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DETERMINATION OF CHLORDIAZEPOXIDE PLASMA CONCENTRATIONS BY ELECTRON CAPTURE GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Chlordiazepoxide (Librium) plasma concentrations were determined in a group of eleven subjects by a gas chromatographic method involving the use of an electron capture detector.

The drug was extracted from 1 ml of alkalinized plasma in *n*-heptane containing 1.5% of isoamyl alcohol. Determination of plasma concentrations following administration of oral doses of 20 mg or more *pro die* was achieved by injecting directly into the chromatograph aliquots of this heptane extract. At lower doses, plasma levels were determined after re-extracting the drug from the heptane into small volumes of aqueous and organic solvents.

The two known metabolites of the drug, the lactam and N-desmethyl chlordiazepoxide, did not interfere with the analysis of the unmetabolized compound, nor did a number of other psychotropic drugs analyzed according to the procedure described.

The response of the electron capture detector to amounts of chlordiazepoxide as low as 0.5 ng permits the determination of the drug in 1-ml plasma samples after administration of a single 5-mg dose.

Chlordiazepoxide plasma concentrations encountered in the samples analyzed ranged from 17 μg-% after ingestion of a single 5-mg dose to 689 μg-% following administration of multiple 50-mg doses.

INTRODUCTION

Chlordiazepoxide**,7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide, is a psychotherapeutic agent widely used as a tranquilizer.

Colorimetric procedures for the analysis of the drug in biological material have been reported by RANDALL[1] and by BÄUMLER AND RIPPENSTEIN[2]. Studies of the metabolism of the compound[3–5] have included the determination of the unmetabolized drug and the two known metabolites, the lactam and N-desmethyl chlordiazepoxide.

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A spectrofluorometric method for the determination of chlordiazepoxide and the lactam compound in plasma has been developed by KOECHLIN AND D'ARCONTE[3]. This procedure was later modified by SCHWARTZ AND POSTMA[5] in order to include in the analysis the determination of the N-desmethyl metabolite; plasma levels of the unmetabolized drug were calculated by difference, after determining by a separate analysis the concentration of the N-desmethyl compound. The sensitivity of these methods does not permit the determination of plasma levels following administration of low therapeutic doses.

A gas chromatographic (GC) method developed by DE SILVA and co-workers[6,7] for the analysis of diazepam and its metabolites in plasma is also applicable, according to the authors, to the determination of unmetabolized chlordiazepoxide. The procedure requires conversion of the benzodiazepine derivatives to their respective benzophenones and necessitates separation by thin-layer chromatography (TLC) as the analysis is not specific for chlordiazepoxide. A similar procedure, also involving separation of the compounds by TLC, has been proposed by DE SILVA[8] for the determination of the unmetabolized drug and the two metabolites.

Procedures for the analysis of the benzodiazepine derivatives by GC using a flame ionization detector have been published by several authors[9-11]. These methods also lack the sensitivity necessary for the detection of the drug at low plasma concentrations.

Unpublished studies from this laboratory had shown that several compounds of the benzodiazepine series can be detected at nanogram levels and in their intact forms by GC utilizing the sensitivity of the electron capture detector to halogenated compounds. While this work was in preparation, a similar procedure for the determination of medazepam and its metabolites was published by DE SILVA AND PUGLISI[12].

The present paper describes a sensitive GC method for the determination of chlordiazepoxide in plasma. The compound is chromatographed in its intact form, is clearly separated from its metabolites and can be determined in 1-ml plasma samples after ingestion of the lowest therapeutic dose. The method was applied to the determination of chlordiazepoxide plasma levels following administration of oral doses ranging between 5 and 150 mg *pro die*.

EXPERIMENTAL

Reagents and reference solutions

Only analytical grade reagents were used. Stock solutions were prepared by dissolving 10 mg of chlordiazepoxide hydrochloride in 0.20–0.30 ml of distilled water and diluting the solution to 100 ml with absolute ethyl alcohol. Aliquots of this solution were used to prepare working standards. After evaporation of the ethyl alcohol under nitrogen, the residue was dissolved either in distilled water or in *n*-heptane containing 1.5% of isoamyl alcohol. The aqueous solutions were prepared at concentrations ranging between 0.03–10 μg/ml; standard solutions in *n*-heptane-isoamyl alcohol contained 0.2–1 ng/μl. Aqueous standard solutions were used only on the day of preparation; solutions of the drug in ethyl alcohol or in *n*-heptane–isoamyl alcohol were stable under refrigeration for several weeks.

Material

Blood samples collected in EDTA tubes were obtained from hospitalized patients 75–130 min after oral administration of morning doses of 10–50 mg. These patients were receiving chlordiazepoxide alone or together with other drugs for periods ranging between 1 and 39 weeks. Blood samples from four volunteers were collected 120 min after ingestion of a single dose of 5 or 10 mg. All the samples were centrifuged as soon as delivered to the laboratory. The plasma was separated and kept frozen until the analysis could be performed.

Extraction procedure

1 ml of plasma was mixed with 1 ml of distilled water; the mixture was made alkaline (pH 9–10) by addition of 0.1 *N* sodium hydroxide and extracted with 5 ml of *n*-heptane–isoamyl alcohol by mechanically shaking the flask for 15 min. After centrifugation, 4.5 ml of the organic phase were transferred to a 5-ml centrifuge tube; 2–9 μl of this extract were injected into the chromatograph.

At low plasma levels, concentration of the drug in the extract was achieved by back-extraction in dilute acid: 1 ml of 0.1 *N* hydrochloric acid was added to the heptane extract; the test tube was shaken by hand for 10 min and centrifuged. The acidic aqueous solution was made alkaline (pH 9–10) by addition of 1 *N* sodium hydroxide and re-extracted with 0.3–0.5 ml of the *n*-heptane–isoamyl alcohol mixture. Following centrifugation, the aqueous layer was removed by aspiration and the heptane phase was collected in the tapered portion of the tube. Aliquots of this extract (3–9 μl) were analyzed by GC. 1-ml aliquots of the aqueous standard solutions containing 0.03–10 μg of chlordiazepoxide were added to 1 ml of water or drug-free plasma and extracted according to the procedure described above.

Extracts for TLC studies were prepared by pooling the heptane extracts from three 1-ml plasma samples at high concentration and by re-extracting the drug in 3 ml of 0.1 *N* hydrochloric acid. The acidic aqueous solution, made alkaline as described above, was re-extracted with 0.3 ml of *n*-heptane–isoamyl alcohol. Synthetic plasma solutions containing comparable amounts of chlordiazepoxide, lactam and N-desmethyl metabolites were extracted according to the same procedure.

Gas chromatography

The analyses were performed in a Beckman GC-4 gas chromatograph equipped with an electron capture detector and a 10-in. potentiometric recorder. Conditions for GC were as follows: Column: glass, 4 ft. long, $\frac{1}{4}$ in. O.D., 2 mm I.D.; 2% OV-17 on Chromosorb W HP, 80–100 mesh. Temperatures: inlet lines 320°; column oven 275°; detector and detector lines 330°. Flow rates: helium carrier gas 80 ml/min; helium discharge gas 100 ml/min; carbon dioxide 3 ml/min. Detector electrical settings: polarizing voltage 620 duodial, bias voltage 120 duodial, source current 7 mA. Electrometer settings: range 100, attenuation 1024, suppression current off (background current equivalent to 80% full-scale); suppression current on, range 100, attenuation settings from 1024 to 128.

