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RAPID DETERMINATION OF DIAZEPAM AND NORDIAZEPAM IN PLASMA BY ELECTRON CAPTURE GAS—LIQUID CHROMATOGRAPHY

APPLICATION IN CLINICAL PHARMACOKINETIC STUDIES

ROBERT E. WEINFELD, HOWARD N. POSMANTER, KO-CHIN KHOO and
CARL V. PUGLISI

*Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, N.J.
07110 (U.S.A.)*

SUMMARY

A rapid method was developed for the determination of diazepam and nordiazepam
(N-desmethyldiazepam) in human plasma using electron capture gas—liquid chromatography
(GLC—ECD). The concentration of diazepam and nordiazepam is determined using 0.5 ml of
plasma extracted with 1.0 ml of benzene containing 25 ng/ml of methylnitrazepam as the
internal standard. The benzene extract is removed and an aliquot is subjected to automated
GLC—ECD analysis. The method has a sensitivity limit of 5 ng diazepam and 10 ng nordi-
azepam per milliliter of plasma. The method was used to determine the plasma levels in man
following the first 5-mg diazepam dose, as well as during chronic oral administration of 5 mg
diazepam three times daily and 15 mg diazepam once a day.

INTRODUCTION

The determination of drug concentration in the numerous plasma samples
generated in bioequivalency and clinical pharmacokinetic studies in man, has
created a need for simple, rapid and sensitive assays. Diazepam (the active drug
substance in Valium, marketed by Hoffmann-La Roche, Nutley, N.J., U.S.A.),
a member of the 1,4-benzodiazepine class of compounds (Fig. 1), is used in the
relief of tension, anxiety and skeletal muscle spasms [1—8]. In clinical practice,
single oral doses of diazepam range typically from 2 to 10 mg and are adminis-
tered 2—4 times daily. In previous studies in man, peak concentrations of
221—400 ng/ml of plasma [9] and 137—189 ng/ml of whole blood [10] were

DIAZEPAM

DESMETHYLDIAZEPAM
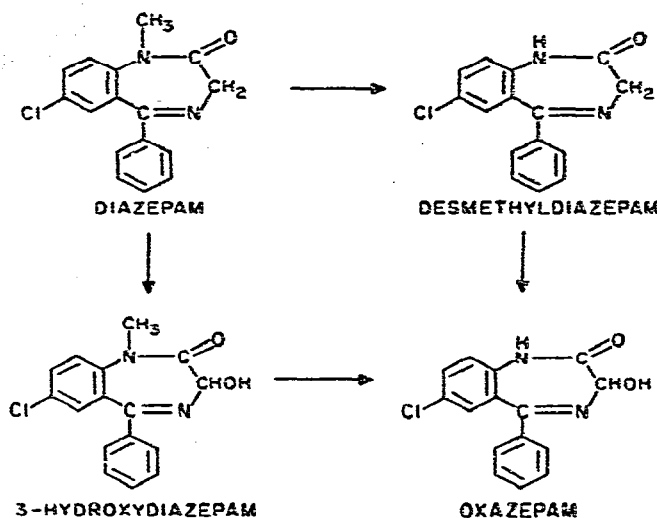
3-HYDROXYDIAZEPAM

OXAZEPAM

Fig. 1. Major pathways of diazepam metabolism in humans.

measured after single oral administration of 10 mg of diazepam. Chronic oral administration of 5 mg of diazepam three times daily resulted in steady state plasma levels of 230—440 ng/ml [11], while chronic oral administration of 10 mg of diazepam once daily resulted in blood levels of about 200 ng/ml [10]. Concentrations of the major metabolite of diazepam, nordiazepam (N-desmethyldiazepam) (Fig. 1), can range from 10 to 20 ng/ml of blood at the time of peak diazepam levels, following single oral administration of 10 mg of diazepam and reach steady-state concentrations of 100—150 ng/ml of blood during chronic administration of 10 mg of diazepam daily [10].

Assays for the determination of diazepam and nordiazepam in blood and plasma following therapeutic doses of the drug have required the sensitivity and specificity of electron capture gas—liquid chromatography (GLC—ECD).

Numerous GLC—ECD procedures have been developed to measure diazepam and nordiazepam [11—18]. The earlier assays required the conversion of diazepam and nordiazepam to their corresponding o-aminobenzophenone derivatives by acid hydrolysis prior to GLC—ECD analysis [12]. The more recent procedures were able to quantitate diazepam and its metabolites by GLC—ECD without derivatization employing the liquid phase OV-17 and a ^{63}Ni electron capture detector (ECD) [11, 13—18].

The previously published procedures are useful in the measurement of diazepam and nordiazepam in studies involving a limited number of specimens for analysis. They are, however, time consuming and impractical when large scale human studies are required which involve the analysis of hundreds of specimens on a routine basis in order to demonstrate bioequivalency of formulations or in clinical pharmacokinetic studies. Moreover, most of the previously reported assays involve many tedious steps including multiple extractions and transfers, clean-up steps, solvent evaporation, and manual injection into the gas chromatograph.

