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IMPROVED GAS CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF CLONAZEPAM LEVELS IN PLASMA USING A NITROGEN-SENSITIVE DETECTOR

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SUMMARY

A gas—liquid chromatographic procedure (GLC) is described for the determination of clonazepam in plasma. The drug is extracted from buffered plasma at pH 9.0 with diethyl ether and then back-extracted into 6 *N* hydrochloric acid—6 *N* sulfuric acid (95:5) and hydrolyzed at 100°C to convert the drug into its benzophenone derivative. The benzophenone derivative of flurazepam is added to plasma as an internal reference standard. Drug derivatives are finally extracted from the neutralized aqueous phase and assayed by GLC. The present procedure makes use of a nitrogen-sensitive detector which is more stable and selective than the commonly employed electron-capture procedure. The sensitivity of the detector for clonazepam is 1 ng/ml.

INTRODUCTION

Clonazepam (Clonopin®, Roche), one of the relatively newer members of the benzodiazepine class of compounds, has been increasingly used in the treatment of seizure disorders. Therapeutic drug level monitoring of various anticonvulsant drugs has been a valuable tool in the management of epilepsy. However, pharmacokinetics of clonazepam, as well as its blood level data during chronic and single-dose administration, are relatively few [1—4]. Clonazepam is usually prescribed along with other common anticonvulsant drugs such as diphenylhydantoin, phenobarbital and succinimides. Data about the possible interactions of these anticonvulsants with clonazepam and its metabolism are very limited.

Gas—liquid chromatography (GLC) has been a valuable tool in therapeutic drug level monitoring because of its versatility in qualitative and quantitative

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analysis of a wide variety of drugs. In routine therapeutic drug level monitoring, a flame ionization detector (FID) is often used for drugs present in μg/ml concentrations in plasma. The sensitivity of FID is unsuitable for those anticonvulsant drugs which are present in plasma only in ng/ml quantities. Electron-capture (EC) detectors have been used for monitoring various drugs present in low concentrations in plasma, including clonazepam [5—10] but their use in routine laboratory work is difficult because of inherent instability and because of extremely high response to halogen-containing compounds which may be present as impurities. Since many drugs contain nitrogen, recently introduced nitrogen—phosphorus-sensitive detectors have been increasingly used in therapeutic drug level monitoring because of their selectivity and stability as compared to EC detectors.

Several investigators have published GLC procedures using EC detectors for clonazepam plasma level determinations [5—10]. These methods can be separated into two groups: (a) those that measure the unchanged drugs [7, 10, 11—13] and (b) those in which clonazepam is modified either by derivatization, e.g., methylation [6, 9, 14] and trimethylsilylation [15] or by degradation by acid hydrolysis [5, 6, 16]. Derivatization of clonazepam by methylation or by trimethylsilylation etc., is often difficult to reproduce and often yields varying products and sometimes decreased product yields. Conversion of benzodiazepinones to their respective benzophenones can be carried out with good yield and therefore is still the method of choice for the benzodiazepinone assay [17].

In our present procedure, we have modified the acid hydrolysis method of De Silva et al. [5] and have used a nitrogen-sensitive detector for routine GLC determination of clonazepam levels in plasma with acceptable accuracy and reproducibility.

EXPERIMENTAL

Reagent

All reagents were of highest grade purity available. Diethyl ether (Fisher Scientific, Pitsburgh, PA, U.S.A.), Spectranalysed was made peroxide free by passing through a column of basic alumina (Alumina Basic, Brockman Activity 1, Fisher Scientific). Ethyl acetate used was glass distilled grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). Hydrochloric and sulfuric acid were of "Ultrex" grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). Borate—KCl—Na_2CO_3 buffer (1 *M*) pH 9.0 was prepared from analytical grade reagents [17]. Bromothymol blue reagent was a 0.1% solution in 50% ethanol. Sodium hydroxide (6 *M*) was prepared from analytical grade pellets (J.T. Baker). Clonazepam [7-nitro-5-(2-chloro-phenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one], flurazepam [7-chloro-1-(2-diethylaminoethyl)-5-(2′-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one] and other reference benzodiazepines were obtained as generous gifts from Hoffman-La Roche (Nutley, NJ, U.S.A.). Clonazepam (10 mg) was dissolved in 100 ml of methanol. One ml of this stock solution was diluted in deionized water to give a solution of 500 ng/ml of working solution. The aqueous solution was placed in an ultrasonic bath to ensure complete dissolution of clonazepam. The working solution was further

diluted in drug-free pooled plasma to concentrations of 10, 25, 50 and 100 ng/ml. Aliquots of prepared plasma standards and patient plasma samples containing various concentrations of clonazepam were kept frozen at −20°C and were used for day-to-day analysis over a period of six months. Freshly prepared spiked plasma standard solutions were used to compare the stability of clonazepam in storage. This comparison served as the basis for day-to-day quality control and for within-run and between-run accuracy determinations of the procedure.

