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GLC Determination of 7-Trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in Plasma

LEONARD C. BAILEY and ARVIN P. SHROFF▲

Abstract □ A GLC assay method is described for the determination of 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in the plasma or serum of dogs, rabbits, or humans. After extraction with hexane from acidified plasma or serum, the compound is analyzed by GLC using a flame-ionization detector. Quantitation is accomplished using a standard curve, which was found to be linear over the 0.002–3.5-mcg. range. The analysis is sensitive to amounts of the drug above 0.1 mcg./ml. Recovery of the title compound added to human plasma averaged 98.7%, with a variation of approximately 7.1% (*RSD*). Plasma data are presented to demonstrate the utility of the method.

Keyphrases □ 7-Trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione—GLC analysis in plasma □ 1,5-Benzodiazepine compounds—GLC analysis of 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in plasma □ GLC—analysis, 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione in plasma

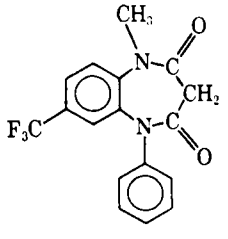
A recently synthesized member of the benzodiazepine class of compounds, 7-trifluoromethyl-1-methyl-5-phenyl-1*H*-1,5-benzodiazepine-2,4-(3*H*,5*H*)-dione[1] (I), is currently being evaluated for its activity as a minor tranquilizer. It differs significantly from other benzodiazepine psychotropic agents in that the carbon and nitrogen atoms in the 4- and 5-positions of the diazepine ring are reversed, giving rise to the 1,5-benzodiazepine structure. The pharmacological action of Compound I on the CNS of various test animals has been reported (1, 2), as well as the metabolism of the drug in animals and man (3).

To assist in correlating pharmacological activity with blood levels of Compound I, it was necessary to develop a sensitive, specific method of analysis that would permit processing of a large number of samples in a relatively short time. Various methods have been described for the determination of benzodiazepines, notably diazepam and chlordiazepoxide, in physiological fluids. In some cases, quantitation is achieved by making use of the UV-absorbing properties of the compound (4), while in others it depends upon hydrolysis of the drug followed by colorimetric determination of a Bratton–Marshall adduct (5–8). Cathode ray polarography has been successfully applied to plasma determinations (9). The most sensitive and specific methods use GLC, either of the intact compounds or of their hydrolysis products, and electron-capture detection (4, 10–12). However, these methods require extensive, time-consuming manipulations to produce an extract clean enough for chromatography.

The method described here used GLC followed by flame-ionization detection for the determination of Compound I in plasma or serum to levels of 0.1 mcg./ml. The analysis is specific for Compound I in the presence of its known metabolites and is applicable to human or animal samples after administration of therapeutic doses.

EXPERIMENTAL

Reagents—The purity of Compound I used was 100% as determined by GLC and TLC. All other materials were analytical reagent quality. The hexane was purified further by shaking with sulfuric acid, washing with water until neutral, and redistilling



I

[1] ORF 8063, obtained from Boehringer Ingelheim G.m.b.H., Ingelheim, Germany.