Thin-layer chromatography

Merck Silica Gel F_{254} plates, 20 × 20 cm, were obtained from Brinkmann Instruments. The plates containing extracts and reference solutions were overrun for

15 min, removed from the tank, air-dried and observed under short-wave UV light. The chromatographic systems were: System I: methanol–12 *N* ammonium hydroxide (100:1); System II: *n*-heptane–chloroform–absolute methanol (70:30:10); System III: ethyl acetate–*n*-heptane–absolute ethanol (70:30:10).

RESULTS AND DISCUSSION

Compounds of the benzodiazepine series have been extracted from biological material in ether[6,9,11–13], chloroform[10,14], ethylene dichloride[15], ethyl acetate[16,17], and ethyl alcohol–ether[18]. Preliminary experiments in this laboratory showed that extraction with these solvents necessitates isolation of the drug by means of re-extraction, tedious washings of the extract and concentration of the final solvent to small volumes. Chromatographic traces obtained from such extracts by using an electron capture detector were still cluttered with extraneous peaks. Although no interfering peaks were noted in the retention area of chlordiazepoxide, injection of aliquots of these

TABLE I

RECOVERIES OF VARIOUS AMOUNTS OF CHLORDIAZEPOXIDE FROM 1-ml PLASMA SAMPLES

Chlordiazepoxide added (μg)	*Number of determinations*	*Recovery (% ± S.D.)*
0.03	5	87.4 ± 5.1
0.2	5	91.3 ± 3.5
0.5	5	90.6 ± 3.2
1	10	89.8 ± 1.5
2.5	10	92.3 ± 2.1
5	10	93.2 ± 2.0
7.5	5	91.1 ± 3.2
10	5	90.2 ± 3.9

extracts resulted in contamination of the detector with a loss of sensitivity and consequently a noticeable decrease of the peak area of the compound. Extraction with the less polar solvent used ensured satisfactory recovery of the substance and at the same time resulted in clean extracts which could be injected directly into the chromatograph.

Recoveries of amounts of chlordiazepoxide ranging between 0.03 and 10 μg dissolved in one volume of buffer solutions at pH above 7.4 and extracted with five volumes of *n*-heptane–isoamyl alcohol were virtually complete. Recoveries of the substance from plasma solutions, under the same experimental conditions, varied from 97 to 99%. Preliminary studies of the partition characteristics of chlordiazepoxide, lactam and N-desmethyl compounds between heptane and buffer solutions at pH above 7.4 showed that extraction in the range 9–10 is more selective for the unmetabolized drug with respect to the two metabolites. Recoveries of various amounts of chlordiazepoxide added to 1 ml drug-free plasma samples and taken through the entire procedure are shown in Table I.

The amount of substance extracted in each step was determined by GC of the organic solvent and by spectrofluorometric analysis of the corresponding aqueous phase according to the procedure developed by KOECHLIN AND D'ARCONTE[3].

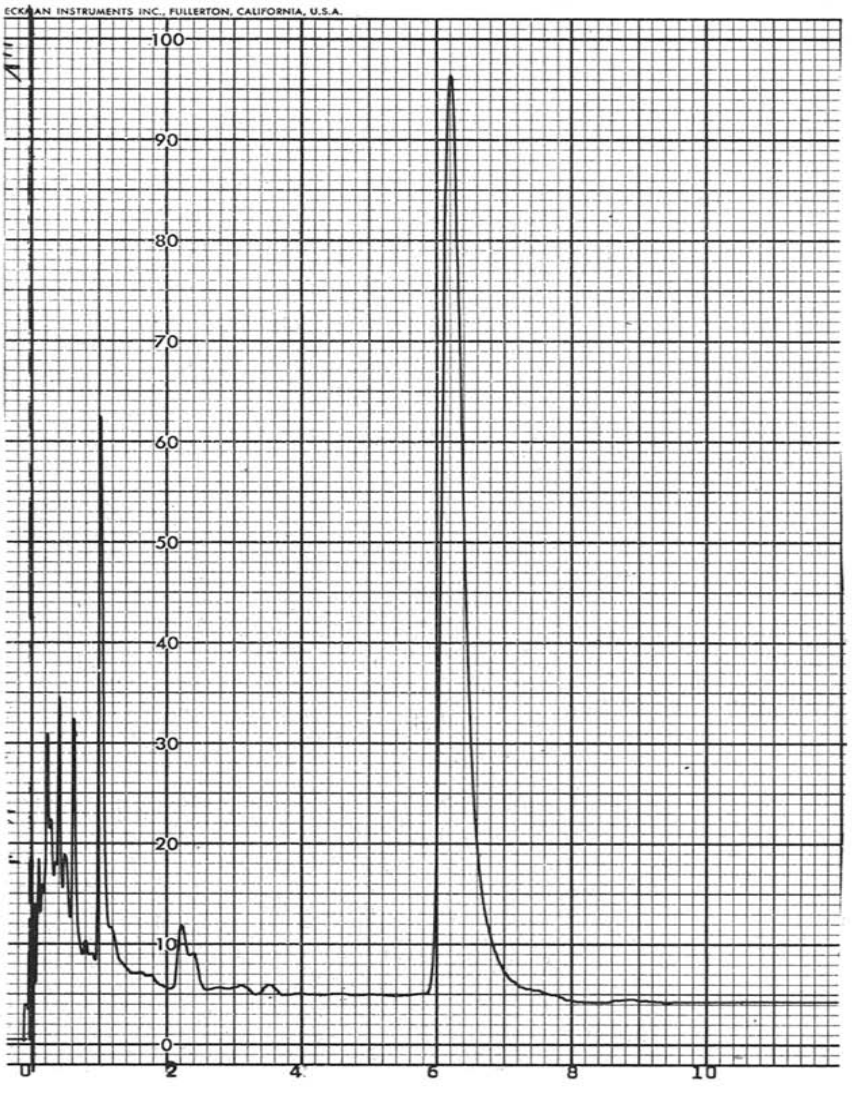

Fig. 1. GLC traces of a standard solution of chlordiazepoxide hydrochloride in *n*-heptane–isoamyl alcohol. Injected: 2.5 μl containing 2.5 ng of substance.

Under the chromatographic conditions described, the response of the detector to injection of nanogram amounts of chlordiazepoxide was very satisfactory. At attenuation 512, injections of 3 ng of substance dissolved in 3 μl of *n*-heptane–isoamyl alcohol gave a full-scale pen response. The minimum amount determinable with accuracy corresponded to 0.5 ng. The sensitivity of the method was established experimentally by adding 1 ml of a 3 μg-% aqueous solution of the drug to 1 ml of drug-free plasma and extracting the sample according to the entire procedure. In the final step, the volume of the heptane was 0.3 ml. Aliquots of this extract corresponding to 0.5 ng of chlordiazepoxide, analyzed at the lower attentuation setting used during the experiments, gave a well-defined peak equivalent to 40% full-scale pen response.

