazocine conjugate in this sample. Mulé *et al.*[5] accounted for the acid-hydrolyzable conjugates of both cyclazocine and norcyclazocine as β-glucuronide conjugates in the dog. Our preliminary results indicate that in the human also cyclazocine and norcyclazocine are excreted as glucuronide conjugates. The total excreted acid-hydrolyzable conjugate of cyclazocine can be accounted for as the glucuronide conjugate whereas for norcyclazocine the partial recovery of conjugate as the glucuronide may indicate that norcyclazocine is excreted in the form of other conjugates in human urine.

The methods we have described should prove valuable in the elucidation of the pharmacokinetics of cyclazocine in man. We are currently applying the method de-

scribed above to study the metabolism and excretion of cyclazocine in a considerably larger number of subjects. The modification of this method to allow the determination of plasma levels of drug and metabolite should provide a means of elucidating the relationship between plasma level and narcotic blockade.

The results of this preliminary study indicate that both N-dealkylation and conjugation are important routes of biotransformation for cyclazocine in man (Fig. 7).

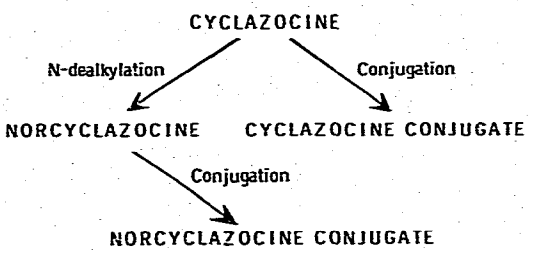

Fig. 7. Metabolism of cyclazocine in the human.

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