The procedure described herein was developed in response to the need for a rapid, sensitive, specific and reproducible assay for diazepam and nordiazepam in plasma, permitting the preparation of large numbers of extracts in a single day for GLC analysis, and the subsequent automation of sample injection, peak area integration and computation of results.

The concentration of diazepam and nordiazepam in plasma following single and during chronic oral administration of diazepam have been determined successfully by this rapid assay.

EXPERIMENTAL

Parameters for GLC analysis

Column. A U-shaped borosilicate glass column, 1.2 m × 4 mm. I.D., containing a pretested preparation of 3% OV-17 on 60—80 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.) was used. The column was conditioned as previously described[13].

Instrumental parameters. A Tracor Model 222 gas chromatograph (Tracor, Austin, Texas, U.S.A.) equipped with a 15-mCi ^{63}Ni ECD, and an automatic sampler (Model 7671A, Hewlett-Packard, Avondale, Pa., U.S.A.) was used. Argon—methane (90:10) gas mixture (Matheson Gas Products, East Rutherford, N.J., U.S.A.), oil-pumped and dry, at 40 p.s.i.g. and a flow-rate of 70 ml/min through the column, was used as carrier gas. The purge gas flow-rate was 20 ml/min through the detector. The temperature settings were: column, 235°; injection port, 275°, detector, 350°. The flow-rate and oven temperature were adjusted so as to obtain retention times (t_R values) of approximately 3.9, 5.5, 8.6 and 13.0 min for diazepam, nordiazepam and the internal standards, methylnitrazepam and griseofulvin, respectively. An electron capture linearizer (Tracor, Model No. 114460-001) operated the detector in the "constant current pulsed mode". The linearizer parameters were set as follows: standing current, 0.3 × 10⁻⁹ A; relative pulse width, 0.18 (0.75 μ sec actual pulse width); attenuator, 8. A 1.0-mV recorder (Model 7127A, Hewlett-Packard) was operated at a chart speed of 0.25 in/min. Under these conditions 1.0 ng of diazepam and 2.0 ng of nordiazepam injected gave nearly full-scale responses, and 0.25 ng methylnitrazepam and 1.0 ng griseofulvin injected gave about 50% f.s.d. The minimum detectable amounts of diazepam and nordiazepam were 5 ng and 10 ng/ml of plasma, respectively.

A mini-computer based data system (Hewlett-Packard, 3352B Laboratory Data System) was interfaced with the electron capture linearizer and automatic sampler.

Reagents

The following reagents were used: Saturated potassium chloride (analytical-reagent grade) (approx. 4.8 M), prepared in distilled, deionized water; benzene (Nanograde, Mallinckrodt, St. Louis, Mo., U.S.A.); and absolute ethanol (Publicker, Linfield, Pa., U.S.A.)

Glassware

Special extraction tubes, 125 mm × 13 mm O.D., round-bottom, 9-ml capacity with a 10/18 ⹋ joint, were constructed (Fig. 2)*. Tubes and stoppers were washed with a non-corrosive surfactant cleaning solution (Micro, International Products Corp., Trenton, N.J., U.S.A.) in a 50° ultrasonic bath, subsequently rinsed with distilled, deionized water and dried in an oven. Sample vials (Hewlett-Packard) were rinsed with distilled, deionized water and dried in an oven prior to use.
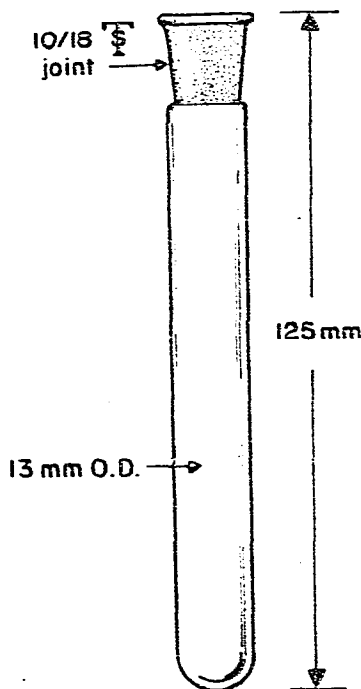

Fig. 2. Special extraction tube.

Standard solutions

Stock solutions of diazepam and nordiazepam were prepared as follows: 10.00 mg of each compound was weighed out in separate 10-ml volumetric flasks, dissolved in 1.0 ml of absolute ethanol and made up to volume with benzene, to yield 1 mg/ml solutions. These stock solutions were diluted 1:100 with benzene to yield 10 μg/ml solutions of diazepam and nordiazepam. A series of mixtures of diazepam and nordiazepam containing 0.05, 0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, 4.5 and 5.0 μg/ml benzene of each compound was prepared by suitable dilutions of the 10 μg/ml standard solutions for use as calibration standards by addition to plasma.