The use of an internal standard was considered unnecessary. Because of the short retention time of the substance, standardization of the instrument was easily accomplished. Repeated injections of aliquots of standard heptane solutions at the

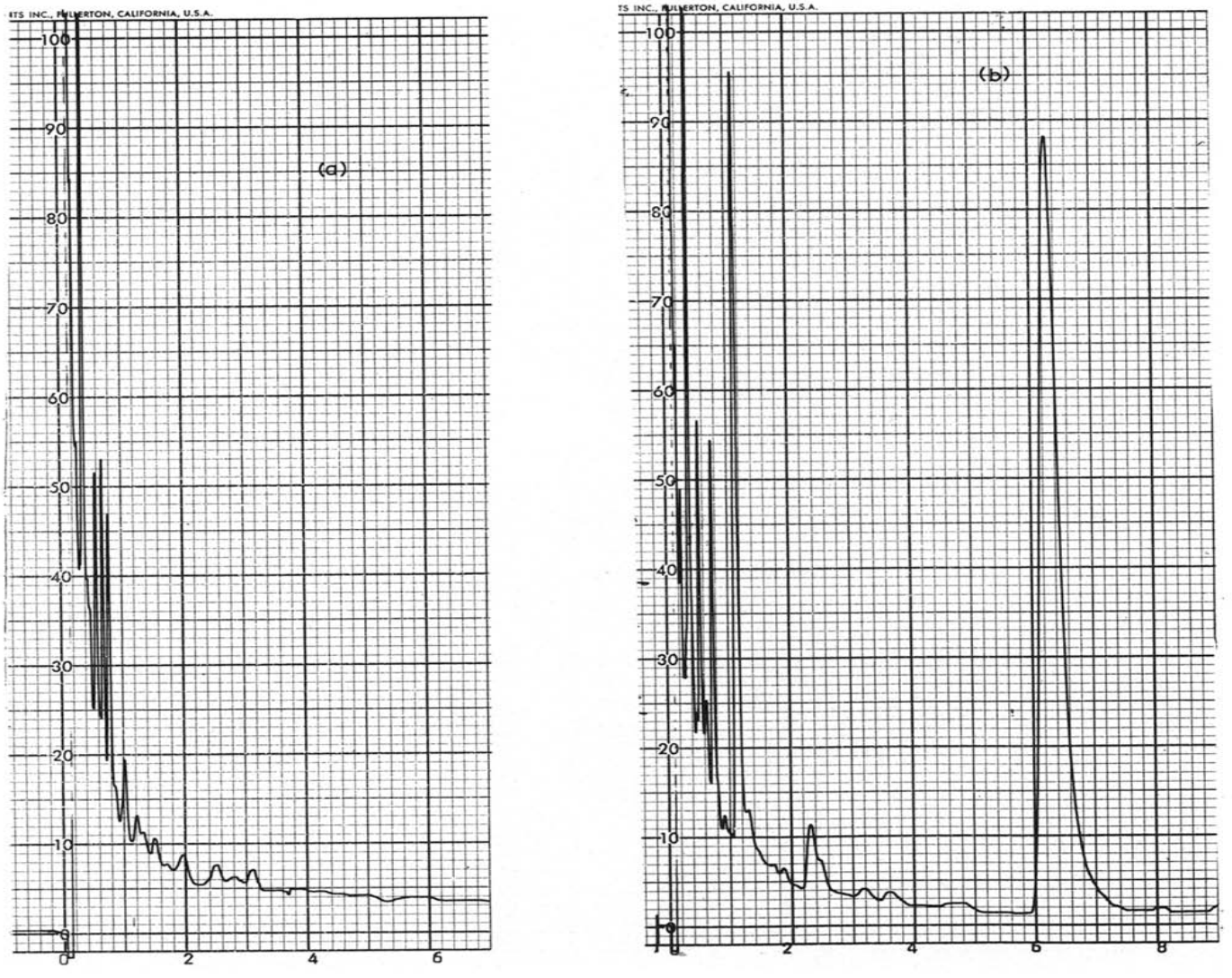

Fig. 2. GLC traces of heptane extracts from (a) 1 ml of drug-free plasma and (b) 1 ml of the same sample to which 5 μg of chlordiazepoxide had been added. Injected: 2.5 μl corresponding to 2.5 ng. Recovery 97.5%.

same concentration showed excellent reproducibility. The response to injections of various amounts of chlordiazepoxide extracted in *n*-heptane–isoamyl alcohol from synthetic plasma solutions was linear over the range 0.5–50 ng. Chlordiazepoxide concentrations in the samples analyzed were calculated by measurement of the peak areas obtained from the unknown and from synthetic plasma solutions extracted according to the procedure.

The retention time of chlordiazepoxide, under the chromatographic conditions outlined, was *ca.* 6 min (Fig. 1). GLC traces of extracts from several drug-free plasma samples showed no peaks emerging after 1 min. Extracts of plasma samples from patients receiving a number of other psychotropic agents were free of interfering peaks in the retention area of chlordiazepoxide. Fig. 2 represents chromatograms of extracts from 1 ml of drug-free plasma and from 1 ml of the same sample to which 5 μg of chlordiazepoxide had been added.

TABLE II

DATA OF CASES STUDIED

Sample No.	Chlordiazepoxide pro die (mg)	Length of therapy (weeks)	Other drugs administered	Time of collection after morning doses (min)	Plasma levels (μg-%)
1	5 (single dose)	—	—	120	17
2	5 (single dose)	—	—	120	19
3	10 (single dose)	—	—	120	39
4	10 (single dose)	—	—	120	42
5	10 b.i.d.	2	Diazepam, benztropine	90	89
6	25 t.i.d.	39	Theophylline, ephedrine, phenobarbital, methyprylon	80	324
7	25 t.i.d.	11	Thioridazine, diphenylhydantoin	75	372
8	25 q.i.d.	7	—	110	201
9	25 q.i.d.	6	—	130	411
10	50 t.i.d.	1	Imipramin	120	689
11*	10 t.i.d.	1	—	—	negative

* Sample 11 was collected 2 weeks after chlordiazepoxide treatment had been discontinued.