All glassware was scrupulously cleaned with detergent in an ultrasonic bath, rinsed with regular glass-distilled water and finally with distilled deionized water and dried in the oven. The water used was distilled in glass, deionized and pretested for any spurious peaks with the nitrogen-sensitive detector.

Preparation of internal standard from flurazepam

The benzophenone of flurazepam (Dalmane®, Roche): 2-amino(diethylaminoethyl)-5-chloro-2′-fluorobenzophenone (ADACB) is prepared by hydrolysing 25 mg of flurazepam in 50 ml of 6 *N* hydrochloric acid—6 *N* sulfuric acid (95:5) in a boiling water bath for 60 min in a stoppered glass tube. The hydrolyzed solution is then cooled on ice, a few drops of bromothymol blue indicator added and neutralized with 6 *N* sodium hydroxide solution until just blue. The neutralized solution is extracted with cold peroxide-free diethyl ether (25 ml) three times. The ether extract is dried over anhydrous sodium sulfate and finally evaporated to dryness under a slow stream of nitrogen. The yellow hydrolysate is a mixture of the benzophenone of flurazepam and unhydrolyzed flurazepam. The material is then passed through a column of alumina (8 cm × 1.5 cm) and eluted with 10% ethyl acetate in benzene. Eight fractions (20 ml each) are collected. The benzophenone of flurazepam is eluted in the middle fractions (Nos. 3, 4 and 5) as indicated by its bright yellow color. The solvent is removed in a rotary evaporator and the residue weighed. The benzophenone of flurazepam (10 mg approx.) is then dissolved in 100 ml of methanol and dilutions are made in distilled deionized water to give a working solution for the internal standard of 50 ng/ml. One ml of this solution is added to the plasma samples as an internal reference standard. The purity of the benzophenone is established by GLC on different columns (3% SE-30 and 3% OV-17) and also by mass spectrometry. Mass spectrometry (electron impact technique) yielded an M^+ peak with an *m/e* value of 348.

Apparatus and GLC conditions

Analyses were performed using a Hewlett-Packard gas chromatograph, Model 5830A, modified and equipped with interchangeable flame ionization and nitrogen—phosphorus-sensitive detectors. Coiled glass columns (Supelco, Bellefonte, PA, U.S.A.), 1.8 m × 2 mm I.D., were packed with 3% SE-30 on 80—100 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) and baked overnight at 260°C, with a helium carrier gas flow-rate of 30 ml/min. The chromatographic conditions for separation of the benzophenones of clonazepam and flurazepam are as follows: carrier gas helium with a flow-rate of 30 ml/min; column oven temperature, 215°C; injection port temperature (on column) 270°C; air flow-rate to collector, 50 ml/min;

hydrogen flow-rate to collector, 3 ml/min; and the collector voltage varied from 12 to 16 V.

General extraction procedure

In a 15-ml PTFE-lined screw-capped centrifuge tube are placed 50 ng of the internal standard, 1.0 ml of plasma (clonazepam standard solutions or patient plasma) and 2 ml of borate buffer. Diethyl ether (10 ml) is added and the tubes are shaken in a reciprocating shaker for 10 min. The tubes are then cooled on ice, and centrifuged for 7 min. The ether phase is then carefully transferred into another tube containing 2 ml of 6 *N* hydrochloric acid—6 *N* sulfuric acid (95:5) and the tubes are shaken for 10 min. After cooling on ice the tubes are centrifuged and the ether layer is siphoned off. The acid layer is washed with 5 ml of diethyl ether which is aspirated and discarded and the tubes are placed in a boiling water bath loosely capped to allow the dissolved ether to escape. After 5 min the caps are tightly closed and the tubes are left in the bath for another 45 min. After hydrolysis the tubes are cooled on ice, a drop of the indicator added and the contents neutralized with 6 *N* sodium hydroxide until just blue. Diethyl ether (4 ml) is then added to the tubes, the tubes are shaken for 10 min, cooled on ice and centrifuged for 10 min. The ether phase is carefully transferred into small (3-ml) conical glass-stoppered tubes. The ether is evaporated under a slow stream of nitrogen at 50°C. The residue is then redissolved in 25 µl of ethyl acetate and 5—7 µl are injected into the gas chromatograph.