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* A similar tube, Corning No. 9810, is commercially available, and would be a suitable substitute.

Methylnitrazepam, 7-nitro-5-phenyl-1,3-dihydro-1-methyl-2H-1,4-benzodi-azepin-2-one, was used as the internal standard. Methylnitrazepam (10 mg) was weighed out and placed in a 100-ml volumetric flask, dissolved in 10 ml of absolute ethanol and made up to volume with benzene to yield a 100 μg/ml stock solution. A 10-μg/ml solution was prepared by diluting a 1.0-ml aliquot of the stock solution to 10 ml in a volumetric flask. This solution was used to prepare a 25-ng/ml methylnitrazepam solution in benzene used for sample extraction, by diluting a 250-μl aliquot to 100 ml in a volumetric flask. The diluted solution was stored in an amber 100-ml conical vessel with a 24/40 $\mathfrak{S}$ ground glass joint. A 1.0-ml dispenser device with a glass joint (Cat. No. 3001-G, Repipet, Lab Industries, Berkeley, Calif., U.S.A.) was used to dispense the extraction solvent.

Griseofulvin (Calbiochem, Los Angeles, Calif., U.S.A.) was used as alternative internal standard. A stock solution was prepared as for methylnitrazepam. The working dilution was prepared by transferring a 200-μl aliquot of the stock solution to a 100-ml volumetric flask and making up to volume with benzene to yield a 200-ng/ml solution for extraction.

Procedure

Preparation of samples and calibration standards. Into the special extraction tube (Fig. 2), 0.5 ml of plasma and 0.5 ml of saturated potassium chloride solution were added and mixed, followed by the addition of 1.0 ml of benzene containing 25 ng of methylnitrazepam/ml. The tube was sealed tightly with a PTFE stopper (Chemplast, Wayne, N.J., U.S.A.) by twisting into the glass joint of the tube. The samples were placed in a suitably sized test tube support and clamped in a mechanical rotator (Model RD-250, Kraft Apparatus, Mineola, N.Y., U.S.A.), and rotated for 10 min at 30 rpm. The samples were centrifuged at 10° for 15 min at 1000 g in a refrigerated centrifuge (Model PR-J with a No. 253 rotor and No. 381 sample cups; Damon/IEC Division, Needham, Mass., U.S.A.) and approximately 0.6—0.75 ml of the organic phase was transferred into a 2-ml glass sample vial using a disposable 9-in. Pasteur capillary pipet. The vial was capped with a Teflon-faced rubber septum aluminum seal (Hewlett-Packard) using a hand-operated crimper (Wheaton Scientific, Millville, N.J., U.S.A.). The prepared samples were then ready for automatic injection into the gas chromatograph. Extracts should be chromatographed the same day they were prepared to avoid the possibility of sample degradation.

Along with unknown samples, five plasma calibration standards containing 50 ng of diazepam and 50 ng of nordiazepam were prepared by evaporating 100 μl of the 0.5-μg/ml standard mixture in benzene to dryness under a nitrogen stream (in a special extraction tube) and adding 0.5 ml of control human plasma. These calibration standards were then processed along with the unknowns.

Calibration of the data system. Using the GLC conditions initially set at the parameters outlined (see *Parameters for GLC analysis*), 10 μl of one of the above prepared calibration standard extracts containing 50 ng of diazepam and 50 ng of nordiazepam added to 0.5 ml control human plasma was injected. A typical chromatogram is shown in Fig. 3. This sample served to establish the re-

tention times of the 3 known peaks (diazepam, nordiazepam and methylnitrazepam) and identify any chromatographic problems (e.g. poor peak shape, poor response, abnormal sample impurities, interfering peaks or other disturbances) that could invalidate the results.

The software of the computer-based data system was used to prepare a method defining the parameters illustrated in Fig. 4.