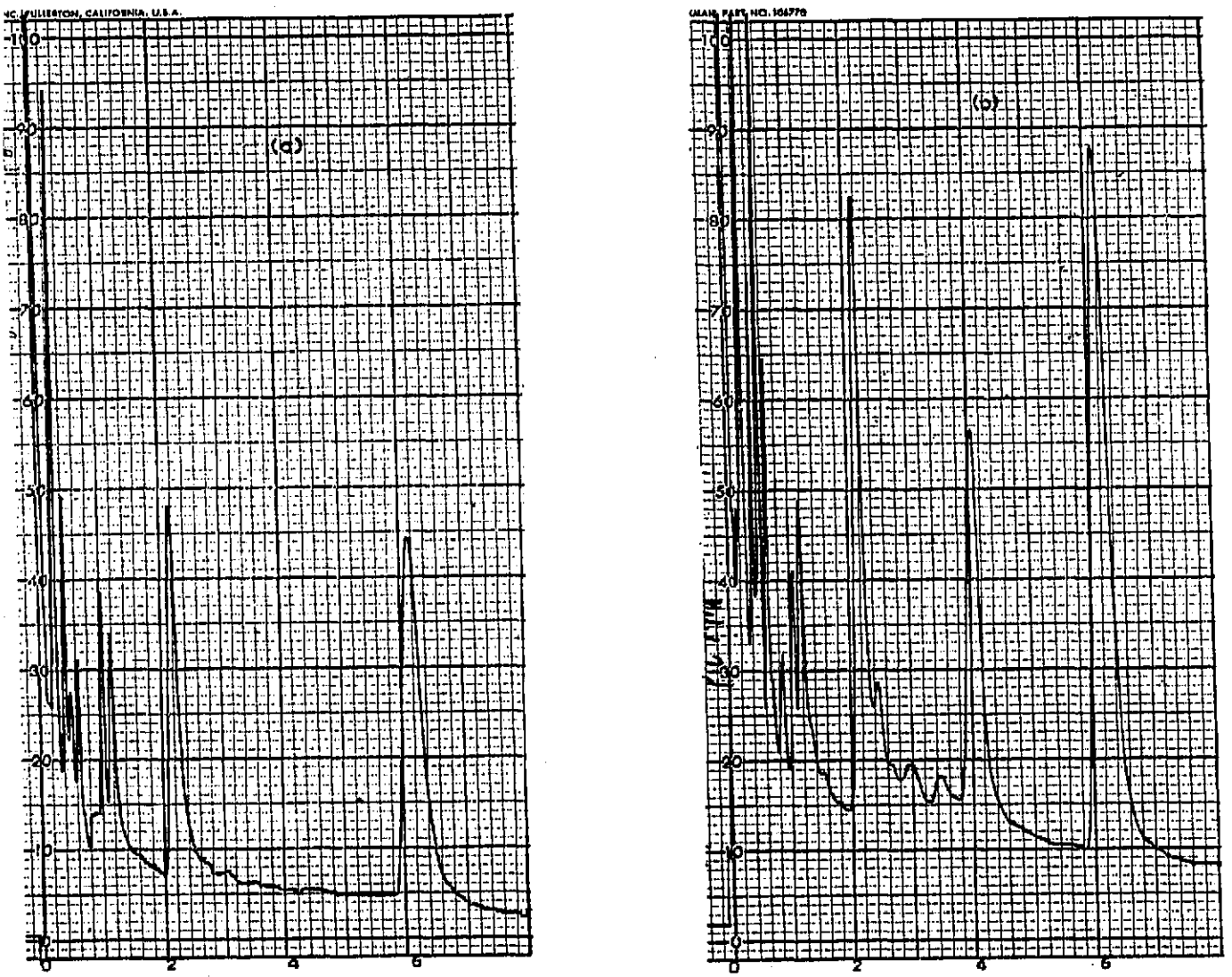

Fig. 3. GLC traces of heptane extracts from 1-ml plasma samples following administration of (a) a single 5-mg dose and (b) multiple 25-mg doses. Chlordiazepoxide was the only drug

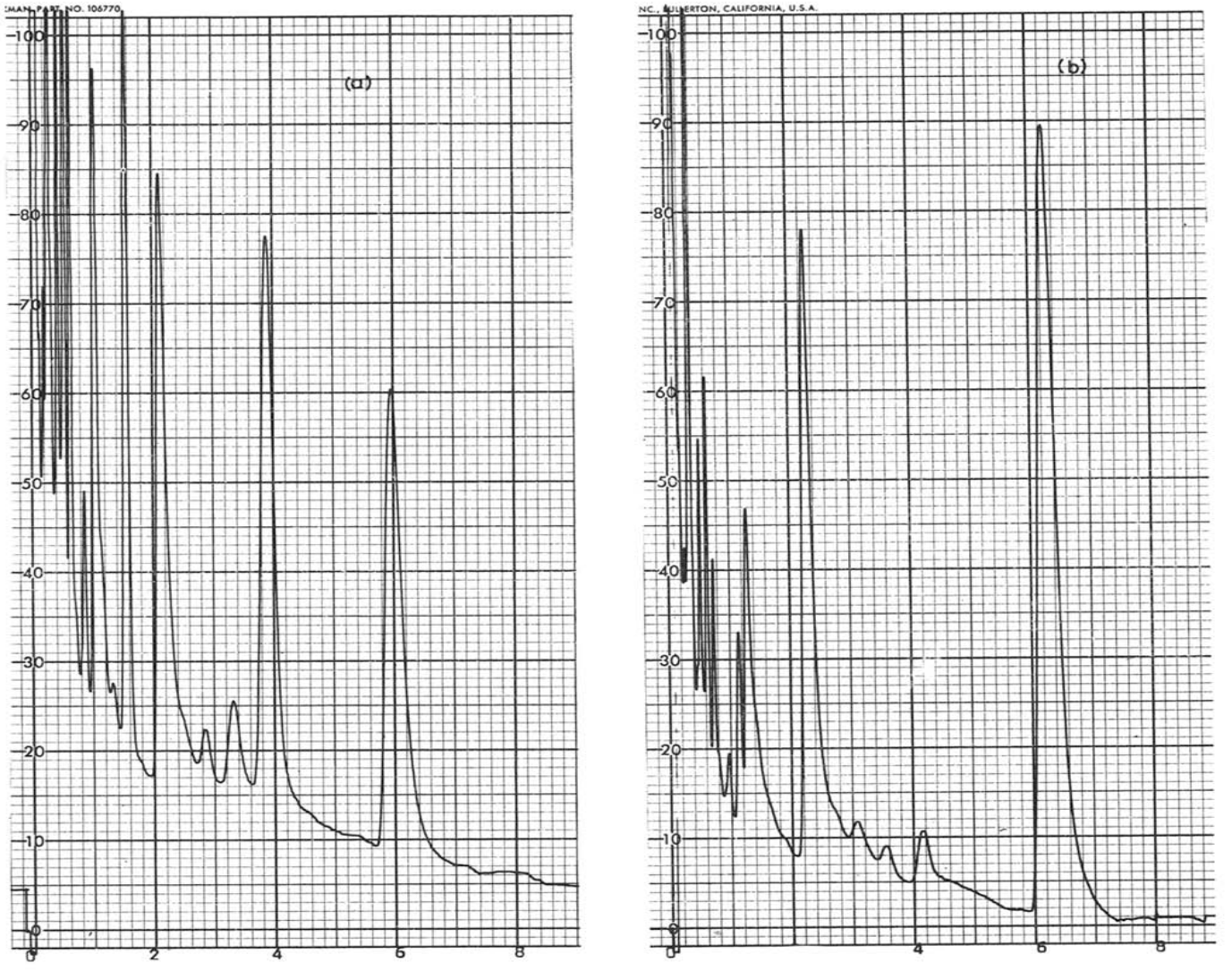

Fig. 4. GLC traces of heptane extracts from plasma samples after administration of chlordiazepoxide together with (a) diazepam and (b) imipramin.

The procedure developed was applied to plasma samples obtained from four volunteers after ingestion of a single dose of 5 or 10 mg, and from a group of patients receiving multiple oral doses of chlordiazepoxide ranging between 10 and 50 mg (20–150 mg *pro die*). As mentioned above, these patients were maintained in chlordiazepoxide therapy for periods varying from 1 to 39 weeks. One sample was collected two weeks after chlordiazepoxide therapy had been discontinued. Plasma concentrations determined in the samples analyzed, dosages, duration of the treatment with the drug, other drugs administered and time of collection of the sample after the morning dose are given in Table II.

Typical chromatograms of extracts of plasma samples after administration of a single 5-mg dose and multiple 25-mg doses are shown in Fig. 3; chlordiazepoxide was the only drug administered. Fig. 4 presents chromatograms of extracts of plasma samples from patients receiving diazepam or imipramin together with chlordiazepoxide.