A standard curve is obtained by analyzing plasma standards of clonazepam in the concentration range of 10, 25, 50 and 100 ng/ml. Following chromatography, the peak height ratios and the integrator printouts for the area are compared in order to determine the accuracy of the procedure. Peak height ratios are used to calculate the concentrations of unknowns and the relative standard deviations of calibrators for within-run and day-to-day run accuracy.

RESULTS AND DISCUSSION

Fig. 1 shows the benzophenone derivatives of the commonly used benzodiazepinones and medazepam (benzodiazepine); the latter is not converted to its benzophenone derivative on treatment with strong acid. The relative retention times (*RRT*) of the benzophenones with respect to medazepam on a 3% SE-30 column are also shown (medazepam retention time is approximately 4 min). Fig. 2 shows the gas—liquid chromatograms of drug-free plasma to which were added 50 ng of clonazepam and 50 ng of the internal standard (ADACB) and then carried through the extraction procedure, and of plasma samples from three patients who are on a combination of anticonvulsant drugs including clonazepam. The early peaks in the chromatograms are possibly due to the acid hydrolysis breakdown products of other drugs (anticonvulsants) and endogenous nitrogen-sensitive substances of the plasma. The findings exemplified in Figs. 1 and 2 and the data in Table I show that commonly prescribed anticonvulsant drugs do not produce any interfering peaks in the assay for clonazepam. Thus, we have used this procedure to analyze a large number of patient plasma samples for clonazepam and found no instance in which the clonazepam peak in