A second extraced plasma calibration standard was chromatographed, and the peak area data were used to establish the response factors for diazepam and nordiazepam required for the internal standard method of calculation of the unknowns, with the results reported in ng/ml of plasma (shown in Fig. 4). The report format for such a calibration standard is shown in Fig. 5.
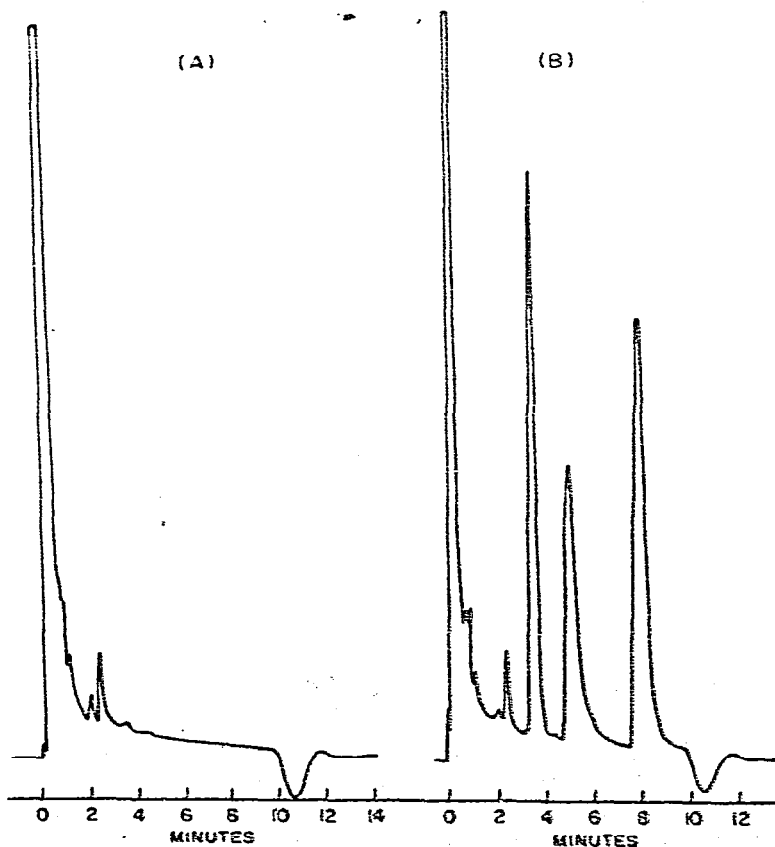

Fig. 3. Chromatograms of the GLC–ECD analysis of plasma benzene extracts. (A) Human control plasma; (B) human control plasma containing 50 ng diazepam (t_R = 3.9 min), 50 ng nordiazepam (t_R = 5.5 min) and 25 ng methylnitrazepam (internal standard, t_R = 8.6 min) per 0.5 ml of plasma. Injection volume: 10 μl of benzene from each extract. Attenuation: × 8.

```
1. CHAN,PROC,RPRT,RDVC
   18, ISTD, ME, T3

2. SAMP,UNTS,TITLE
PLASMA   , NG/ML , PLASMA DIAZEP-NORDIAZEP

3. #PKS,RTM,PRG
   15, 16.00, NO,

4. MIN AR,MV/M,DLY,DVT,DIL-FTRI
   100,   .100, 3.00, 0.00, 100.00

5. REF-RTW,IRTW,ID-LVL,RF-UNK
   .50, 10,      100, 1.000

6.# KWN PKS
   3

#    TIME  AMT          FACTOR        NAME
1    3.90  5.0000E  1  =3.1617E  0  DIAZEPAM
2    5.49  5.0000E  1  =3.1232E  0  NORDIAZEP
3    8.64  2.5000E  1  =1.0000E  0  #METHYLNIT

7. # EVENTS
   0
```

Fig. 4. The format of a typical computer-based data system method showing the parameters used in the analysis of diazepam and nordiazepam in plasma. The parameters defined and illustrated above included: method of calculation (PROC), format of report (RPRT), reporting device (RDVC), type of sample (SAMP), concentration units to be used for reporting results (UNTS), length of chromatographic run time (minutes) prior to reporting of data (RTM), minimum area (μV-sec) which must be exceeded before a peak will be reported (MIN AR), slope sensitivity (mV/min) threshold for the detection of peaks (MV/M), integration delay (minutes) which should expire after sample injection before integration should begin (DLY), parameters relating to identification of peaks by comparison with retention times of known peaks (REF-RTW), identification level for peaks having an area greater than this level to be considered for identification as a known peak (ID-LVL), retention times, amounts, response factors and names of known peaks (#KWN PKS).

The remaining plasma calibration standards were interspersed among the unknown samples in the automatic sampler tray and served to monitor the GLC—ECD system for accuracy, precision and changes in response during the unattended run.

Procedure for processing samples using the automatic liquid sampler (ALS). The ALS sequence was prepared using the computer-based software of the data system. The sequence contained the information defining instructions for processing the sample vials, and included the parameters illustrated in Fig. 6. In this manner, up to 36 sample vials could be processed unattended per run and the results were reported via teleprinter (Hewlett-Packard Model HP 2752A) for each vial processed. The series of sample vials prepared were then placed in the sampler tray in the assigned position defined in the ALS sequence, including three benzene wash vials and the system was then placed in the automated mode by user command. The system continued to operate unattended by automatic sample injection in the sequence specified and reported the results in ng of diazepam and nordiazepam per ml of plasma in each vial. The teletype report format for an unknown sample is illustrated in Fig. 5.

```
REPORT NO.    606                              PLASMA DIAZEP-NORDIAZEP

CHAN# 10        METHOD: DIAZEP        SAMPLE: RC100        BOTTLE  1

ISTD =  25.000    NG/ML

   RT          AREA       NG/ML       NAME

   3.96       11329 BB    99.779     DIAZEPAM
   5.49       12052 BB   100.491     NORDIAZEP
   8.64       16411 BB               &METHYLNIT

TOTAL AREA =           39792
```

```
REPORT NO.    622                              PLASMA DIAZEP-NORDIAZEP

CHAN# 10        METHOD: DIAZEP        SAMPLE: A3242        BOTTLE  17

ISTD =  25.000    NG/ML

   RT          AREA       NG/ML       NAME

   3.88       17269 BB   155.101     DIAZEPAM
   5.63       14469 BB   123.032     NORDIAZEP
   8.58       16092 BB               &METHYLNIT

TOTAL AREA =           47830
```

