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# DIAZEPAM METABOLISM DURING CHRONIC MEDICATION

## UNBOUND FRACTION IN PLASMA, ERYTHROCYTES AND URINE

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SUMMARY

The metabolism of diazepam was studied in a group of patients receiving 4 to 60 mg of the drug *pro die* for periods of time ranging from 2 days to 106 weeks.

Chronic administration of high therapeutic doses may result in a considerable accumulation of the drug in blood; following administration of 60 mg *pro die* for an 11-week period, plasma levels as high as 194 $\mu$g% of diazepam and 267 $\mu$g% of demethyldiazepam were detected in one patient.

In the majority of cases, the plateau level attained after a few weeks of medication remained constant during the following months of therapy. In four cases, an increasing accumulation of the drug in plasma was observed during the course of treatment; in two patients, the plasma level of unmetabolized drug increased, throughout a period of several months, to twice the value attained after the first weeks of medication.

Demethyldiazepam was the major metabolite detected in plasma. The two hydroxylated metabolites, oxazepam and 3-hydroxydiazepam, were present in the majority of samples irrespective of the length of treatment. Absorption of diazepam and demethyldiazepam by the erythrocyte occurred after 11 or more weeks of therapy. Upon discontinuance of the treatment, diazepam compounds disappeared from the plasma more rapidly than from the erythrocytes. Free forms of diazepam and metabolites appeared in the urine following a few weeks of medication.

Ninety minutes after ingestion of a 2- to 20-mg dose, the plasma levels of unmetabolized diazepam encountered in the samples analyzed ranged from 9 to 194 $\mu$g%. The concentration of the metabolites varied as follows: demethyldiazepam, 3–275 $\mu$g%; oxazepam, 0–29 $\mu$g%; 3-hydroxydiazepam, 0–18 $\mu$g%.

The average plasma concentration of diazepam and demethyldiazepam in each group of patients receiving identical dosages (total 52 samples) reflected the amount of drug administered in a 24-h period as well as the dosage interval.

The unpredictable accumulation of the drug in blood during long-term therapy and the excessive concentration attained after administration of high doses illustrate the necessity of evaluating plasma levels as a guide to administration of diazepam during chronic medication.

Specific analytical procedures were developed for the simultaneous determination of the four compounds and for the isolation of each metabolite by fractiona-

tion of plasma extracts. The method adopted requires the use of a gas chromatograph equipped with an electron capture detector.

## INTRODUCTION

Diazepam*, 7 chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, is a member of the benzodiazepine series widely used for the symptomatic relief of tension and anxiety states. The drug is also clinically effective in treatment of the acute agitation associated with alcohol withdrawal, in neuromuscular disorders and as premedication in patients undergoing surgery. Recently, diazepam has been recommended for treatment of hallucinogenic crises[1,2] and successfully used in the management of heroin withdrawal in adolescence[3] and in a case of strychnine poisoning[4]. In psychiatric institutions the drug is largely used in reducing tension in hospitalized patients as well as in treatment of anxiety states in ambulatory patients.

Data on the metabolism of diazepam *in vitro*[5], in animals[6-16] and in humans[7,10,15-18] indicate that N-demethylation and hydroxylation represent the major pathways of degradation. In man, diazepam is metabolized to the N-demethyl analog, the 3-hydroxy analog and the 3-hydroxylated N-demethyl analog (oxazepam). Demethyldiazepam represents the major metabolite in blood[7,10-22]; glucuronide conjugation appears to be the most important pathway of degradation[7].

DE SILVA *et al.*[20] have determined the concentration of diazepam and demethyldiazepam in blood samples, following continuous administration of therapeutic doses of the drug for a few weeks. The results obtained by these authors showed a close correlation between doses administered and plasma concentration of the unmetabolized compound. GARATTINI *et al.*[22] have noted large variations in blood level of the intact drug in a group of patients receiving identical dosage of diazepam for a few days. Considerable accumulation of demethyldiazepam has been reported by VAN DER KLEIJN *et al.*[21] during a 14-day treatment with 30 mg of diazepam *pro die*. Free hydroxylated metabolites have not been detected in blood, nor has the excretion of free diazepam been reported in human urine.

From an evaluation of the data published, it is apparent that more information is needed in order to establish a relationship between doses administered and concentration of the drug in plasma. Because of expected interpatient differences, more reliable data can be obtained by analyzing a large number of samples from patients receiving different doses of diazepam for various periods of time. The data obtained could be also used to: (1) evaluate, on the onset of the treatment, the rate of absorption; (2) determine the dosage necessary to attain an effective level; (3) maintain, during long-term therapy, a safe concentration of the drug in blood; (4) ascertain if the prescribed doses have been regularly ingested. In the case of ambulatory patients, the knowledge of the plasma level of the drug (reflecting the patient's cooperation in taking the medication), in addition to its economic value, will provide the physician with a basis for designing subsequent treatments. In suspected cases of poisoning, the toxicologist could use this information in establishing whether the blood level determined is related to administration of repeated doses or to acute intake. A high ratio

* Marketed as Valium by Hoffman-La Roche, Inc., Nutley, N.J., U.S.A.

of the concentration of unmetabolized diazepam to diazepam metabolites is expected in cases of poisoning.

A review of the literature also shows that no data are available on the metabolism of diazepam in patients receiving commonly used therapeutic doses for periods of time longer than two weeks. As the drug is excreted slowly, a rapid increase in blood concentration is expected during the first days of medication until a plateau level is reached. When the drug is administered repeatedly over weeks or over several months, as in chronic cases, differences may occur not only with respect to the concentration of the unmetabolized drug in blood but also with respect to the formation and accumulation of the metabolites. Since two of the known diazepam metabolites, demethyldiazepam and oxazepam*, have shown pharmacologic properties similar to those of the parent compound[23], therapeutic responses to diazepam treatment cannot be related only to the concentration of the unmetabolized drug in blood. The contribution of the two active metabolites to this response should also be evaluated. Consequently, an evaluation of the activity of the drug in relation to its concentration in blood will require the determination of levels of unmetabolized diazepam as well as demethyldiazepam and oxazepam.

This investigation was undertaken in order to elucidate the metabolism of diazepam during chronic administration of the drug. Accordingly, studies were carried out to: (1) determine the concentration of diazepam and demethyldiazepam in a large number of plasma samples, following continuous administration of various therapeutic doses for different periods of time; (2) establish a relationship between plasma levels and doses administered; (3) study the influence of different dosage intervals on these levels; (4) ascertain whether a plateau level is maintained throughout the duration of the treatment; (5) attempt the detection of free forms of the two hydroxylated metabolites in blood; (6) investigate the distribution of diazepam and its metabolites between plasma and erythrocytes; (7) ascertain whether excretion of free forms of diazepam and metabolites occurs during long-term therapy.

It was necessary to develop a specific analytical procedure of sufficient sensitivity to determine diazepam and its metabolites at subnanogram levels. The method developed is based on selective solvent extraction of the four compounds and requires the use of a gas chromatograph equipped with an electron capture detector.

## EXPERIMENTAL

### *Reagents and reference solutions*

Analytical grade reagents were used. The extracting solvent was a mixture of toluene and *n*-heptane in the ratio 80:20; the toluene contained 2 % of isoamyl alcohol. The buffer solution was prepared by adjusting the pH of a saturated ammonium chloride solution to 9.5 with concentrated ammonium hydroxide. The following compounds were used as reference substances: diazepam, demethyldiazepam 3-hydroxydiazepam and oxazepam. Stock reference solutions were prepared by dissolving 10 mg of each compound in 10 ml of 95 % ethyl alcohol. Aliquots of these solutions were successively diluted with absolute ethanol to final concentrations of 1 ng per $\mu$l of diazepam and demethyldiazepam and 0.1 ng per $\mu$l of oxazepam and

* Marketed as Serax by Wyeth Laboratories, Philadelphia, Pa., U.S.A.

3-hydroxydiazepam. Working standards were prepared by evaporating aliquots of the stock solutions under a stream of nitrogen and re-dissolving the residues in the extracting solvent or in drug-free plasma, hemolysate or urine samples. Solutions of the compounds in ethanol were stable under refrigeration for several months.

*Material*

Blood samples were obtained from hospitalized patients 90 min after oral administration of morning doses of 2 to 20 mg of diazepam. A few samples were collected after the treatment had been discontinued. These patients had been receiving continuous oral doses of diazepam for a minimum period of 48 h to a maximum of 106 weeks prior to the onset of sampling. Blood samples, collected in EDTA tubes, were centrifuged immediately after arrival in the laboratory and the plasma fraction separated. The erythrocytes from a few blood samples were washed according to a procedure previously described[24]. Urine samples, collected on the same day, represented morning specimens. Plasma, erythrocytes and urine samples were kept below 0° until analyzed.

*Extraction procedures*

*Plasma*. One milliliter of plasma was mixed with 2 ml of the buffer solution at pH 9.5 and extracted with 5 ml of the extracting solvent by mechanically shaking the mixture for 10 min. Following centrifugation, the organic phase was separated and evaporated to dryness under a stream of nitrogen.