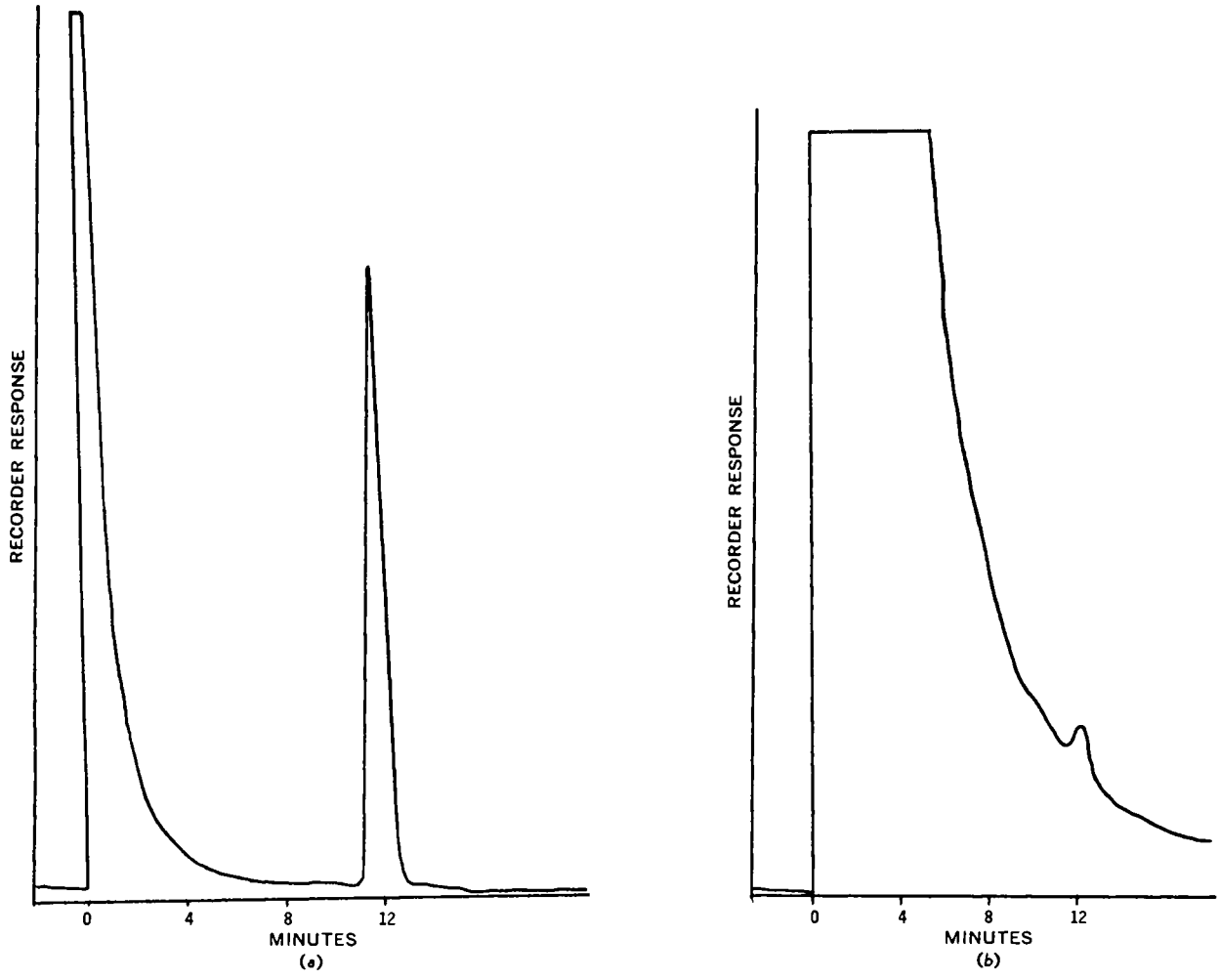


Figure 1—*Representative chromatograms of Compound I:* (a) *0.27 mcg. (attenuation* × *16), and* (b) *0.8 ng. (attenuation* × *1).*

twice through a 30.48-cm. (12-in.) Vigreux column. This was necessary to eliminate interferences which became significant after concentration of the hexane.

Apparatus—A gas chromatograph[2] with a dual flame-ionization detector was used. The output of the chromatograph was recorded on a recorder[3] with a full-scale range of 1.0 mv.

Analyses were performed using a glass column, 0.61 m. (2 ft.) × 4 mm. i.d., packed with 6% Carbowax 20M on Gas Chrom Q (100–120 mesh). The column was conditioned for 24 hr. at 250° with a helium flow of 60 ml./min. The inner surface of the column and the glass wool used to retain the packing were previously deactivated by treatment with dichlorodimethylsilane (13).