Fig. 5. The typical format of the data system report is illustrated for a diazepam and nordia-zepam calibration standard (100 ng/ml of plasma) prepared as described in the text and injected by the automatic sampler (Report No. 606, upper) and a typical report for an unknown plasma extract is shown in Report No. 622 (lower).

RESULTS AND DISCUSSION

Numerous sensitive and specific GLC—ECD assays have been developed for the determination of diazepam and its metabolites in human plasma and other biological fluids [11, 13—18]. These assays are based on the intrinsic electron-capturing properties of diazepam and its metabolites, which permit detection of picogram quantities under the proper gas chromatographic (GC) conditions. The assay described herein also uses GLC—ECD and has a sensitivity limit comparable to that of previously reported procedures, but has significantly reduced the biological sample preparation time by modification of the extraction step and elimination of solvent evaporation steps. The procedure is essentially a one-tube, one-step extraction, requiring only one transfer into a sample vial for injection into the gas chromatograph. As an illustration of the ease of performing this assay, one person can prepare 36 samples for chromatography in 4 h. The only limitation on the number of samples prepared for GLC is the automatic sampler capacity (i.e., 36 vials). Following sample preparation the remaining analytical procedures, including sample injection, integration of peak areas, and data reduction reporting nanograms diazepam and nordiazepam per milliliter of plasma have been automated.

Several significant instrumental and electronic developments have recently become available that made this method practicable. ALS devices for GC have

```
*LI,ALS,9

1. 1ST BTL,#BTLS,RCAL, INJ/RC
       1,  33,   1,  3T

2. WSHS,PMPS,STOP,#INJ
       1,  6,   3,   1

3. CTM,WBTL,ISO
     18.8, YES, YES

4. METHD
   DIAZEP

5. NAMES,WGHTS
BTL  1: RC100      ,  25.800
BTL  2: CONT PRE,    25.800
BTL  3: A3158     ,  25.800
BTL  4: A3165     ,  25.800
BTL  5: A3172     ,  25.800
BTL  6: A3179     ,  25.800
BTL  7: A3186     ,  25.800
BTL  8: A3193     ,  25.800
BTL  9: A3200     ,  25.800
BTL 10: A3207     ,  25.800
BTL 11: A3214     ,  25.800
BTL 12: A3221     ,  25.800
BTL 13: IS100     ,  25.800
BTL 14: CONT PL   ,  25.800
BTL 15: A3228     ,  25.800
BTL 16: A3235     ,  25.800
BTL 17: A3242     ,  25.800
BTL 18: A3256     ,  25.800
BTL 19: A3263     ,  25.800
BTL 20: A3270     ,  25.800
BTL 21: A3277     ,  25.800
BTL 22: A3284     ,  25.800
BTL 23: A3291     ,  25.800
BTL 24: A3298     ,  25.800
BTL 25: A3305     ,  25.800
BTL 26: IS100     ,  25.800
BTL 27: A3312     ,  25.800
BTL 28: A3319     ,  25.800
BTL 29: A3326     ,  25.800
BTL 30: A3253     ,  25.800
BTL 31: A3260     ,  25.800
BTL 32: IS100     ,  25.800
BTL 33: CONT PL   ,  25.800
```

Fig. 6. The format of a typical computer-based data system automatic liquid sampler sequence is illustrated listing the parameters used in the analysis of diazepam and nordiazepam. The parameters defined are: first vial in the sequence (1 ST BTL), total number of vials in the sequence (#BTLS), location of the calibration standard vial used for automatic recalibration of response factors (RCAL), number of wash cycles before each sample injection (WSHS), number of pump cycles before each sample injection (PMPS), injection volume (STOP), number of injections to be performed on each vial (#INJ), total cycle time (minutes) between sample injections.

been available for a number of years [19] and are generally reliable, reproducible and capable of sampling from microliter capacity vials. However, without development of a reliable electron capture linearizer [20, 21] automated GLC—ECD would be limited to accurate quantitation of only a narrow range of concentrations of injected samples due to the non-linear response characteristics of ECDs at high concentrations injected [22]. The electron capture linearizer has extended reliable quantitation of electron capturing compounds, including diazepam and nordiazepam, over a range of at least 10^4 in sample concentration injected and eliminates the need for sample dilution and re-injection, which would otherwise defeat the purpose of automatic injection systems. By interfacing the ^{63}Ni ECD, electron capture linearizer and ALS with a mini-computer based automatic data acquisition and analysis system, the means of fully automating the chromatographic and computation processes were accomplished. The method described was developed for the analysis of diazepam and nordiazepam levels following single or during chronic oral administration of therapeutic doses of diazepam for the purpose of demonstrating bioequivalency of formulations and defining pharmacokinetic profiles. The method was designed to specifically quantitate diazepam and nordiazepam in plasma. However, 3-hydroxydiazepam and oxazepam (Fig. 1), if present, are chromatographically resolved from diazepam and nordiazepam, having t_R values of 2.8 and 8.7 min, respectively, and can also be quantitated by this method.