*Erythrocytes*. Three milliliters of packed red cells were hemolyzed by shaking with a few drops of distilled water. Six milliliters of the buffer solution were added to the hemolysate and the mixture extracted with 30 ml of the extracting solvent. Following separation, the organic phase was re-extracted with 3 ml of 6 *N* hydrochloric acid. After centrifugation, the acidic aqueous phase was separated, made alkaline by addition of 6 *N* sodium hydroxide (pH 9.5) and re-extracted with 5 ml of the toluene–*n*-heptane–isoamyl alcohol mixture. During neutralization the test tube was kept in an ice-water bath. The organic phase was separated and evaporated to dryness under nitrogen.

*Urine*. The pH of the sample was adjusted to 9.5 with diluted sodium hydroxide. One-milliliter aliquots were extracted as described for plasma samples.

The residues from plasma extracts were re-dissolved in 0.1–2 ml of the extracting solvent, the residues from erythrocyte or urine samples in 50 $\mu$l. Aliquots of these extracts (1–5 $\mu$l) were injected into the chromatograph. Synthetic plasma solutions were prepared by adding 1-ml aliquots of drug-free plasma to 0.01-2 $\mu$g of diazepam and demethyldiazepam and to 0.01–0.2 $\mu$g of oxazepam and 3-hydroxydiazepam. For the preparation of erythrocyte or urine synthetic solutions, 3-ml aliquots of drug-free hemolysate or 1 ml of drug-free urine were added to 0.01–0.2 $\mu$g of each of the four compounds. The mixtures were extracted according to the procedures described for authentic samples. After gas chromatography, plasma extracts representing 15–20 ml of plasma samples were pooled and re-extracted with 6 *N* hydrochloric acid according to the procedure adopted for extraction of erythrocyte samples. The extracts obtained were analyzed by thin-layer chromatography (TLC).

*Selective extraction of diazepam and metabolites*

The procedure, applied to plasma samples from patients, included the following steps:

(1) Extraction of 5 ml of plasma with 10 ml of toluene–*n*-heptane–isoamyl alcohol.
(2) Gas chromatography (GC) of the organic phase (Extract A).
(3) Re-extraction of Extract A with 2 ml of sodium hydroxide*.
(4) GC of the organic phase (Extract A1) from step 3.
(5) Re-extraction of Extract A1 with 2 ml of 0.1 *N* hydrochloric acid*.
(6) GC of the organic phase (Extract A2) from step 5.
(7) Re-extraction of Extract A2 with 2 ml of 1 *N* hydrochloric acid*.
(8) GC of the organic phase (Extract A3) from step 7.
(9) Re-extraction of Extract A3 with 2 ml of 6 *N* hydrochloric acid*.
(10) GC of the organic phase (Extract A4) from step 9.
(11) Separate re-extraction of the four aqueous phases from steps 3, 5, 7 and 9, after adjusting the pH to 9.5, with 5 ml of the extracting solvent.
(12) GC and TLC of the four extracts (Fractions 1, 2, 3 and 4) from step 11.

*Conditions for gas chromatography*

The instrument employed in this work was a Beckman GC-4 gas chromatograph equipped with an electron capture detector and a 10-in. potentiometric recorder. The column was a U-shaped glass tube, 2 mm I.D., 120 cm long, packed with 2 % OV-17 on Chromosorb W, HP, 80–100 mesh. The column was conditioned at 300° for 49 h with a helium flow-rate of 25 ml/min. The column oven temperature was maintained at 235°, the detector and detector lines at 330°, and the inlet lines at 320°. The flow-rates were as follows: helium carrier gas, 85 ml/min; helium discharge gas, 100 ml/min; carbon dioxide, 3 ml/min. The detector electrical settings were: polarizing voltage, 675 duodial; bias voltage, 350 duodial; source current, 7 mA. The electrometer settings were: range, 100; attenuation, from 64 to 512.

*Thin-layer chromatography*

Merck Silica Gel $F_{254}$ plates containing extracts from plasma samples and from synthetic plasma solutions were developed in the two following systems: System I—chloroform–*n*-heptane–ethyl alcohol (50:50:5); System II—chloroform–acetone (90:10). A one-dimensional technique was used to develop plates containing the extracts obtained from the selective extraction of diazepam and metabolites (Fractions 1, 2, 3 and 4); the plates were developed in System I, removed from the tank, dried and re-developed in the same direction in System II. Plates containing the pooled extracts were analyzed by two-dimensional TLC using System I in the first direction and System II in the second direction. After drying, the plates were re-developed in the first direction using System II. The plates were sprayed with 50 % sulfuric acid and observed under long-wave UV light.

## RESULTS AND DISCUSSION

*Extraction of diazepam and metabolites*

The optimal conditions for the simultaneous extraction of diazepam and the

* Save the aqueous phase for subsequent analysis.

three metabolites were determined experimentally by studying the partition characteristics of each compound between aqueous solutions at various pH values and different organic solvents. In a preceding work concerning the analysis of chlordiazepoxide (Librium)[25], a compound chemically related to diazepam, *n*-heptane containing 1.5 % isoamyl alcohol was used for the extraction of the drug from plasma samples. In our experience, extracts in ether[19–22,26] or in chloroform[27,28], even if followed by re-extraction in diluted acid, are not suitable for GC when using an electron capture detector. Naturally occurring substances, present in the extract, interfere with the chromatographic analysis of the compounds analyzed. Extraction of diazepam and its metabolites into *n*-heptane–isoamyl alcohol from a buffer solution at pH 9.5 yielded a complete recovery of diazepam, demethyldiazepam and 3-hydroxydiazepam; only the 23 % of the added oxazepam was extracted into the organic phase. By using toluene–isoamyl alcohol, the recoveries of diazepam, demethyldiazepam and oxazepam were complete, but 40 % of the added 3-hydroxydiazepam remained in the aqueous phase. The toluene–*n*-heptane mixture in the ratio 80:20 completely extracted the four compounds and was adopted as extracting solvent in the analyses. Extraction of diazepam and demethyldiazepam was complete in either toluene or *n*-heptane or in mixtures of the two solvents in any proportion. Fig. 1 shows the recovery of oxazepam and 3-hydroxydiazepam in *n*-heptane, toluene and mixtures of these two solvents in different ratios. Isoamyl alcohol was always added to the extracting solvent at a concentration of 1.6 % in order to prevent absorption on to glasses.

Studies on the distribution of diazepam and the three metabolites between the extracting solvent adopted and aqueous solutions showed the different partition characteristics of the four compounds. The data presented in Figs. 2 and 3 were obtained by dissolving 100 $\mu$g of each substance in 100 ml of toluene–*n*-heptane–isoamyl alcohol and extracting 5-ml aliquots of this solution with 3 ml of buffer solutions at various pH values (Fig. 2) or with 1 ml of hydrochloric acid at different concentrations (Fig. 3). Quantitation of the amounts recovered was achieved by GC of the organic phase before and after extraction. Recovery of the four compounds

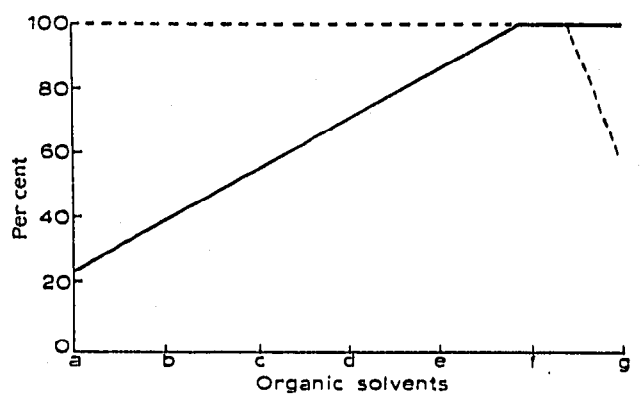


Fig. 1. Recoveries of oxazepam and 3-hydroxydiazepam from a buffer solution at pH 9.5 into *n*-heptane, toluene and mixtures of the two solvents at various ratios. a = *n*-Heptane; b = *n*-heptane–toluene (80:20); c = *n*-heptane–toluene (65:35); d = *n*-heptane–toluene (50:50); e = toluene–*n*-heptane (65:35); f = toluene–*n*-heptane (80:20); g = toluene. ———, Oxazepam; – – – –, 3-hydroxydiazepam.

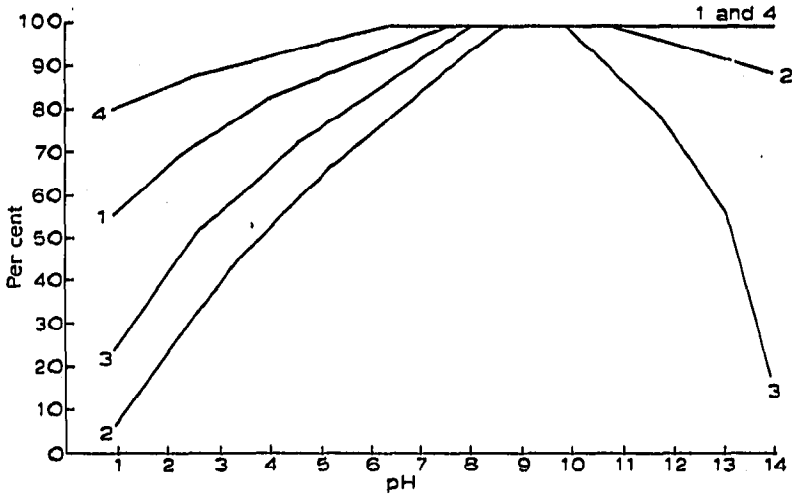


Fig. 2 Partition characteristics of diazepam (1), demethyldiazepam (2), oxazepam (3) and 3-hydroxydiazepam (4) between toluene–*n*-heptane–isoamyl alcohol and buffer solutions at various pH values. Per cent reported represents amount of substance present in the organic phase after each extraction.