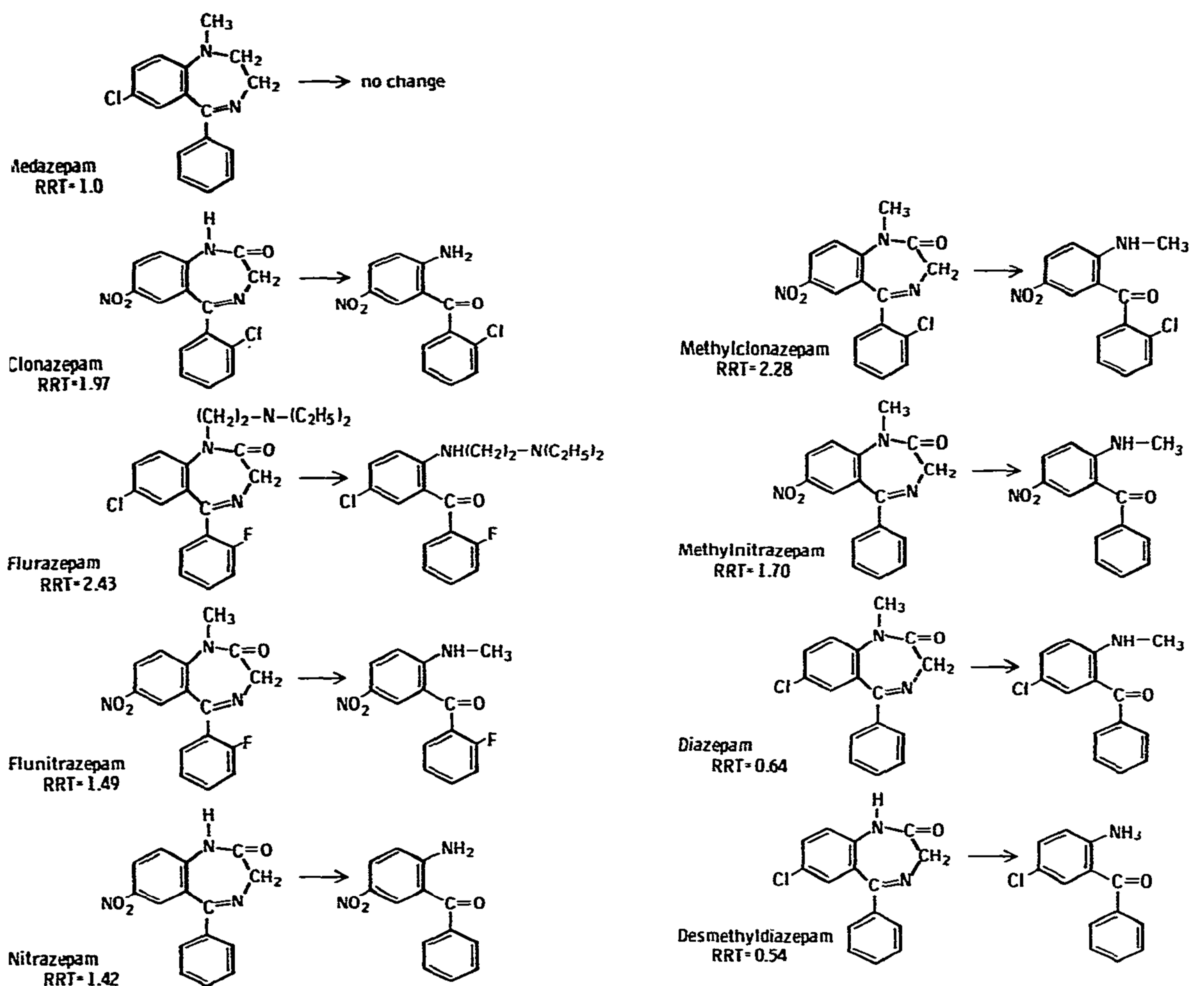

Fig. 1. Benzophenone derivatives of commonly used benzodiazepinone drugs and their *RRT* with respect to medazepam on a 3% SE-30 column.

the chromatogram is obscured by the presence of another drug or by any other endogenous nitrogen-containing compound.

It is evident from Figs. 1 and 2 and Table I that several benzodiazepinones other than clonazepam could easily be detected and estimated in plasma by the present procedure but this was not investigated further. The results of clonazepam analyses are shown in Table I which also includes the quantitative data of the three chromatograms shown in Fig. 2b, c and d. The recoveries of the benzophenone of clonazepam and the internal standard were satisfactory (86 ± 4%, n = 6 ± S.E.M.). The retention time for the clonazepam peak in the chromatogram is about 8 min and for the internal standard is about 10 min under standard assay conditions. Other benzodiazepinones (methylclonazepam) could also, in theory, be used as internal reference standards but we have found that the benzophenone of flurazepam consistently produced the most sym-

TABLE I

CLONAZEPAM CONCENTRATION IN PLASMA OF PATIENTS WHO ARE ON MULTIPLE ANTICONVULSANT DRUG THERAPY

Patient	Clonazepam* (ng/ml)	Phenobarbital (µg/ml)	Dilantin (µg/ml)	Tegretol (µg/ml)	Mysoline (µg/ml)	Valproic acid (µg/ml)	Tridione** (µg/ml)	Zarontin (µg/ml)
A	22.5	—	11.9	—	—	—	—	—
B	12.7	23.5	11.3	—	—	—	—	—
C	27.3	—	—	5.2	—	—	—	—
D	24.6	21.4	—	—	—	1.0	800.0	—
E	16.5	42.8	14.2	14.2	8.0	69.7	—	—
F	46.0	—	19.0	—	—	—	—	—
G	—	—	—	—	—	—	—	—
H	2.0	—	—	—	—	79.7	—	65.7
I	54.0	19.2	—	—	—	—	980.0	—
J	32.8	41.2	—	—	—	—	46.8	—
K	14.0	15.8	5.6	—	—	—	—	22.9
L	26.0	20.2	7.8	—	8.1	113.6	—	—
M	15.5	16.3	9.3	—	8.2	—	—	—
N	20.1	31.0	30.3	4.0	12.0	—	—	—
O	12.1	30.3	12.1	8.1	—	81.4	—	—
P	17.1	—	10.3	—	—	102.0	—	82.2
Q	80.0	—	—	—	—	—	—	—
b	17.3	—	10.1	—	—	94.0	—	94.0
c	11.2	18.3	13.8	—	4.1	99.3	—	—
d	2.05	—	3.7	—	—	4.2	—	—

* All drugs except clonazepam were assayed by GLC with FID using a modified Kupferberg procedure [18].
** Values for dimethadione.