In the development of a rapid assay procedure that required minimal sample handling prior to GLC analysis using an automatic liquid sampler, it was necessary to establish certain guidelines at the outset. A great deal of work has been established by other workers regarding simplified extraction techniques [11, 16, 18, 20, 23, 24], thus, one objective of this work was to further simplify the assay by extraction with a minimal volume of solvent and to eliminate the need to evaporate the solvent prior to GC analysis. Benzene was ideally suited as the extraction solvent since pesticide-grade solvent gave an extremely low ECD response when injected on to the OV-17 phase and more importantly extracted very few impurities and no interfering substances from plasma as shown in Fig. 3. Although a benzene extract of plasma had a yellowish coloration, a 10-μl aliquot injected into the gas chromatograph gave rise to very little ECD responsive material (Fig. 3). Equally important was the fact that diazepam and nordiazepam, although they are relatively polar basic compounds, partition into benzene from plasma to an extent exceeding 90% at physiological pH [11, 16—18, 25]. This property of diazepam and nordiazepam was used to great advantage in the development of a rapid procedure for determining the compounds in plasma by GLC—ECD.

In order to process all samples using a fixed volume of solvent, it was necessary to establish the linearity and recovery of the extraction of diazepam and nordiazepam from plasma into 1.0 ml of benzene under the conditions described. The recoveries were determined from a comparison of the peak areas of ten concentrations (in duplicate) of the standards (5—500 ng) added to plasma prior to extraction with the areas of the same ten concentrations of standards added to control plasma extract residue after extraction. This technique was used to overcome the pronounced tailing and loss of response of in-

jected pure nordiazepam standards in benzene under the GC conditions described. The occurrence of such peak tailing with polar compounds is common in spite of the use of highly inert, silane-treated column supports to minimize such effects. By use of this procedure, "apparent" recoveries of over 100% of added compound from plasma extracts was eliminated. This phenomenon was presumably related to the deactivation of adsorption sites on the column support [26], similar to the phenomenon reported using lecithin, a synthetic phospholipid, as a priming agent in the GLC—ECD analysis of steroids [27]. The average recoveries for diazepam and nordiazepam are summarized in Table I and are shown to be 98.2 ± 3.3% and 94.8 ± 1.5%, respectively. The extraction was found to be linear over this concentration range as shown in Fig. 7. The precision of the extraction procedure was determined based on the analysis of six separate samples of control plasma containing 100 ng diazepam and 100 ng nordiazepam per milliliter and found to be 0.98 ng/ml for diazepam and 2.07 ng/ml for nordiazepam.

Concentrations exceeding 800 ng/ml plasma are very seldom encountered in controlled clinical studies using normal subjects, even during chronic oral administration of up to 15 mg diazepam daily [11]. The steady state plasma levels of patients during chronic oral administration of 30 mg of diazepam daily, were reported to be between 1.0 and 2.0 μg/ml [28]. In such cases, an aliquot of 100 μl (or less) of plasma can be taken for assay, or the volume of benzene used to extract the unknown sample can be increased to 2.0 ml or greater. Whole blood and serum were also tried by the method described and found to work equally well. Slight differences in the chromatograms of control blood compared with plasma were seen, but there were no interferences in the region of diazepam and nordiazepam. In addition, a volume of plasma or blood of up to 1.0 ml can be used in this procedure with no modification. However, plasma is the preferred biological specimen since plasma concentrations of diazepam are nearly twice the corresponding blood concentrations (the blood/plasma concentration ratio is reported to be 0.58). [9].