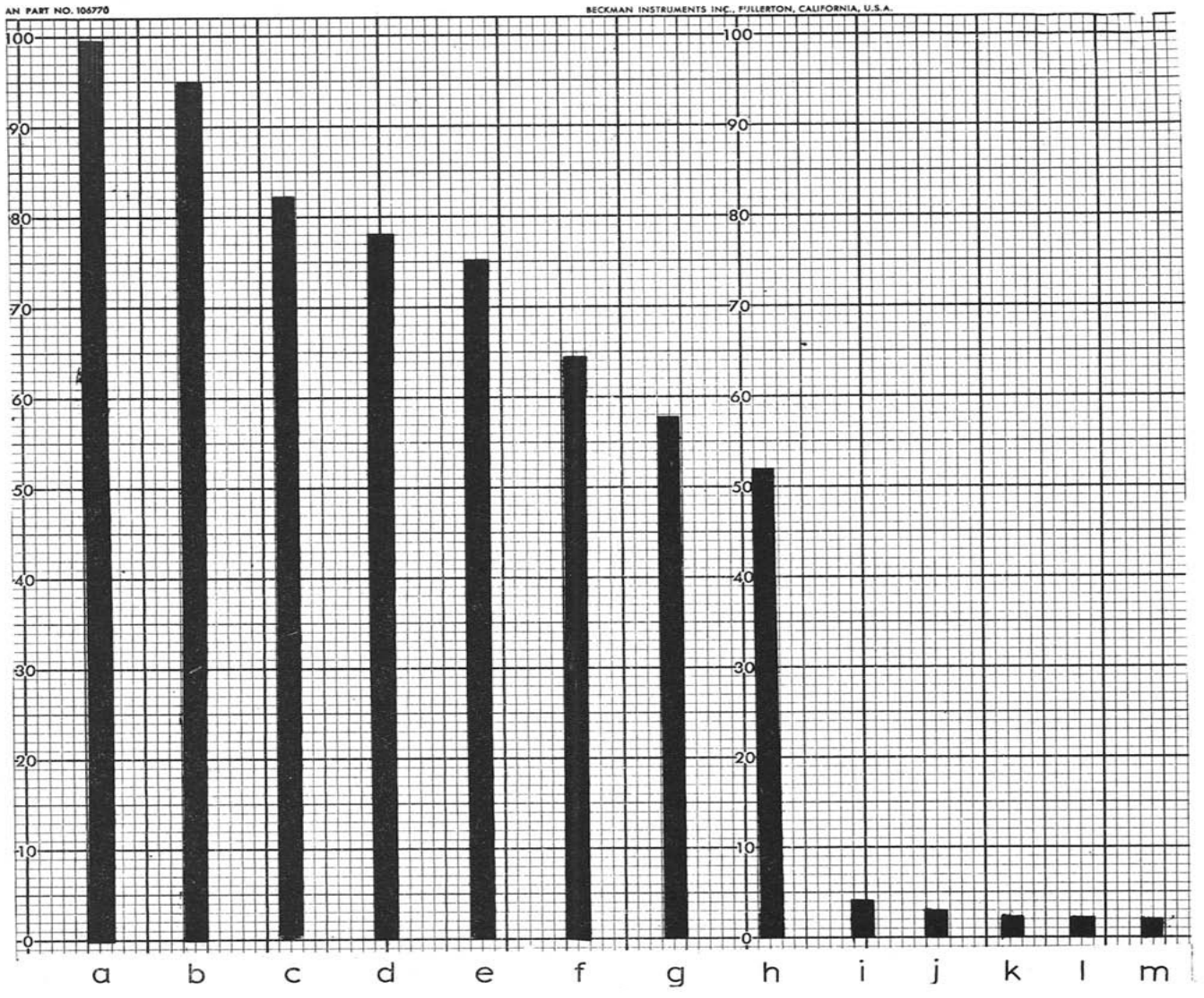

Fig. 5. Responses to injections of 5 ng of chlordiazepoxide dissolved in 5 μl of various solvents expressed in peak heights. a = toluene containing 15% of isoamyl alcohol; b = *n*-heptane containing 1.5% of isoamyl alcohol; c = toluene; d = benzene; e = *n*-heptane; f = hexane; g = hexane containing 20% of acetone; h = acetone; i = absolute ethyl alcohol; j = acetonitrile; k = diethyl ether; l = ethyl acetate; m = absolute methyl alcohol.

The results given in Table II were obtained by using the entire extraction procedure described. In all cases, aliquots of the extracts resulting from the one-step extraction with 5 ml of *n*-heptane-isoamyl alcohol were analyzed by GC; observation of the resulting chromatograms permitted the determination of plasma levels in samples Nos. 5–10. The extraction procedure was continued for all samples; the volume of the final heptane mixture varied between 0.5 and 3 ml. By injecting aliquots of these extracts at attenuation settings ranging from 128 to 1024, the determination of plasma levels was achieved in all samples except No. 11. Chlordiazepoxide plasma concentrations for samples 5–10 as determined by either extraction procedure were identical.

The necessity of re-extracting the drug in acid and then in small volumes of

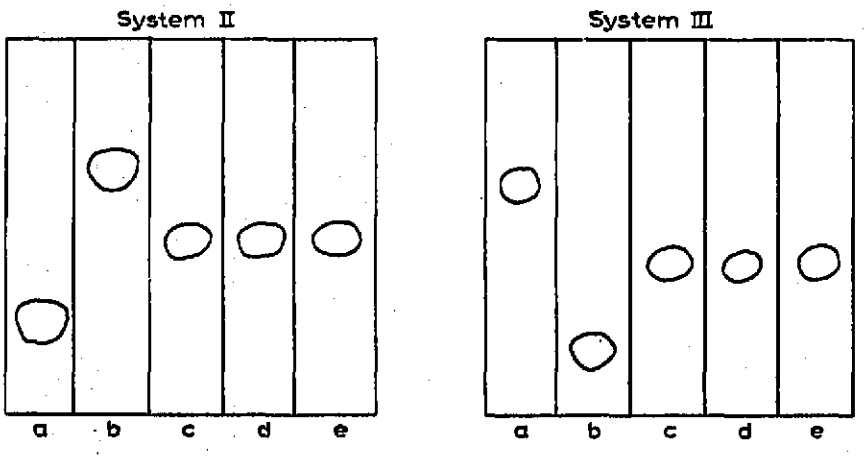

Fig. 6. TLC of chlordiazepoxide and metabolites in two systems. Standard heptane solutions of (a) lactam compound; (b) N-desmethyl chlordiazepoxide; (c) chlordiazepoxide; and heptane extracts of (d) plasma samples from patients receiving chlordiazepoxide; and (e) synthetic plasma solutions to which the three compounds have been added.

heptane when analyzing samples at low concentrations was dictated by the susceptibility of the electron capture detector to contaminations. Attempts to concentrate the drug in the heptane extract by evaporation of the solvent resulted in contamination of the detector and loss of sensitivity to such an extent that no presence of chlordiazepoxide could be detected in samples Nos. 1–4. The chromatography of such extracts was not improved by re-dissolving the residues after evaporation of the heptane in a number of other solvents. In addition, the response to injections of the same amount of chlordiazepoxide depended considerably upon the solvent used. With the exception of toluene containing 15% of isoamyl alcohol, a decrease of the peak area of the compound was noted when the same amount of substance dissolved in several other solvents was chromatographed under the same chromatographic conditions. The data presented in Fig. 5 could be obtained either by diluting 0.10-ml aliquots of an

TABLE III

R_F VALUES (× 100) OF CHLORDIAZEPOXIDE AND METABOLITES

Compound	*System I*	*System II*	*System III*
Chlordiazepoxide	76	45	38
Lactam compound	71	31	54
N-Desmethyl chlordiazepoxide	59	58	23

ethanolic solution of the drug to 5 ml with the solvent, or by re-dissolving in the various solvents the residues obtained by evaporation of heptane extracts from authentic or synthetic plasma solutions.