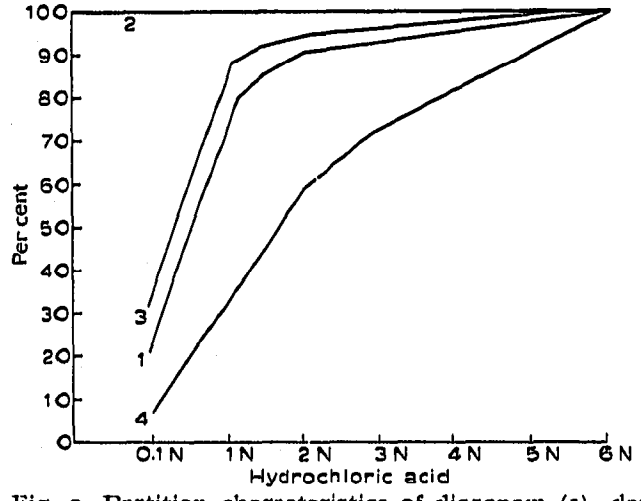


Fig. 3. Partition characteristics of diazepam (1), demethyldiazepam (2), oxazepam (3) and 3-hydroxydiazepam (4) between toluene–*n*-heptane–isoamyl alcohol and hydrochloric acid at various concentrations. Per cent reported represents amount of substance extracted into the aqueous phase.

from pH 1 varied from 5 % of demethyldiazepam to 80 % of 3-hydroxydiazepam (Fig. 2). By increasing the alkalinity of the buffer solution above pH 10, the recovery of oxazepam rapidly decreased. After extraction with 1 *N* sodium hydroxide, only 0.5 % of the added oxazepam is detectable in the organic phase, the concentration of demethyldiazepam is reduced to 90 %, while diazepam and 3-hydroxydiazepam are still present at the original concentration. Simultaneous recovery of the four compounds was complete only in the range of pH 8.5–10. The conditions for concentration of diazepam and metabolites in the extracts, by re-extraction in diluted acid, were

also studied. As shown in Fig. 3, 0.1 *N* hydrochloric acid extracted completely demethyldiazepam from the extracting solvent, while the recovery of the other three compounds ranged between 5 and 30 %. When 1 *N* hydrochloric acid was used, recoveries of diazepam and oxazepam were in the acceptable range, but 70 % of 3-hydroxydiazepam was left in the organic phase. Re-extraction with 6 *N* hydrochloric acid was necessary in order to remove this metabolite completely from the extracting solvent. These findings formed the bases for the development of the extraction procedures adopted in this work and were also used to confirm the identity of the four compounds detected in the sample analyzed. An evaluation of these data also permits the following observations: the single-step solvent extraction in either toluene or *n*-heptane containing 1.6 % isoamyl alcohol represents a rapid procedure for the determination of diazepam in toxicological analysis and offers, in routine clinical assays, the advantage of determining diazepam and the major blood metabolite, demethyldiazepam, in the same extract without any chemical modification or re-extraction steps; concentration of the four compounds in 6 *N* hydrochloric acid, permitting the determination of diazepam and its metabolites at picogram levels, offers the sensitivity needed for metabolic studies; oxazepam (Serax) can be isolated from the extracting solvent into 1 *N* sodium hydroxide or extracted from the original sample directly into toluene–isoamyl alcohol (Table I).

Recoveries of the four compounds, added to plasma, hemolysate or urine samples in various amounts, are shown in Tables II, III and IV, respectively. The residues, after evaporation of aliquots of standard alcoholic solutions, were dissolved in 1 ml of drug-free plasma or urine or in 3 ml of hemolysate and extracted as described above. The amount of substance recovered was determined by GC analysis of the extracts obtained and corresponding aliquots of standard solutions of the compounds in toluene–*n*-heptane–isoamyl alcohol.

*Determination of optimal conditions for GC*

Diazepam and metabolites were separated on a 2 % OV-17 column at isothermic temperature. When operated at 235° under the conditions described, this column allowed an efficient separation of the four compounds in a relatively short period of time and was preferred to the more conventional 3 % OV-17. The column temperature was adjusted to obtain a retention time of 4 min for oxazepam. At this temperature, blank extracts from plasma, hemolysate or urine samples did not show any peak with a retention time longer than 3 min.

The response of the detector to injections of aliquots of a mixture containing similar amounts of the four compounds was different for each substance, depending mainly on the detector electrical settings and on the carrier gas flow. High flow-rates of the carrier gas resulted in a less tailing peak of demethyldiazepam and also improved the sensitivity for the detection of 3-hydroxydiazepam. Preliminary experiments with plasma samples from patients had shown that the concentrations of oxazepam and 3-hydroxydiazepam were significantly lower than those of diazepam and demethyldiazepam. Accordingly, the operating parameters were adjusted to favor the maximal sensitivity for the two minor metabolites. Fig. 4 shows the linear response obtained when 5 $\mu$l of toluene–*n*-heptane–isoamyl alcohol containing the four compounds in various amounts ranging between 0.5 and 5 ng were chromatographed using the parameters selected. The data were obtained at the same electro-

TABLE I

APPLICATIONS OF THE PARTITION CHARACTERISTICS OF DIAZEPAM AND METABOLITES TO ANALYTICAL PROCEDURES

100 µg of each compound were dissolved in 100 ml of the organic solvent containing 1.6% of isoamyl alcohol. Five-milliliter aliquots were extracted with either 3 ml of buffer solution at pH 9.5 or 1 ml of 1 *N* sodium hydroxide or 1 ml of hydrochloric acid at the various concentrations.

| *Analysis* | *Conditions for extraction* | *Extracted into the organic phase* | *Extracted into the aqueous phase* |
|---|---|---|---|
| (A) Toxicological and clinical routine assays | 1. From pH 9.5 into *n*-heptane (Extract A1) | 100% diazepam, 100% demethyldiazepam, 100% 3-hydroxydiazepam | 77% oxazepam |
| | 2. From pH 9.5 into toluene | 100% diazepam, 100% demethyldiazepam, 100% oxazepam, 60% 3-hydroxydiazepam | 40% 3-hydroxydiazepam |
| (B) Detection of oxazepam (Serax) | 1. From pH 9.5 into toluene | 100% oxazepam | |
| | 2. From the aqueous phase, after Extract A1, into toluene | 77% oxazepam | |
| (C) Simultaneous detection of diazepam and metabolites | From pH 9.5 into toluene–*n*-heptane (80:20) (Extract C) | 100% diazepam, 100% demethyldiazepam, 100% oxazepam, 100% 3-hydroxydiazepam | |
| (D) Isolation of oxazepam from diazepam, demethyldiazepam and 3-hydroxydiazepam | Re-extraction of Extract C with 1 *N* sodium hydroxide | 100% diazepam, 90% demethyldiazepam, 100% 3-hydroxydiazepam | 100% oxazepam |
| (E) Isolation of demethyldiazepam from diazepam, oxazepam and 3-hydroxydiazepam | Re-extraction of Extract C with 0.1 *N* hydrochloric acid | 80% diazepam, 70% oxazepam; 95% 3-hydroxydiazepam | 100% demethyldiazepam |
| (F) Isolation of 3-hydroxydiazepam from diazepam, demethyldiazepam and oxazepam | Re-extraction of Extract C with 1 *N* hydrochloric acid | 70% 3-hydroxydiazepam | 81% diazepam, 100% demethyldiazepam, 88% oxazepam |
| (G) Detection of the four compounds at subnanogram levels | Re-extraction of Extract C with 6 *N* hydrochloric acid | | 100% diazepam, 100% demethyldiazepam, 100% oxazepam, 100% 3-hydroxydiazepam |

meter settings used for the analysis of all the plasma extracts (range 100, attenuation, 512). Correction for increasing sensitivity was often needed for the determination of 3-hydroxydiazepam. Because of the long retention time of this metabolite, this was easily achieved by electrometer adjustments. Under the chromatographic

TABLE II

RECOVERIES OF DIAZEPAM AND METABOLITES FROM 1-ml PLASMA SAMPLES

| *Compound* | *Amount added (μg)* | *Number of determinations* | *Recovery (% ± S.D.)* |
|---|---|---|---|
| Diazepam | 0.1 | 10 | 95.1 ± 3.2 |
| | 0.5 | 10 | 97.2 ± 2.1 |
| | 1 | 7 | 96.3 ± 2.9 |
| | 2.5 | 5 | 95.9 ± 4.6 |
| Demethyldiazepam | 0.1 | 10 | 92.6 ± 3.3 |
| | 0.5 | 10 | 95.1 ± 2.9 |
| | 1 | 7 | 97.2 ± 1.3 |
| | 2.5 | 5 | 96.6 ± 3.5 |
| Oxazepam | 0.01 | 10 | 83.6 ± 2.9 |
| | 0.05 | 10 | 86.5 ± 3.7 |
| | 0.1 | 7 | 85.8 ± 2.5 |
| | 0.3 | 5 | 87.0 ± 3.7 |
| 3-Hydroxydiazepam | 0.01 | 10 | 92.1 ± 3.1 |
| | 0.05 | 10 | 93.2 ± 2.9 |
| | 0.1 | 7 | 92.9 ± 3.3 |
| | 0.3 | 5 | 96.1 ± 2.4 |