The analytical parameters used were: oven temperature, 240°; injector and manifold temperature, 230°; hydrogen inlet pressure, 1 kg./cm.^{2}; oxygen inlet pressure, 2 kg./cm.^{2}, and helium flow, 60 ml./min. With these conditions, Compound I was eluted as a symmetrical peak at 12.03 min. (Fig. 1*a*). The minimum detectable quantity of standard is less than 1 ng. (Fig. 1*b*).

Procedure—The glassware was washed with detergent and water, rewashed with chromic acid cleaning solution, rinsed with water and methanol, and dried overnight at 110°.

Blood samples were routinely collected in heparinized tubes, and the plasma was separated by centrifugation. However, in the case of human samples, it was found that less contamination occurred and greater sensitivity was possible if serum was analyzed instead of plasma. Either plasma or serum samples could be stored at 5° for at least 2 months without adversely affecting the analysis.

Exactly 1 ml. of serum was pipeted into a 25-ml. glass-stoppered extraction tube, and 0.05 ml. of concentrated hydrochloric acid was added. The tube was agitated to redissolve the small precipitate, 7 ml. of hexane was added, and the sample was extracted with vigorous shaking for 5 min. The layers were allowed to separate for 5 min., and the hexane layer was transferred with a Pasteur pipet to another extraction tube containing 2 ml. of 10% aqueous sodium carbonate. This was shaken for 1 min. and then allowed to separate for 1 min.; the hexane layer was transferred to a 12-ml. centrifuge tube for evaporation to dryness at 50° under a gentle stream of nitrogen.

The sample was extracted twice more with 6-ml. portions of hexane. These, in turn, were back-extracted with the carbonate solution and transferred to the centrifuge tube. After evaporation of the hexane, the residue was concentrated at the tip of the centrifuge tube with the aid of small portions of hexane–methylene chloride (50:50). The residue was quantitatively transferred to a tube[4] and concentrated in the tip. Exactly 20 μl. of hexane was added, and an accurately measured quantity (~2.5 μl.) was injected into the chromatograph. Figures 2*a* and 2*b* show representative chromatograms of control blood and of a sample containing 0.3 mcg./ml., respectively.

A calibration curve prepared by injecting known amounts of standard Compound I was found to be linear over the range of interest (0.002–3.5 mcg.) and to pass through the origin. To minimize the effects of variations in instrument parameters, standards were run on a daily basis.

Quantitation of the samples was achieved by drawing the baseline and determining the area by multiplying the height times the width at half-height. Sample areas were normalized by dividing by the volume injected, and the quantity of Compound I per microliter

[2] Perkin-Elmer model 900.
[3] Honeywell Electronik 194 or Hewlett-Packard 7123A.

[4] Kontes Microflex.