TABLE I

RECOVERY OF DIAZEPAM AND NORDIAZEPAM FROM PLASMA

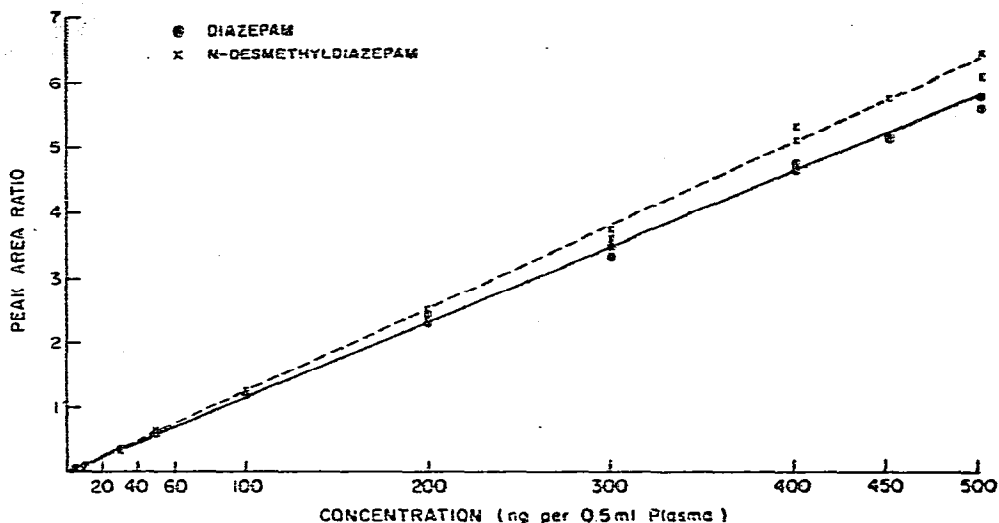| Amount of diazepam and nordiazepam added (ng/0.5 ml plasma)	Recovery (%)	
	Diazepam	Nordiazepam
5.0	92.6	N.D.*
10.0	92.7	97.8
30.0	97.5	94.3
50.0	99.5	96.0
100.0	101.2	94.8
200.0	100.6	93.8
300.0	99.9	93.8
400.0	101.6	96.1
450.0	96.6	92.9
500.0	99.6	93.9
Mean ± S.D.	98.2 ± 3.3	94.8 ± 1.5

*N.D. = Non-detectable.

Fig. 7. Calibration curves for the GLC—ECD analysis of diazepam and nordiazepam (N-des-methyldiazepam) added per 0.5 ml of plasma and analyzed by the rapid method described.

A radioimmunoassay (RIA) was reported for diazepam in blood and plasma [29]. The advantages cited for the RIA technique was speed of analysis, ability to perform a large number of determinations in a single day by one person and sensitivity of the assay, requiring as little as 50 μl of plasma. The disadvantage of the RIA procedure was that it required two separate antibodies in order to determine both diazepam and nordiazepam in plasma. The GLC—ECD procedure described here has all of these advantages and measures both compounds simultaneously.

The extraction tubes used were designed (Fig. 2) to allow for efficient mixing of the plasma sample and organic solvent, and avoidance of emulsion formation. The tube is made so that 1.0 ml of benzene occupies approximately 1.5 cm in height, thus allowing for easy transfer of the benzene extract (following centrifugation) uncontaminated by interface material or aqueous phase.

During the development of this method, several problems became apparent. The glass vials used in the automatic injection system are capped with a Teflon-faced rubber septum aluminum seal, which is penetrated by the syringe needle for injection into the gas chromatograph. The vial can be used for only one injection. Repeated injections from the same vial showed a contaminant with a retention time almost identical to diazepam. The source of this contaminant was traced to the septa of the vials, which are susceptible to chemical attack by benzene once penetration of the Teflon liner has occurred. The identity of this interfering substance was not investigated, but several instances of similar interferences have been reported and traced to various plasticizers present in rubber products [30—32]. To avoid erroneous results due to an increased integrated area for diazepam caused by the contaminant, each sample vial is injected only once. It is extremely important to avoid contamination of the benzene solvent and extracts by contact with materials such as rubber and plastics. Only Teflon

and glass should come in contact with the benzene. Another septum material (black septum, Hewlett-Packard) was evaluated and found to be unsuitable due to interfering peaks in the area of 5.5—6.5 min.

Specificity of the assay

Diazepam is known to undergo the metabolic pathways shown in Fig. 1. The parent compound and nordiazepam are the major measurable plasma components following single and chronic oral administration of diazepam in man [10, 11, 14, 25, 33, 34]. However, the presence of the two hydroxylated metabolites (Fig. 1), 3-hydroxydiazepam and oxazepam (3-hydroxynordiazepam) in plasma after administration of diazepam has been reported [14, 34]. The reported plasma concentrations of 3-hydroxydiazepam and oxazepam were as high as 180 ng/ml and 290 ng/ml, respectively, during chronic administration of 20 mg diazepam three times daily for 10 weeks [14]. To determine if the presence of these hydroxylated metabolites caused any interference in the analysis of diazepam and nordiazepam, known amounts of authentic standards of these two compounds were added to control human plasma and assayed by the method described, with the exception of the internal standard. Both hydroxylated metabolites are chromatographically resolved from diazepam and nordiazepam, having t_R values of 2.8 min (oxazepam) and 8.7 min (3-hydroxydiazepam). The recovery of the two hydroxylated metabolites from plasma was determined to be approximately 100% (3-hydroxydiazepam) and 50% (oxazepam). The sensitivity limits would be only 50—100 ng of each compound per milliliter of plasma under conditions described.