TABLE III

RECOVERIES OF DIAZEPAM AND METABOLITES FROM 3-ml HEMOLYSATE SAMPLES

| *Compound* | *Amount added (μg)* | *Number of determinations* | *Recovery (% ± S.D.)* |
|---|---|---|---|
| Diazepam | 0.03 | 5 | 85.3 ± 4.1 |
| | 0.06 | 10 | 86.9 ± 3.7 |
| | 0.15 | 10 | 88.1 ± 4.1 |
| Demethyldiazepam | 0.03 | 5 | 81.5 ± 3.7 |
| | 0.06 | 10 | 83.7 ± 2.9 |
| | 0.15 | 10 | 85.1 ± 4.1 |
| Oxazepam | 0.03 | 5 | 69.9 ± 4.2 |
| | 0.06 | 5 | 73.1 ± 2.9 |
| 3-Hydroxydiazepam | 0.03 | 5 | 76.6 ± 2.8 |
| | 0.06 | 5 | 75.9 ± 3.8 |

conditions described, oxazepam has a retention time of 4 min, diazepam 5.5 min, demethyldiazepam 8 min, and 3-hydroxydiazepam 13.4 min. Fig. 5 shows the separation of a mixture of the four compounds extracted from a synthetic plasma solution. One microgram of each compound was added to 1 ml of drug-free plasma and the mixture extracted as described above. The residue, after evaporation of the organic phase, was redissolved in 1 ml of the extracting solvent. Chromatograms of extracts from the plasma and red cell fractions of a blood sample from a patient receiving 60 mg of diazepam *pro die* are shown in Figs. 6 and 7, respectively. Fig. 8 represents GLC traces of an extract from a urine sample from the same patient.

TABLE IV

RECOVERIES OF DIAZEPAM AND METABOLITES FROM 1-ml URINE SAMPLES

| *Compound* | *Amount added* ($\mu g$) | *Number of determinations* | *Recovery* (% ± *S.D.*) |
|---|---|---|---|
| Diazepam | 0.01 | 5 | 97.6 ± 2.7 |
| | 0.02 | 5 | 95.9 ± 3.4 |
| | 0.05 | 10 | 97.2 ± 2.5 |
| | 0.1 | 5 | 95.5 ± 3.6 |
| Demethyldiazepam | 0.01 | 5 | 90.6 ± 4.1 |
| | 0.02 | 5 | 90.2 ± 3.2 |
| | 0.05 | 10 | 94.7 ± 2.4 |
| | 0.1 | 5 | 95.2 ± 3.1 |
| Oxazepam | 0.01 | 5 | 82.7 ± 3.8 |
| | 0.02 | 5 | 80.9 ± 4.2 |
| | 0.05 | 10 | 84.3 ± 2.6 |
| | 0.1 | 5 | 85.6 ± 3.3 |
| 3-Hydroxydiazepam | 0.01 | 5 | 80.8 ± 3.7 |
| | 0.02 | 5 | 79.9 ± 3.9 |
| | 0.05 | 10 | 83.6 ± 2.5 |
| | 0.1 | 5 | 83.9 ± 3.8 |

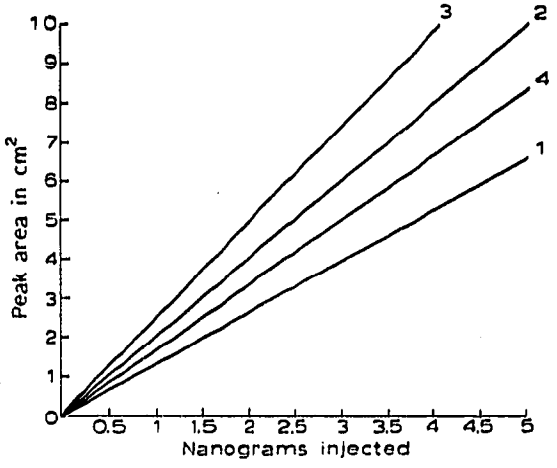


Fig. 4. Response of the electron capture detector to injections of 5 $\mu$l of the extracting solvent containing various amounts of diazepam (1), demethyldiazepam (2), oxazepam (3) and 3-hydroxydiazepam (4).

*Analysis of plasma, erythrocytes and urine samples*

The results from the analyses of plasma samples are tabulated in Table V, together with the doses the patients were receiving and the length of treatment. As mentioned above, plasma samples from patients undergoing diazepam therapy were collected 90 min after ingestion of the morning dose. Seven blood samples were obtained at various times after discontinuance of the drug. A few samples, representing one week or less of treatment, were included in the study for comparison purposes. In Table V, the samples are arranged according to the total amount of diazepam received in a 24-h period and, within the limits of each dose group, according to

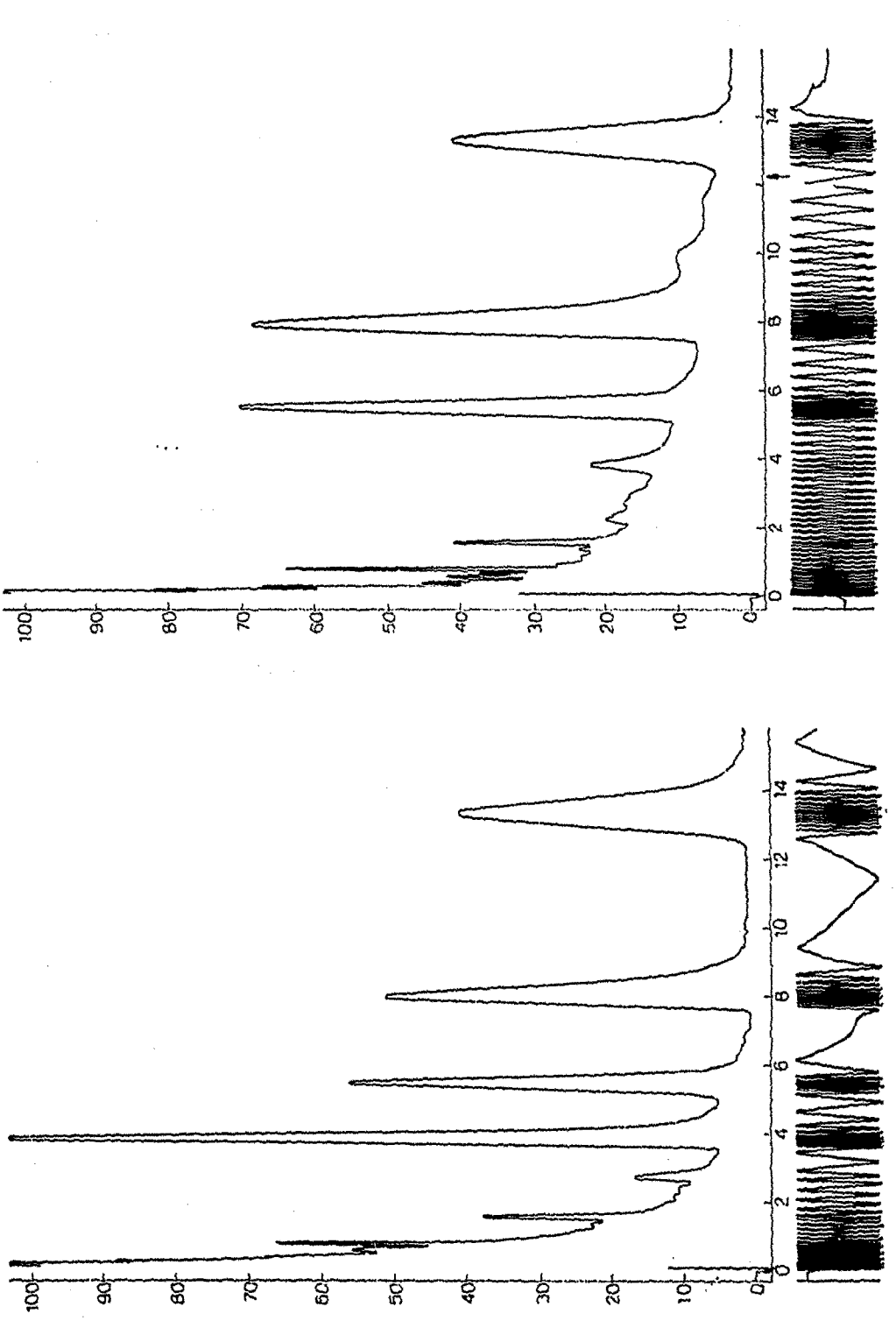

Fig. 5. GLC traces of an extract *from* a synthetic plasma solution containing diazepam and metabolites. 1 *µl*, corresponding to 1 ng of each compound, was injected. Retention times: oxazepam, 4 *min*; diazepam, 5.5 *min*; demethyldiazepam, 8 *min*; 3-hydroxydiazepam, 13.4 *min*.