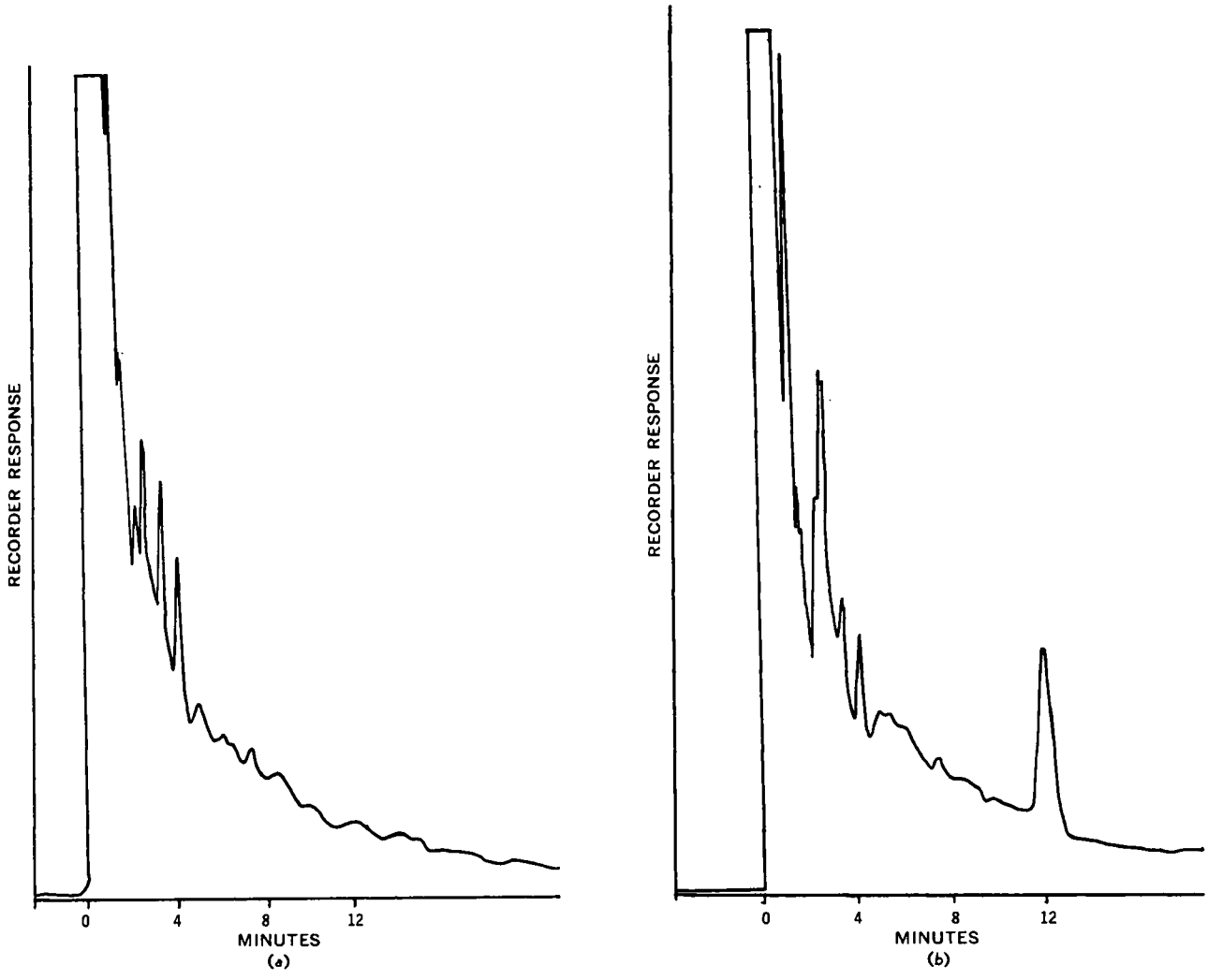


Figure 2—*Typical chromatograms of human plasma extracts:* (a) *control plasma (attenuation ×8), and* (b) *plasma containing 0.30 mcg./ml. Compound I.*