Characterization of chlordiazepoxide in the extracts was confirmed by TLC (Fig. 6). Extracts from plasma samples prepared for TLC as described above were chromatographed in three different systems together with standard heptane solutions of chlordiazepoxide, lactam and N-desmethyl metabolites and with extracts of the

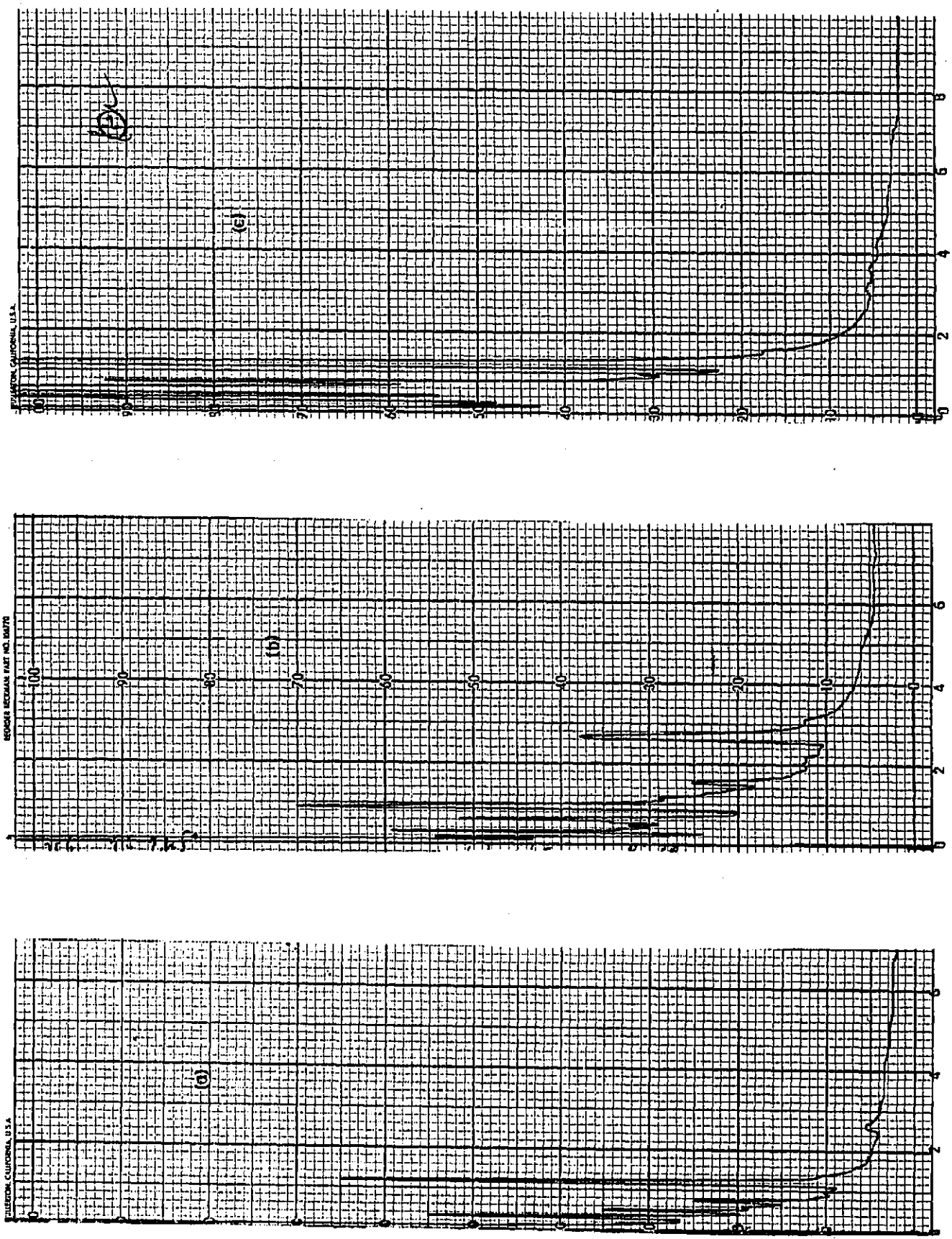

Fig. 7. GLC traces of standard heptane solutions of (a) lactam compound; (b) N-desmethyl chlordiazepoxide; and (c) traces of a heptane extract of the two substances from synthetic plasma solutions.

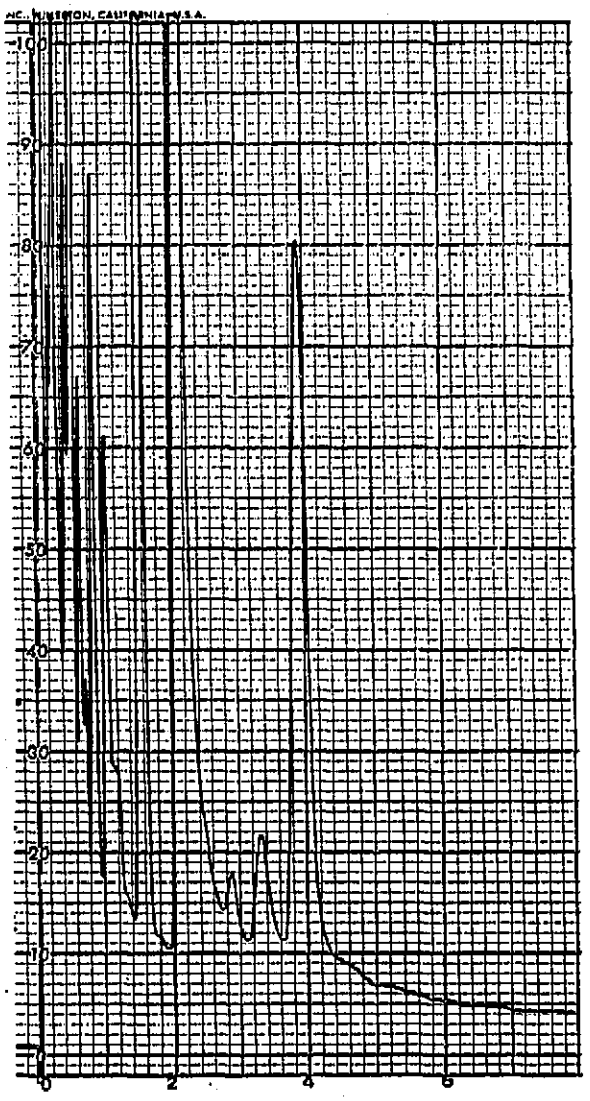

Fig. 8. GLC traces of a plasma extract from a patient receiving diazepam. Column temperature 275°.

three compounds added to drug-free plasma in comparable amounts. The R_F values of chlordiazepoxide and the two metabolites are given in Table III.

The minimum amount of chlordiazepoxide detectable on the plate corresponded to 2.5 μg. Extracts from authentic plasma samples and from drug-free plasma samples, to which the three compounds had been added, showed the presence of only unmetabolized chlordiazepoxide.