Fig. 6. GLC traces of an extract of a plasma sample *from* a *patient* receiving 60 mg of diazepam *pro die*. 5 *µl*, corresponding to 2.5 *µl* of plasma were injected. Arrow indicates electrometer adjustment.

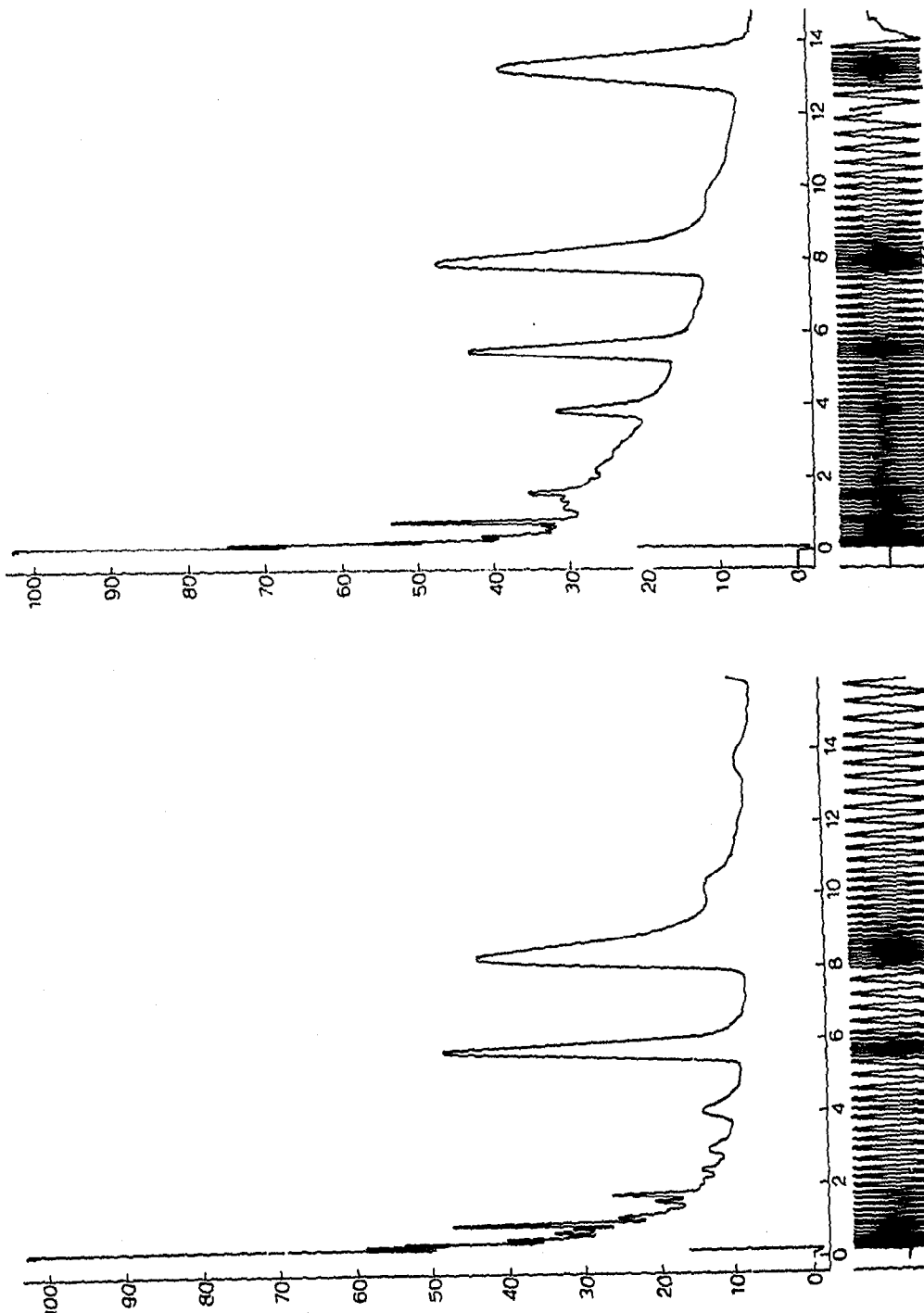


Fig. 7. GLC traces of an extract from the red cell fraction of the same blood sample as in Fig. 6.

Fig. 8. GLC traces of an extract of a urine sample from the same patient.

TABLE V

PLASMA LEVELS OF DIAZEPAM AND ITS METABOLITES FOLLOWING ADMINISTRATION OF VARIOUS DOSES FOR DIFFERENT PERIODS OF TIME[a]

| *Sample No.*[b] | *Diazepam pro die (mg)* | *Length of treatment (weeks)*[c] | *Plasma levels (μg %)* | | | |
|---|---|---|---|---|---|---|
| | | | *Oxazepam* | *Diazepam* | *Demethyl-diazepam* | *3-Hydroxy-diazepam* |
| 1 | 2 *b.i.d.* | 8 | — | 9.3 | 17.3 | — |
| 2 | 2 *b.i.d.* | 10 | — | 9.8 | 6.8 | — |
| 3 | 2 *b.i.d.* | 32 | — | 12.5 | 14.8 | 1.6 |
| 4 | 5 *o.d.* | 2 | — | 10.1 | 9.7 | — |
| 5 | 5 *o.d.* | 2 | — | 17.6 | 17.2 | — |
| 6 | 5 *o.d.* | 16 | — | 10.4 | 8.3 | 2.4 |
| 7 | 5 *o.d.* | 32 | — | 16.2 | 13.9 | 2.9 |
| 8 (6) | 5 *o.d.* | 53 | — | 10.4 | 9.6 | 2.7 |
| 9 | 5 *o.d.* | 60 | — | 16.8 | 15.1 | 1.8 |
| 10 (4) | 5 *o.d.* | 2[d] | — | 7.2 | 9.7 | — |
| 11 (7) | 5 *o.d.* | 32[d] | — | 8.1 | 10.2 | — |
| 12 | 2 *t.i.d.* | 1 | — | 11.4 | 11.1 | — |
| 13 | 2 *t.i.d* | 10 | — | 21.7 | 8.9 | — |
| 14 | 2 *t.i.d.* | 10 | — | 12.7 | 17.4 | — |
| 15 (14) | 2 *t.i.d.* | 60 | 1.7 | 25.1 | 46.1 | 3.0 |
| 16 | 5 *b.i.d.* | 48h | — | 31.1 | 3.3 | — |
| 17 | 5 *b.i.d.* | 2 | — | 29.1 | 34.4 | 4.8 |
| 18 | 5 *b.i.d.* | 4 | — | 18.5 | 21.5 | — |
| 19 (17) | 5 *b.i.d.* | 6 | 2.8 | 27.9 | 35.4 | 3.7 |
| 20 | 5 *b.i.d.* | 18 | — | 9.3 | 13.0 | 2.6 |
| 21 | 5 *b.i.d.* | 24 | — | 21.3 | 30.3 | — |
| 22 | 5 *b.i.d.* | 28 | 3.1 | 28.1 | 28.9 | 1.9 |
| 23 (18) | 5 *b.i.d.* | 36 | 1.9 | 18.4 | 24.3 | 1.3 |
| 24 | 5 *b.i.d.* | 48 | 1.7 | 19.6 | 21.9 | 2.8 |
| 25 (24) | 5 *b.i.d.* | 50 | 1.9 | 22.0 | 25.2 | 3.0 |
| 26 (20) | 5 *b.i.d.* | 53 | — | 18.2 | 31.4 | 2.2 |
| 27 (21) | 5 *b.i.d.* | 60 | — | 21.2 | 21.8 | 1.3 |
| 28 (25) | 5 *b.i.d.* | 80 | 1.6 | 25.0 | 36.1 | 2.3 |
| 29 (28) | 5 *b.i.d.* | 84 | 1.5 | 25.8 | 37.5 | 2.1 |
| 30 | 5 *b.i.d.* | 106 | 2.3 | 18.1 | 19.8 | 1.9 |
| 31 | 5 *b.i.d.* | 106 | 1.7 | 37.1 | 46.4 | — |
| 32 (26) | 5 *b.i.d.* | 53[e] | — | 2.1 | 3.2 | — |
| 33 (29) | 5 *b.i.d.* | 84[e] | — | 1.7 | 5.3 | — |
| 34 | 10 *o.d.* | 3 | 2.1 | 23.3 | 42.4 | 2.1 |
| 35 | 5 *t.i.d.* | 5 days | 3.2 | 51.2 | 27.3 | 4.0 |
| 36 | 5 *t.i.d.* | 1 | — | 16.5 | 9.6 | 2.9 |
| 37 | 5 *t.i.d.* | 1 | 1.5 | 28.4 | 22.5 | 5.2 |
| 38 | 5 *t.i.d.* | 2 | 2.1 | 22.5 | 21.8 | 2.3 |
| 39 | 5 *t.i.d.* | 4 | 2.2 | 43.2 | 35.4 | 3.2 |
| 40 (37) | 5 *t.i.d.* | 16 | 1.9 | 28.6 | 21.0 | 2.1 |
| 41 | 5 *t.i.d.* | 18 | 3.1 | 21.7 | 19.2 | 1.9 |
| 42 (38) | 5 *t.i.d.* | 28 | 1.7 | 22.2 | 20.4 | 2.2 |
| 43 | 5 *t.i.d.* | 40 | 5.3 | 55.6 | 48.2 | 4.5 |
| 44 | 5 *t.i.d.* | 56 | 3.4 | 40.0 | 29.3 | 3.6 |
| 45 (42) | 5 *t.i.d.* | 28[f] | 2.3 | 18.4 | 26.2 | 2.1 |
| 46 (41) | 5 *t.i.d.* | 18[g] | — | — | — | — |
| 47 (45) | 5 *t.i.d.* | 28[g] | — | — | — | — |
| 48 | 10 *b.i.d.* | 3 | 8.0 | 37.8 | 55.2 | 4.2 |
| 49 | 10 *b.i.d.* | 4 | 4.5 | 29.9 | 42.1 | 2.3 |
| 50 | 10 *b.i.d.* | 61 | 1.5 | 25.2 | 31.9 | 3.2 |
| 51 | 5 *q.i.d.* | 1 | 1.5 | 49.2 | 26.9 | 2.2 |
| 52 | 5 *t.i.d.*[h] | 1 | 2.1 | 60.5 | 35.2 | 9.8 |