The presence of 3-hydroxydiazepam (t_R 8.7 min) in an extract would interfere with the internal standard used in this method. However, in the routine analysis of two thousand plasma samples collected following single and during chronic oral administration of diazepam in normal, healthy male subjects, no evidence of either hydroxylated metabolite was detected. The use of griseofulvin as the internal standard (t_R 13.0 min), is suggested to avoid possible interference from 3-hydroxydiazepam.

Application of the method to biological specimens

The plasma levels of diazepam and nordiazepam were determined in six healthy male subjects in a study comparing the steady state pharmacokinetic profiles of diazepam and nordiazepam when the total daily dose of diazepam was divided into three oral administrations (5 mg three times daily at 7 a.m., 12 noon and 5 p.m.) for a period of 14 days followed by 15 mg single oral daily dosing for a period of 10 days [35]. During the three times daily dosing period, plasma levels were measured following all three doses on day 1 and following the first and third doses during the remainder of that dosing regimen. During the once a day dosing treatment, plasma levels were measured following each administration. The diazepam and nordiazepam plasma concentration-time curves for one subject are shown in Fig. 8.
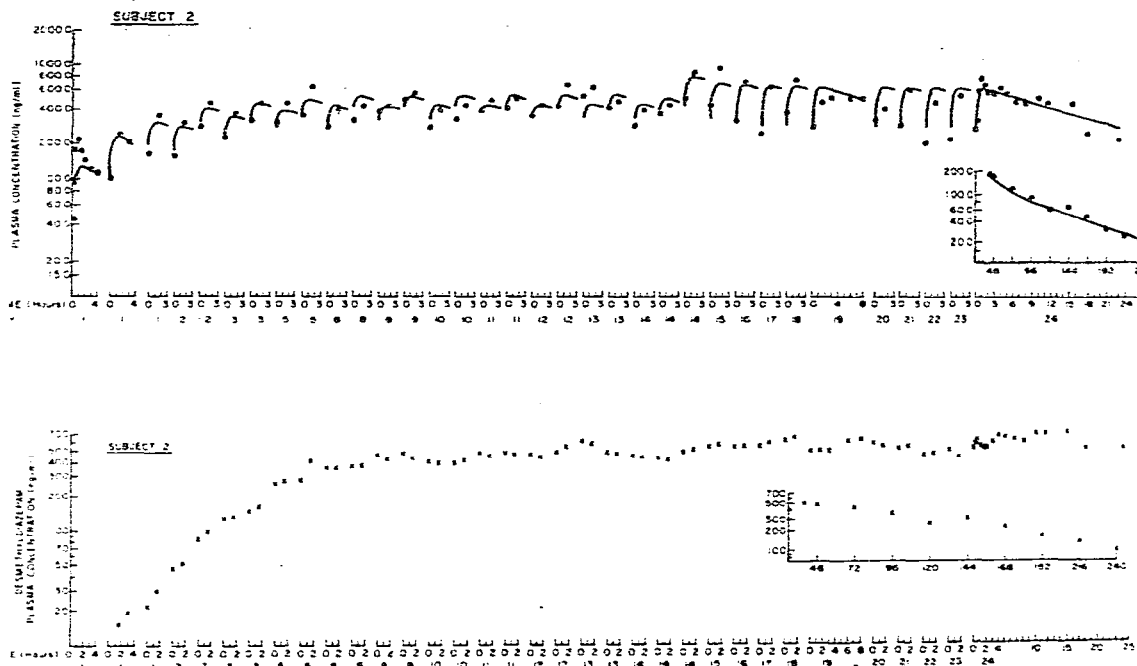
Fig. 8. Experimental plasma concentration—time profile curves of diazepam (upper) and nordiazepam (desmethyldiazepam, lower) following oral administration of Valium[TM] to one subject (No.2). On days 1 to 13, the dose was 5 mg at 0, 5, and 10 h (three times daily regimen). On day 14, at the time for the third dose, the regimen was crossed over to 15 mg every 24 h for an additional ten days. Abscissa represents time after each dose. Insert at bottom right portion of each curve illustrates the fall-off profile of diazepam and nordiazepam at times following the last administered dose. For diazepam, computer-generated theoretical curves assuming a linear two-compartment open model system with first order absorption, is also shown (see ref. 35 for complete details).

CONCLUSION

A rapid method for the determination of diazepam and nordiazepam in human plasma was developed. The method incorporates many desirable aspects of previously reported assays, but has significantly reduced the complexity of the sample preparatory steps by elimination of many sample manipulation steps, including multiple extractions, transfers, and solvent evaporation. The assay is essentially a one-step, one-tube extraction procedure utilizing methylnitrazepam as the internal standard, followed by GLC analysis through interfacing an electron capture linearizer, [63]Ni ECD, ALS and a mini-computer based data system to provide a nearly totally automated assay for diazepam and nordiazepam.

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