In order to ascertain that structurally related compounds do not interfere with the determination of chlordiazepoxide, several benzodiazepine derivatives were analyzed according to the procedure described. Fig. 7 shows chromatographic traces of heptane solutions of the two metabolites, lactam and N-desmethyl chlordiazepoxide, together with heptane extracts of the two substances recovered from plasma solutions buffered at pH 9–10. A chromatogram of the extract of a plasma sample from a patient receiving only diazepam (30 mg *pro die*) is shown in Fig. 8. Fig. 9(a,b,c)

represents chromatographic traces of extracts of plasma samples from patients receiving diazepam, or chlordiazepoxide, or the two drugs simultaneously. In order to separate the various compounds the column temperature was programmed from 250° to 265°; under these conditions, retention time of chlordiazepoxide was *ca.* 14 min. Peaks A and B in Fig. 9(a) and (c) were identified as diazepam and N-desmethyl diazepam, respectively, by comparison with extracts of reference solutions of the two substances. Peak C in Fig. 9(a), (b) and (c) represents an unknown compound. Its retention time at 275° was *ca.* 4 min (Fig. 3(b) and Fig. 4(a)). This peak was noted in all the extracts of plasma samples from patients receiving multiple doses of chlordiazepoxide, either alone or together with other drugs, as well as in extracts of plasma samples following administration of diazepam (Fig. 8). It was not present in extracts of plasma samples after ingestion of single 5- or 10-mg doses, or in extracts from drug-free plasma or from samples containing a number of other drugs. With the excep-

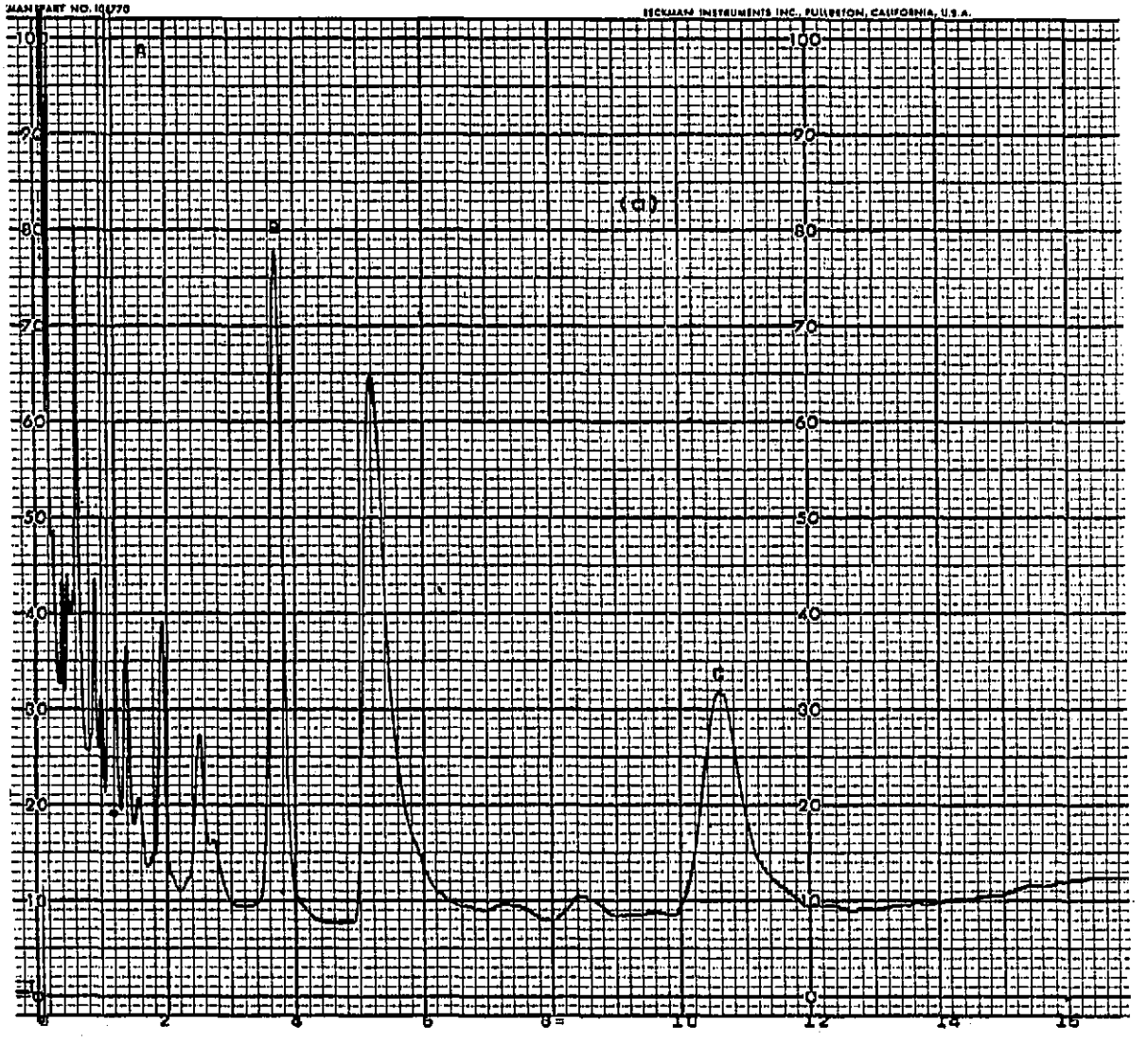

Fig. 9(a). GLC traces of a plasma extract from a patient receiving diazepam. Initial column temperature 250°, isothermal for 8 min, then programmed for an increase of 15° in 8 min. Peak A = diazepam; peak B = desmethyl diazepam; peak C = unidentified compound.

tion of sample No. 8, this peak was relatively small for all the chlordiazepoxide plasma samples mentioned; chromatographic traces of extracts from sample No. 8 showed for this unknown compound a peak of significant magnitude (Fig. 3(b)). It is interesting to note that the plasma concentration of chlordiazepoxide in this patient is considerably lower than expected after administration of 100 mg of the drug *pro die*. It is conceivable that this compound represents an unknown metabolite of chlordiazepoxide.

Within the limits of the number of samples analyzed and with the exception of the sample mentioned above, the plasma concentrations determined by the method developed are in close accordance with the doses received. Apparently, long-term therapy does not influence noticeably the concentration of the unmetabolized drug in plasma (see sample No. 6). In accordance with this observation are the results of the analyses performed on the plasma sample obtained two weeks after chlordiazepoxide