TABLE V (*continued*)

| *Sample No.*[b] | *Diazepam pro die (mg)* | *Length of treatment (weeks)*[c] | *Plasma levels (μg %)* | | | |
|---|---|---|---|---|---|---|
| | | | *Oxazepam* | *Diazepam* | *Demethyl-diazepam* | *3-Hydroxy-diazepam* |
| 53 | 10 *t.i.d.* | 16 | 3.1 | 52.0 | 47.1 | 2.1 |
| 54 | 10 *t.i.d.* | 60 | 2.2 | 44.5 | 50.9 | 1.1 |
| 55 (53) | 10 *t.i.d.* | 62 | 2.4 | 51.4 | 49.4 | 2.3 |
| 56 | 20 *b.i.d.* | 60 | 5.9 | 79.2 | 106.2 | 5.6 |
| 57 (56) | 20 *b.i.d.* | 64 | 12.1 | 83.2 | 102.5 | 4.2 |
| 58 (57) | 20 *b.i.d.* | 106 | 11.5 | 96.9 | 109.2 | 5.2 |
| 59 | 20 *t.i.d.* | 10 | 29.2 | 84.5 | 146.5 | 16.0 |
| 60 | 20 *t.i.d.* | 11 | 18.2 | 193.9 | 266.6 | 18.5 |
| 61 (60) | 20 *t.i.d.* | 28 | 16.7 | 194.2 | 274.9 | 13.9 |
| 62 | 20 *t.i.d.* | 40 | 9.9 | 143.7 | 182.1 | 11.1 |

[a] 90 min after ingestion of morning dose, unless otherwise indicated.
[b] Numbers in parentheses indicate samples obtained from the same patient.
[c] Unless otherwise indicated.
[d] Sample obtained 24 h after administration of the last dose.
[e] Sample obtained 6 days after the drug had been discontinued.
[f] Sample obtained 9 h after administration of the last dose.
[g] Sample obtained 10 days after the drug had been discontinued.
[h] An additional 10-mg dose was administered at bed time.

TABLE VI

LEVELS OF DIAZEPAM AND METABOLITES IN THE RED CELL FRACTION OF FIFTEEN BLOOD SAMPLES (μg %)

| *Sample No.*[a] | *Oxazepam* | *Diazepam* | *Demethyl-diazepam* | *3-Hydroxy-diazepam* |
|---|---|---|---|---|
| 15 | — | 1.3 | 2.4 | — |
| 17 | — | — | — | — |
| 22 | — | 1.5 | 3.1 | — |
| 23 | — | 1.4 | 2.1 | — |
| 25 | — | 1.3 | 3.6 | t |
| 36 | — | — | — | — |
| 39 | — | — | — | — |
| 42 | — | 0.9 | 1.7 | — |
| 43 | — | 1.9 | 3.5 | — |
| 46 | — | — | 1.9 | — |
| 47 | — | — | 2.7 | — |
| 53 | t[b] | 1.7 | 2.4 | — |
| 54 | — | 2.6 | 5.2 | t |
| 58 | t | 2.4 | 4.9 | t |
| 60 | t | 3.6 | 5.6 | t |

[a] Corresponding designation in Table V.
[b] t = traces.

the duration of the treatment. Samples obtained after the therapy had been discontinued are located at the end of the respective dose group. The results from the analyses of the red cell fraction of fifteen blood samples are tabulated in Table VI. Table VII shows the data obtained from the analyses of urine specimens. Synthetic plasma, erythrocyte and urine solutions were extracted and chromatographed in

parallel with the relative authentic samples. Quantitation was carried out by measurement of peak areas.

TABLE VII

LEVELS OF DIAZEPAM AND METABOLITES IN URINE ($\mu$g %)

| *Sample No.*[a] | *Oxazepam* | *Diazepam* | *Demethyl-diazepam* | *3-Hydroxy-diazepam* |
|---|---|---|---|---|
| 14 | — | 0.8 | 5.2 | 2.1 |
| 20 | 1.0 | 1.4 | 9.2 | 7.3 |
| 29 | — | 2.1 | 5.3 | 3.3 |
| 38 | — | 2.2 | 2.5 | 1.5 |
| 39 | — | 2.0 | 9.2 | 1.9 |
| 43 | — | 1.2 | 2.4 | 2.0 |
| 54 | — | 1.1 | 8.1 | 2.3 |
| 56 | 4.5 | 1.6 | 4.1 | 9.4 |
| 60 | 1.1 | 1.4 | 5.7 | 4.4 |
| 62 | 1.1 | 1.9 | 5.2 | 3.2 |

[a] Corresponding designation in Table V.

With the exception of chlorpromazine, chromatograms of extracts of plasma, hemolysate or urine samples from patients receiving a number of other drugs were free of interfering peaks. Extracts of samples from patients known to receive chlorpromazine (sample No. 16 and sample No. 36) and from subjects recently admitted were also chromatographed using programmed temperature. The initial temperature was 210°, programmed for an increase of 30° in 32 min. Under these conditions, an adequate separation of chlorpromazine metabolites from diazepam metabolites was achieved.

The data presented in Table V show evident variations in plasma concentration of unmetabolized diazepam in patients receiving identical dosages. No consistent correlation can be seen with the length of therapy or with the total amount of drug received. Apparently, these variations represent interpatient differences reflecting the relative influences of the rate of absorption and metabolism. A similar observation, concerning a group of patients receiving 15 mg of the drug *pro die* for a few days, has been reported by GARATTINI *et al.*[22]. From the present study, it appears that interpatient differences exist at any dose level, during short as well as long-term therapy. The concentration of unmetabolized diazepam in sample No. 16 (48 h of treatment) and in sample No. 35 (five days of treatment), remarkably high with respect to the average level in the corresponding dose groups, shows the accumulation of the drug in blood during the first days of medication. Apparently, after one to two weeks of therapy, the plasma level drops to lower values; thereafter it again reflects individual differences. In order to ascertain whether the plasma concentration attained after a few weeks of medication represents a plateau level or whether the accumulation of the drug in blood increases further during the following weeks of therapy, two or more samples of blood were collected from each of twelve patients at various times. The analyses of these samples gave the following results: in eight cases the plasma level of diazepam achieved during the first period of medication (from 1 to 24 weeks) was maintained constant throughout the following months (sample Nos. 6 and 8, 17 and

19, 18 and 23, 21 and 27, 37 and 40, 38 and 42, 53 and 55, 60 and 61); in two cases it increased considerably (from 48 to 84 weeks in samples Nos. 24, 25, 28 and 29; from 60 to 106 weeks in samples Nos. 56, 57 and 58); in two cases it doubled its value: from 10 to 60 weeks (samples Nos. 14 and 15) and from 18 to 53 weeks (samples Nos. 20 and 26). The concentration of demethyldiazepam, in the majority of cases, showed a moderate or remarkable increase. After administration of 25 mg of the drug during a 48-h period, demethyldiazepam was present in a plasma sample at a concentration equivalent to 11 % of the unmetabolized diazepam present (sample No. 16). Following one week of treatment, the concentration of demethyldiazepam was still lower than the level of diazepam in four cases (samples Nos. 36, 37, 51 and 52) and equal in only one case (sample No. 12). Ninety minutes after ingestion of a given dose, irrespective of the duration of treatment and of the amount of drug received, the ratio of the concentration of diazepam to demethyldiazepam was $< 1$ in 31 cases and $> 1$ in 24 cases. The seven samples collected after discontinuance of the treatment are not included in this evaluation. With the exception of sample No. 16 (48 h of therapy), in all the samples in the group of patients receiving 5 mg of diazepam twice a day, demethyldiazepam was consistently present at a higher concentration than the unmetabolized drug. This ratio was reversed in the samples collected after administration of a 5-mg dose repeated three times a day. Because of the longer half-life of demethyldiazepam in respect to the parent compound[29, 30], these differences can conceivably be explained in relation to the different dosage intervals. Six days after discontinuance of the treatment, an average 9 % of diazepam and 12 % of demethyldiazepam were still detectable in plasma (samples Nos. 32 and 26 and samples Nos. 33 and 29). The two compounds were not detectable in samples collected ten days after the drug had been discontinued following several months of medication (samples Nos. 46 and 47). The concentration of oxazepam and 3-hydroxydiazepam in the plasma samples analyzed ranged between 0 and 29 $\mu$g % and 0 and 18 $\mu$g %, respectively. At low doses (4–6 mg *pro die*), 3-hydroxydiazepam was detected in many samples following sixteen or more weeks of treatment; oxazepam was present only in one sample corresponding to sixty weeks of medication (sample No. 15). With respect to the duration of therapy, the two metabolites were simultaneously detected in plasma after administration of 15 mg *pro die* for a five-day period (sample No. 35).