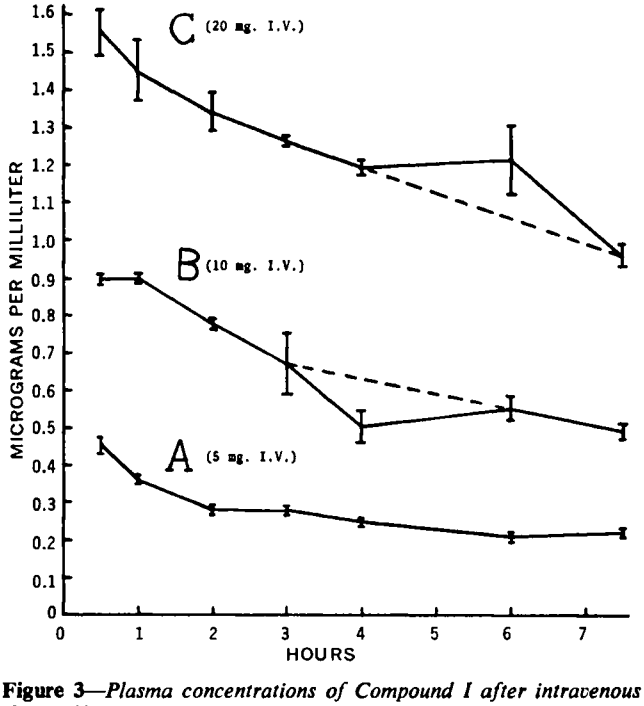


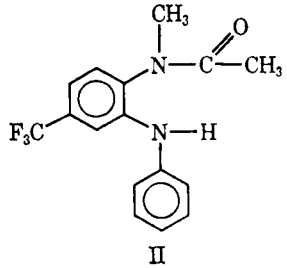
Figure 3—*Plasma concentrations of Compound I after intravenous doses of (A) 5, (B) 10, and (C) 20 mg. to rabbits.*

injected was determined by interpolation of the standard values. This result was multiplied by 20 to arrive at the final concentration in micrograms per milliliter.

RESULTS AND DISCUSSION

Sadée and Van der Kleijn (14) reported that certain diazepine compounds are decomposed during GLC as indicated by GLC–mass spectrometry. To verify the identity of the species being analyzed, mass spectra were obtained on a solid sample of Compound I and on the material eluted from the GLC column. Both mass spectra were identical, indicating the stability of Compound I to GLC.

Although Compound I does not form salts with mineral acids, it was determined in this investigation that recoveries of the drug were enhanced if the plasma or serum was acidified before extraction. Interfering substances that are also extractible from acid solution are removed by the carbonate back-extraction.



II

Table I—Accuracy and Precision of GLC Analysis of Compound I Added to Human Plasma

Added, mcg./ml.	Number of Analyses	Recovered, $\bar{x}$ (Range)	Recovery, %	*RSD*[a], %	*RE*[b], %
10	2	10.15 (9.8–10.5)	101.5	5.29	+1.5
5	13	5.06 (4.7–5.8)	101.2	5.89	+1.2
2	15	2.00 (1.8–2.2)	100.0	7.05	0
1	9	0.96 (0.9–1.1)	96.0	5.31	−4.0
0.5	13	0.48 (0.44–0.56)	96.0	9.79	−4.0

[a] *RSD* = relative standard deviation. [b] *RE* = relative error.

Table II—Plasma Levels[a] of Compound I in Humans

Dose, mg./day	Patient					
	R.E.B.	R.H.	R.B.	J.D.	M.K.	P.A.
10	0.169	0.182	0.099	0.176	0.107	0.193
20	0.286	0.300	0.300	0.414	0.214	0.375
30	0.371	0.376	0.403	0.442	0.328	0.470

[a] Values are micrograms per milliliter.

It was reported[5] that Compound I undergoes hydrolytic degradation in strong acid to form N^1-acetyl-N^1-methyl-N^2-phenyl-(4-trifluoromethyl)-2,1-phenylenediamine (II). However, the rate constants indicate that a negligible amount of hydrolysis should occur at room temperature during the time necessary to extract the plasma. Residues from sample analyses were chromatographed on silica gel thin-layer plates using various solvent systems. Comparison with concurrently chromatographed authentic Compound II indicated no degradation of Compound I. Elution of the sample zone and subsequent GLC confirmed this observation.

The accuracy and precision of the analysis, determined by adding known amounts of Compound I to plasma or serum, are shown in Table I.

To demonstrate the suitability of the method for actual biological samples, rabbits were dosed intravenously with 5, 10, or 20 mg. of Compound I and plasma samples were taken at appropriate intervals for analysis. The plasma concentrations (mean ± *SE*) shown in Fig. 3 illustrate the applicability of the method.

Blood levels were also determined in human plasma samples obtained from a group of patients participating in a clinical study. Plasma concentrations ranging from 0.099 to 0.47 mcg./ml. were found in samples taken 24 hr. after successive daily doses of 10, 20, and 30 mg. (Table II). These results confirm the utility of the GLC analysis for clinical samples.

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