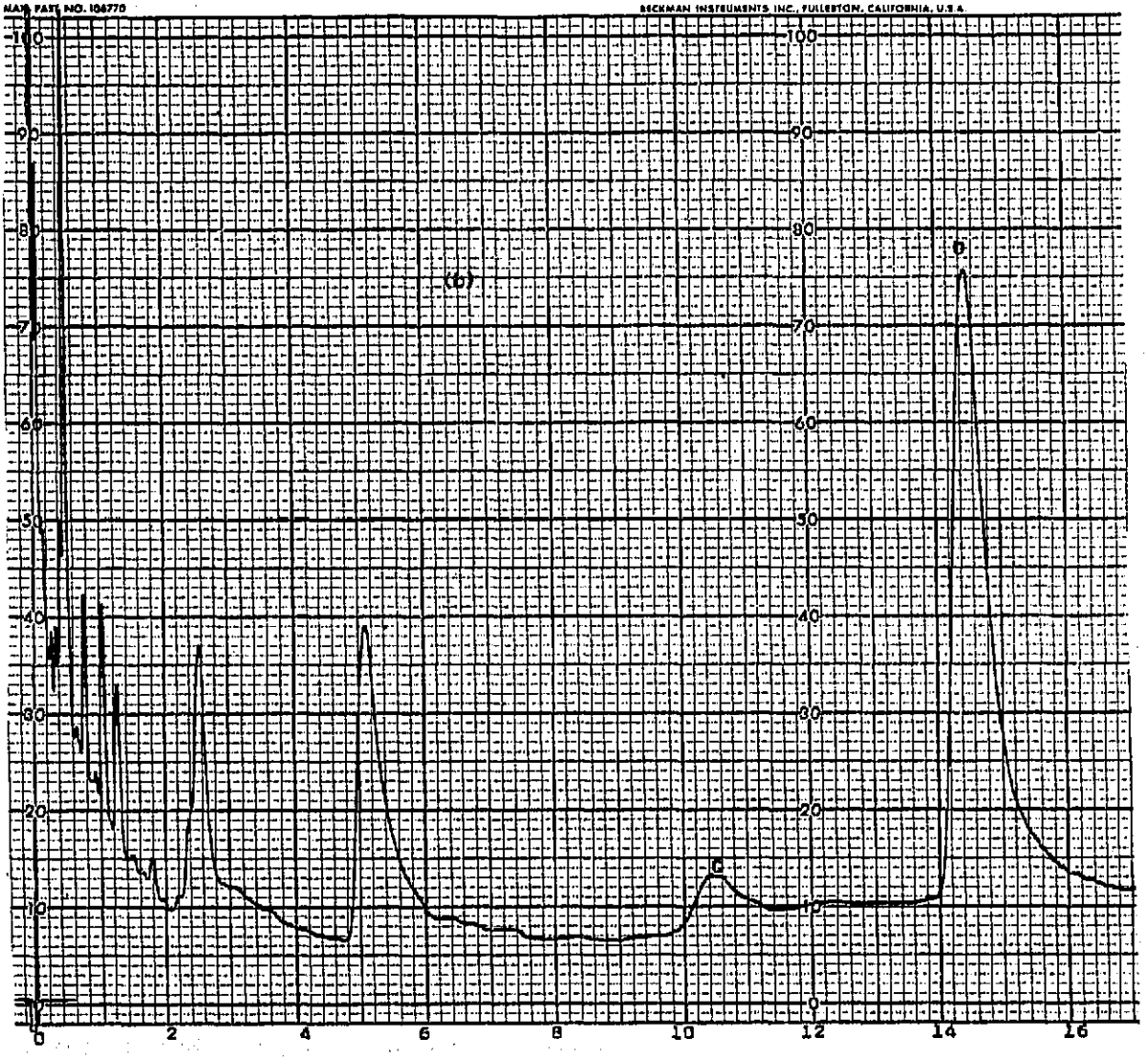

Fig. 9(b). GLC traces of a plasma extract from a patient receiving chlordiazepoxide. Column temperature as in Fig. 9(a). Peak C = unidentified compound; peak C = chlordiazepoxide.

therapy had been discontinued; aliquots of the extract from 7.5 ml of this sample, chromatographed at the maximum sensitivity permitted by the instrumental parameters, showed no presence of chlordiazepoxide.

Plasma concentrations determined in the present study, as shown in Table II, are rather lower than those reported in the literature[1,5,19] for samples analyzed by other techniques. This could be attributed to the greater specificity of the GC method.

The results obtained demonstrate the validity of the method for the determination of chlordiazepoxide in plasma. The one-step extraction in *n*-heptane–isoamyl alcohol provides a simple and specific method for the analysis of plasma samples at concentrations expected after administration of therapeutic doses most frequently encountered or at toxicological levels; when the entire extraction procedure is applied, the method is sufficiently sensitive to determine the concentration of the drug in 1-ml plasma samples following ingestion of a single 5-mg dose.

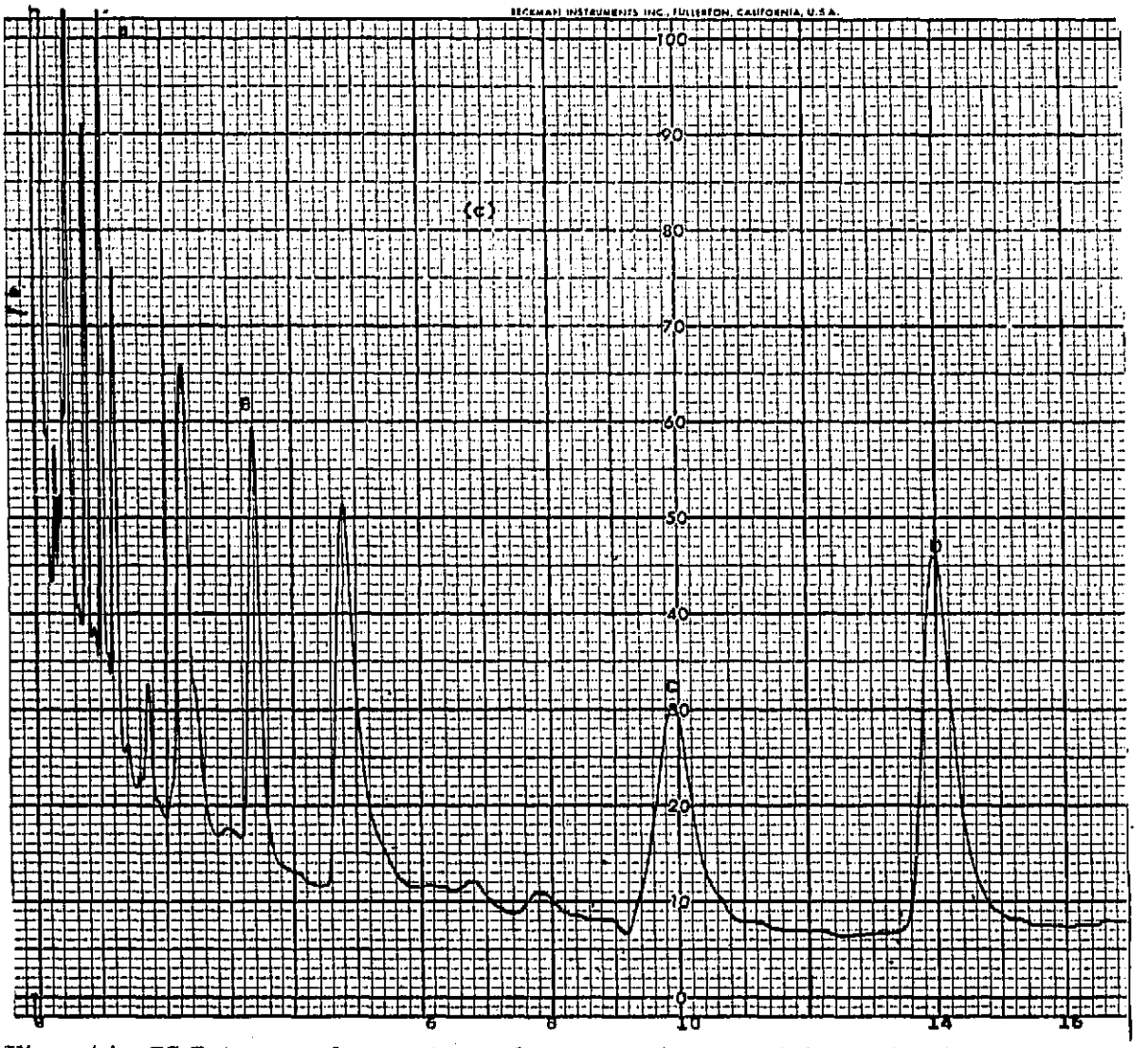

Fig. 9(c). GLC traces of an extract from a patient receiving chlordiazepoxide together with diazepam. Column temperature as in Fig. 9(a). Peak A = diazepam; peak B = desmethyl diazepam; peak C = unidentified compound; peak D = chlordiazepoxide.

Preliminary experiments in this laboratory have shown that the GC method described has potential applicability to extensive studies of the metabolism of the drug.

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