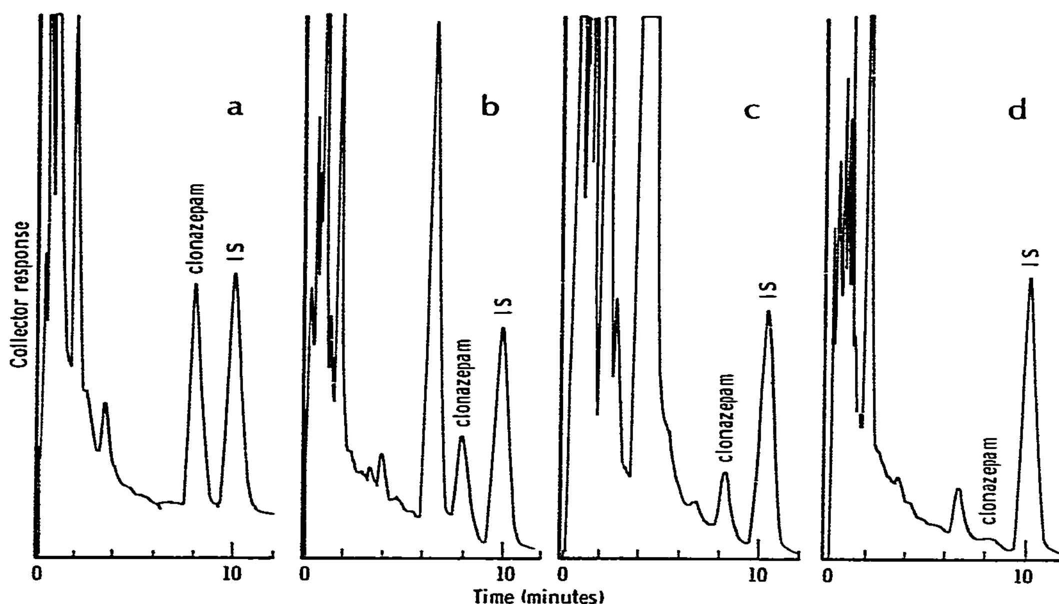

Fig. 2. Chromatograms of (a) blank plasma with added clonazepam (50 ng) and (b, c, d) three patient plasmas all assayed in the same manner after adding 50 ng internal standard, IS (see Table I).

metrical peak. The linearity of the entire procedure was demonstrated by replicate analyses of the standard plasma solutions of clonazepam containing 10, 25, 50 and 100 ng/ml. The ratio of peak heights of clonazepam benzophenone (ANCB)/internal standard was plotted against concentrations. The range of linearity was excellent from 10 to 100 ng/ml. The precision data for the assay were obtained using spiked plasma samples as well as patient plasma samples containing clonazepam. The within-day reproducibility of the method was obtained with plasma standards containing 25, 50, and 100 ng/ml of clonazepam. The day-to-day reproducibility for the procedure was obtained using similar concentrations in control plasma as well as in patient plasma samples. These control and patient plasma samples contained 16.5, 28.2 and 69.5 ng/ml of clonazepam. The results are summarized in Table II.

At concentrations less than 5 ng/ml the accuracy is poor and not clinically useful. Plasma samples containing concentrations over 100 ng/ml can be determined accurately by repeating the assay using 0.5 ml of the plasma or less. Although the relationship between plasma concentration of clonazepam and therapeutic response is not fully established, 20—70 ng/ml in plasma is usually accepted as the optimal range for clinical effectiveness.

Benzodiazepinones are usually converted to their respective benzophenone derivatives upon mineral acid hydrolysis [5, 6]. We found that benzodiazepinones with a long side-chain attached to nitrogen I of the ring (as in the case of flurazepam) undergo only incomplete hydrolysis even under the most rigorous conditions. This finding has not been reported by previous investigators. Therefore, it is important to use the benzophenone of flurazepam as the internal standard instead of the drug itself.