Demethyldiazepam was the only metabolite present in measurable amount in the hemolysates analyzed. Traces of oxazepam and 3-hydroxydiazepam were noted in a few samples. Unmetabolized diazepam and demethyldiazepam were present in the red cell fraction of all the blood samples representing eleven or more weeks of continuous medication with daily doses ranging between 6 and 60 mg. The three samples representing one to four weeks of treatment gave negative results. The analyses of sample No. 46 and sample No. 47, obtained ten days after discontinuance of the treatment, were also negative for unmetabolized diazepam. It is of interest to note that demethyldiazepam was still detectable in the red cell fraction of these two blood samples while the analysis of the corresponding plasma fraction exhibited negative results (Table V, samples Nos. 46 and 47). In all the hemolysates analyzed, the ratio of demethyldiazepam to diazepam was $> 1$. *In vivo* absorption of some phenothiazine derivatives by the erythrocytes has been reported by various authors[24, 31, 32] and related by AHTEE AND PAASONEN[31] to the tranquilizing effect of the drugs. No information is available at this moment on the significance of the

presence of diazepam compounds in the erythrocytes. It may be meaningful that diazepam and demethyldiazepam were present only in samples from patients undergoing long-term therapy (11–106 weeks). The persistence of the major blood metabolite in the erythrocytes after any diazepam compounds had disappeared from the plasma also could be significant.

Free diazepam, demethyldiazepam and 3-hydroxydiazepam were detected in all the urine specimens analyzed (2–84 weeks of medication). Free oxazepam was present only in four samples. The concentrations of the four compounds in the ten samples analyzed were: demethyldiazepam > 3-hydroxydiazepam > diazepam > oxazepam, in seven cases; demethyldiazepam > diazepam > 3-hydroxydiazepam > oxazepam, in two cases; 3-hydroxydiazepam > oxazepam > demethyldiazepam > diazepam in one case.

Because of the difficulties encountered in collecting 24-h specimens, the determination of the daily excretion was not possible.

*Evaluation of the method*

In order to ascertain the reproducibility of the method when applied to authentic specimens, 1-ml aliquots of a plasma sample (frozen in pre-measured amounts) were analyzed over a period of several months. In three series of eight analyses each, the plasma concentrations of the four compounds determined in each assay varied between 96 and 101 % in respect to the values obtained in the first analysis. Apparently, the long storage in the freezer did not affect the results of the analysis.

The influence of the pH value on the recovery of free compounds from biological material was investigated by analyzing authentic plasma samples. Lower recoveries of substances added to drug-free plasma in respect to those obtained from aqueous solutions have been reported by many authors involved in the detection of drugs in blood. This phenomenon had been generally attributed to "*in vitro*" binding of the drug to plasma proteins. In order to verify if the pH value of the sample during extraction plays an important role either on binding free metabolites to plasma proteins or on releasing bound metabolites, three 1-ml aliquots of a patient's plasma sample were extracted from physiological pH, from pH 9.5 and from pH 11, respectively. Synthetic plasma solutions and aqueous standard solutions, buffered at the same pH value, were extracted in parallel. Although in any case the recovery of the four compounds from drug-free plasma was lower than from aqueous solutions, GC analysis of the three extracts obtained from the authentic plasma samples showed no difference in the concentration of diazepam and metabolites.

The possible influence of the 6 *N* hydrochloric acid on the stability of the compounds studied and on naturally occurring substances present in plasma was also investigated. The toluene–*n*-heptane–isoamyl alcohol extract from 2 ml of plasma from a patient was divided in two equal portions. One aliquot was processed for GC analysis. The other portion was extracted with 6 *N* hydrochloric acid according to the procedure described for erythrocyte samples. Evaluation of the two extracts from the authentic plasma sample and from extracts of synthetic plasma solutions processed in an identical manner showed that no decomposition of the compounds or interference with other substances had occurred.

The method permits the determination of diazepam and metabolites at con-

centrations corresponding to 0.2 $\mu$g %, when 1 ml of plasma is extracted as described, the extract concentrated to 50 $\mu$l, and 5-$\mu$l aliquots are injected into the chromatograph.

*Characterization of diazepam and metabolites*

The identity of the four compounds, detected by GC in the samples analyzed, was confirmed by selective extraction of each substance from authentic plasma samples according to the partition characteristics shown by the corresponding reference compounds (Table I, Figs. 2 and 3). Fig. 9 represents superimposed chromatograms of an extract from 5 ml of plasma (Extract A) and the same extract following successive re-extractions with 1 *N* sodium hydroxide (Extract A1), 0.1 *N* hydrochloric acid (Extract A2), 1 *N* hydrochloric acid (Extract A3), and 6 *N* hydrochloric acid (Extract A4). Extract A represents the four compounds present in the toluene–*n*-heptane–isoamyl alcohol extract from the plasma sample (Sample No. 59). After re-extraction with 1 *N* sodium hydroxide (Extract A1), only traces of oxazepam are left in the organic phase, a 10 % decrease is noted in the peak area of demethyldiazepam, while diazepam and 3-hydroxydiazepam are still present at the original concentrations. GC of Extract A2 shows that demethyldiazepam has been completely extracted into 0.1 *N* hydrochloric acid together with 15 % of diazepam. Successive re-extraction of the organic phase with 1 *N* hydrochloric acid removed 78 % of diazepam and 25 % of 3-hydroxydiazepam (Extract A3). No traces of the four compounds are noted in Extract A4. Diazepam and metabolites, re-extracted from the four aqueous solutions (Fractions 1, 2, 3 and 4), were identified by GC and TLC.

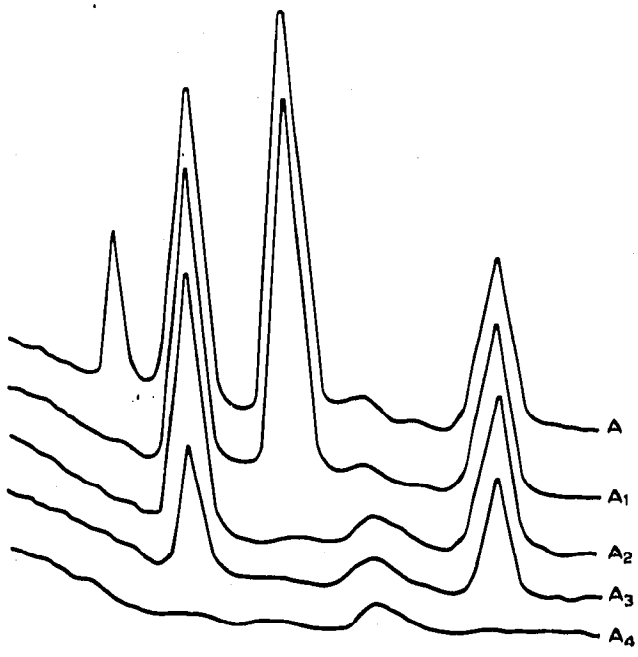


Fig. 9. Superimposed chromatograms of an extract from 5 ml of plasma (A) successively re-extracted with 1 *N* sodium hydroxide (A1), 0.1 *N* hydrochloric acid (A2), 1 *N* hydrochloric acid (A3) and 6 *N* hydrochloric acid (A4).

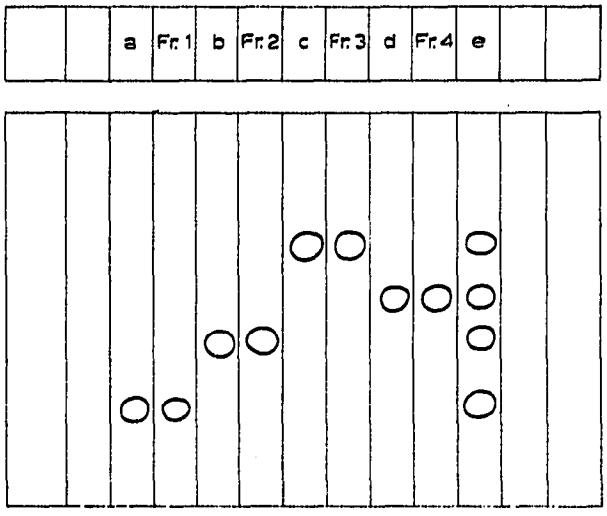


Fig. 10. TLC of extracts from 5 ml of plasma (Fractions 1, 2, 3 and 4) obtained by selective re-extraction of oxazepam from 1 *N* sodium hydroxide (Fr. 1), demethyldiazepam from 0.1 *N* hydrochloric acid (Fr. 2), diazepam from 1 *N* hydrochloric acid (Fr. 3) and 3-hydroxydiazepam from 6 *N* hydrochloric acid (Fr. 4) and extracts from synthetic plasma solutions containing oxazepam (a), demethyldiazepam (b), diazepam (c), 3-hydroxydiazepam (d) and a mixture of the four compounds (e).