TABLE II

PRECISION DATA FOR CLONAZEPAM ASSAY

Plasma standards are 25, 50 and 100 ng/ml; patient plasma values 16.5, 28.2 and 69.5 ng/ml.

	n	Level 1			Level 2			Level 3		
		Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)	Mean (ng/ml)	S.D.	CV (%)
Within-day										
Fresh plasma	20	24.6	0.14	0.56	49.5	0.19	0.38	97.8	0.83	0.85
Frozen plasma	10	24.3	0.16	0.65	49.2	0.23	0.46	98.0	0.80	0.81
Between-day										
Fresh plasma	12	24.7	0.13	0.52	49.0	0.26	0.53	98.1	0.80	0.81
Frozen plasma	6	24.1	0.18	0.74	48.8	0.31	0.63	97.5	0.93	0.95
Patient plasma frozen	6	15.8	0.05	0.36	27.6	0.10	0.36	68.5	0.13	0.20

The specificity of the present procedure has been well tested in the presence of other anticonvulsant drugs, and virtually no interference has been found. Clonazepam levels found in patient plasma varied from several ng/ml to 80 ng/ml; the latter value was from a patient (child) who had been prescribed clonazepam for two years without any other co-medication and whose seizures are under complete control.

The benzophenone of flurazepam, prepared for use as internal reference standard, was found to be pure and devoid of any contamination from the parent drug. Once prepared (about 10 mg), the internal standard is sufficient for analyzing 2000 samples.

Flurazepam, if co-administered with clonazepam will interfere with the present assay procedure for clonazepam. However, it is very unlikely that the two drugs will be prescribed for concurrent use. Of the 500 or more patient plasma samples analyzed so far in our laboratory none was found to contain flurazepam as a co-medication.

In a separate series of experiments the benzophenone of clonazepam was prepared; the response of the nitrogen-sensitive detector was found to be linear with increasing concentrations.

The procedure is specific for clonazepam and no other commonly prescribed benzodiazepinones will interfere. The metabolites of clonazepam (Fig. 3), i.e., the 7-amino derivative and 3-hydroxyclonazepam will produce the same benzophenone derivative as that produced by clonazepam, upon hydrolysis. However, under the conditions of the assay these metabolites are not extracted; thus after addition of 150 ng/ml of 7-amino clonazepam and 7-acetamidoclonazepam to drug-free plasma no peak corresponding to the benzophenone breakdown product of clonazepam was noted.

Our findings substantiate the observations of previous authors [5, 6]. The 3-hydroxy metabolite of clonazepam is not found in significant amounts in plasma [6, 19] and washing of the acid phase by diethyl ether eliminates most of these metabolites [5] and reduces the changes of substantial interference.

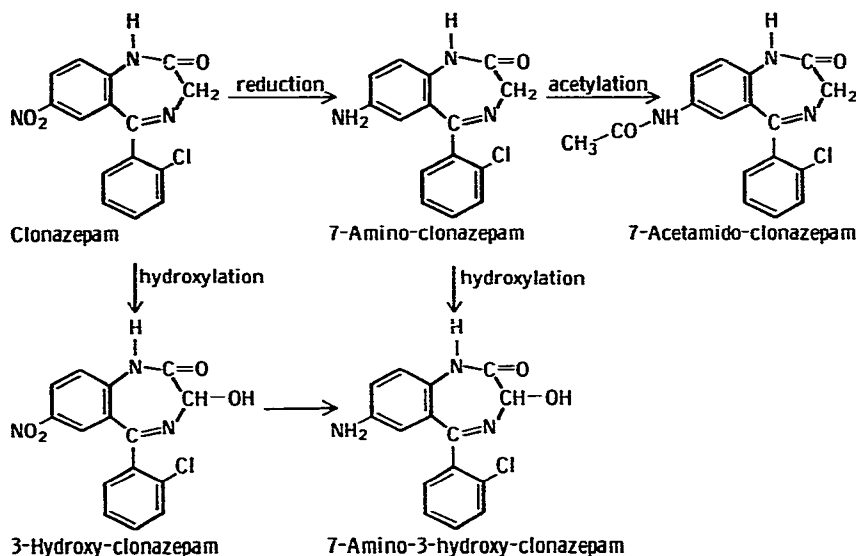

Fig. 3. Possible metabolic pathway of clonazepam in man (Eschenhof [19]).

Our present method based on the technique of mineral acid hydrolysis developed by De Silva et al. [5], is relatively simple and uses the nitrogen-sensitive detector for GLC analysis. The method may be used for routine analysis since it eliminates the problems associated with the more commonly employed EC technique.

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