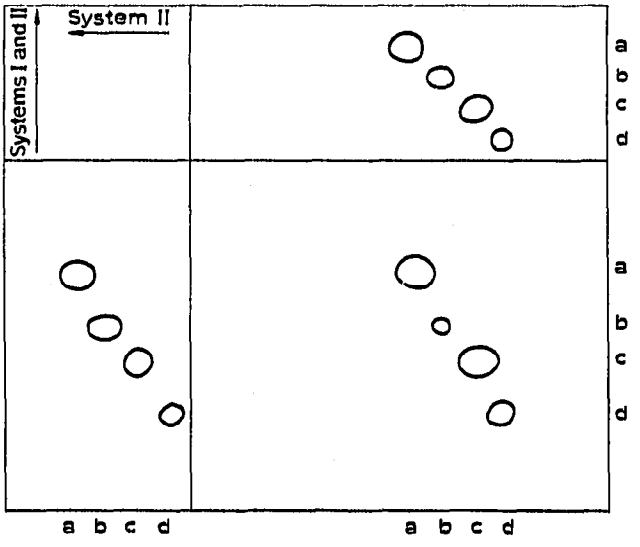


Fig. 11. Two-dimensional TLC of pooled plasma extracts corresponding to 20 ml of plasma (average daily dose 28 mg). a = Diazepam, b = 3-hydroxydiazepam, c = demethyldiazepam, d = oxazepam.

Since the compounds were isolated in each extract, Fractions 1, 2, 3 and 4 were analyzed by one-dimensional TLC. The two-dimensional technique was used for the chromatography of the pooled plasma extracts. Concentration of these extracts by evaporation of the solvent resulted in the presence on the plate of interfering fluorescent spots with the same $R_F$ value of diazepam. Re-extraction with 6 *N* hydrochloric

acid removed these unknown substances without appreciable loss of any metabolites. Separation of the four compounds in the two chromatographic systems used in this work has been accomplished by DE SILVA AND PUGLISI[33]. In our hands, a clearer separation of oxazepam from demethyldiazepam and of 3-hydroxydiazepam from diazepam was achieved when the two systems were used successively. Figs. 10 and 11 reproduce chromatographic plates containing the four fractions and the pooled extracts, respectively, together with extracts from synthetic plasma solutions. The $R_F$ values of diazepam and metabolites in System I, System II and after developing the plate successively in the two systems are given in Table VIII. After spraying the plate with 50 % sulfuric acid, the intensity of fluorescence in spots representing similar amounts of each substance was: oxazepam > diazepam > 3-hydroxydiazepam > demethyldiazepam.

TABLE VIII

$R_F$ × 100 VALUES OF DIAZEPAM AND METABOLITES

| | *System I* | *System II* | *System I and II*[a] |
|---|---|---|---|
| Diazepam | 44 | 34 | 60 |
| 3-Hydroxydiazepam | 27 | 25 | 44 |
| Demethyldiazepam | 18 | 16 | 31 |
| Oxazepam | 7 | 8 | 13 |

[a] The two systems were used in succession.

## CONCLUSION

In spite of the interpatient differences noted in each dose group, plasma levels of diazepam in the samples analyzed reflected the amount of drug administered. The average values reported in Table IX show the close correlation between the doses received and the plasma concentrations of diazepam and demethyldiazepam and, to a lesser extent, of the two minor metabolites. This evaluation includes the results from all the plasma samples obtained 90 min after administration of a given dose, independent of the length of treatment. Apparently, the large number of samples analyzed compensated for the variations in diazepam plasma levels noted in patients receiving identical dosages as well as for the increasing accumulation of drug in blood observed in some cases during long-term therapy.

The influence of the dosage interval on the plasma levels of diazepam and demethyldiazepam, and consequently on the ratio of their concentrations, was more apparent in the dose group corresponding to administration of 15 mg *pro die* with a dosage interval of 8 h. In this group, ninety minutes after ingestion of the morning dose, the ratio of diazepam to demethyldiazepam was > 1. Following administration of higher doses with the same dosage interval of 8 h, this ratio approached the value of 1 (10 mg *t.i.d.*) and then decreased to < 1 (20 mg *t.i.d.*). When the drug was administered with a dosage interval of 12 h, the ratio of diazepam to demethyldiazepam was consistently < 1, independent of the amount ingested before sampling (2, 5, 10 or 20-mg doses). The concentration of unmetabolized diazepam in 100 ml of plasma

TABLE IX

AVERAGE PLASMA LEVELS OF DIAZEPAM AND METABOLITES FOLLOWING ADMINISTRATION OF VARIOUS DOSES

| *Diazepam pro die* (*mg*) | *Dosage interval* (*h*) | *Number of samples* | *Average plasma levels* (*μg* %) | | | |
|---|---|---|---|---|---|---|
| | | | *Oxazepam* | *Diazepam* | *Demethyl-diazepam* | *3-Hydroxy-diazepam* |
| 4 | 12 | 3 | — | 10.5 | 13.0 | 0.5 |
| 5 | 24 | 6 | — | 13.6 | 12.3 | 1.6 |
| 6 | 8 | 4 | 0.4 | 17.7 | 20.8 | 0.7 |
| 10 | 12 | 16 | 1.1 | 23.1 | 26.9 | 1.9 |
| 15 | 8 | 10 | 2.4 | 33.0 | 25.5 | 3.2 |
| 20 | 12 | 3 | 4.7 | 31.0 | 43.0 | 3.2 |
| 30 | 8 | 3 | 2.6 | 49.3 | 49.1 | 1.8 |
| 40 | 12 | 3 | 9.8 | 86.4 | 105.9 | 5.0 |
| 60 | 8 | 4 | 18.5 | 154.0 | 217.5 | 14.9 |

ranged between 0.15 and 0.29 % of the total amount of drug received in a 24-h period. The higher figure in this evaluation corresponded to administration of a 2-mg dose repeated three times a day; when compared to the different doses administered, this dosage form yielded the maximal plasma concentration of free unmetabolized drug available for distribution into the tissues. It is of interest to note that higher average plasma levels of diazepam were attained after administration of a 5-mg dose repeated every 8 h than after a 10-mg dose repeated every 12 h, while the concentration of demethyldiazepam continued to reflect the different amount of drug received. In order to supplement this observation, it would be desirable to determine the minimum plasma level of diazepam between two consecutive doses and establish whether optimal concentrations are constantly maintained with either of the two dosages. Unfortunately, such studies, requiring a large number of blood samples from the same patient, were not possible during the course of this investigation.

A comparison of the results obtained in the present study from the analysis of plasma samples with data reported in the literature is limited to cases of patients receiving therapeutic doses of diazepam for two weeks or less. In these cases, compatible with variations in doses, dosage intervals and time of sampling, our results confirmed the findings of other authors[19–22]. A good correspondence was observed between the plasma levels reported by DE SILVA *et al.*[20] in a case concerning a patient receiving 150 mg of the drug *pro die* for a few weeks and the plasma concentrations determined in the present study in samples from patients given 60 mg *pro die* for several weeks. To our knowledge, the presence of free forms of the two hydroxylated metabolites in plasma had not been reported prior to this study, nor had the distribution of diazepam and metabolites between plasma and erythrocytes been investigated. Free diazepam had not been detected before in human urine. The uptake of diazepam and demethyldiazepam by the erythrocytes appeared to be the most apparent characteristic of samples from patients undergoing long-term therapy. During chronic administration of the drug, in the majority of cases, the plasma level of unmetabolized diazepam attained after a few weeks of medication remained constant throughout the following months of treatment. In these patients, an equilibrium had been reached between absorption of the drug, distribution to the various tissues and elimination.

In other patients, an increasing accumulation of the drug in blood resulted in plasma levels progressively higher throughout the course of treatment, indicating that the rate of elimination and/or the degree of binding of diazepam to tissues or plasma proteins had been minimal in respect to the absorption.

These observations suggest the necessity of determining plasma levels of diazepam and its active metabolites from the onset of the treatment. Dosage regimens can be developed for each patient, according to individual metabolic patterns, in order to compensate for the different ratios of absorption and metabolism. Minimum effective levels can be maintained during long-term therapy by periodically determining the concentration of the drug in plasma. This procedure, when correlated with clinical observations, will insure a continuous therapeutic response without increasing the risk of undesirable side-effects likely to occur at high blood concentration[22]. Before a complete picture of the metabolism of diazepam during chronic medication can be obtained, the degree of binding of the drug and its metabolites to plasma proteins and the entire excretion pattern need to be investigated. The influence of the protein-bound fraction on the disappearance of the drug from the blood, upon discontinuing the treatment, must also be clarified.

This investigation is currently in progress in this laboratory.

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