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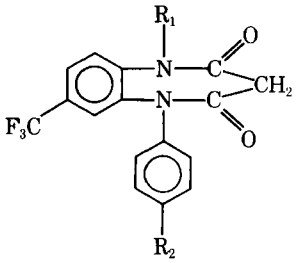
High-Pressure Liquid Chromatographic Analysis of Triflubazam and Its Metabolites in Human and Animal Blood and Urine

RICHARD E. HUETTEMANN [x] and ARVIN P. SHROFF

Abstract □ A high-pressure liquid chromatographic method is described for analyzing triflubazam [1-methyl-5-phenyl-7-trifluoromethyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione] and its primary metabolites in blood and urine. Adsorption chromatography, using pellicular silica gel as the stationary phase and dioxane–isooctane as the mobile phase, permitted rapid sample analysis. After extraction of blood and urine samples with toluene, quantitation is achieved using liquid chromatography with an internal standard. The method is sensitive above 50 ng/ml of triflubazam and its known metabolites. Recoveries for all compounds from blood or urine averaged above 95%. The specificity of the method was established by collecting samples separated by liquid chromatography and characterizing them by mass spectrometry. Human and animal data are presented to illustrate the utility of the method.

Keyphrases □ Triflubazam (a 1,5-benzodiazepine) and its metabolites—high-pressure liquid chromatographic analysis in human and animal blood and urine □ 1,5-Benzodiazepines—high-pressure liquid chromatographic analysis of triflubazam and its metabolites in human and animal blood and urine □ High-pressure liquid chromatography—analysis, triflubazam (a 1,5-benzodiazepine) and its metabolites in human and animal blood and urine

The quantitative analysis of drugs and their metabolites in physiological fluids is important for correlating pharmacological activity. Various methods requiring preliminary purification and/or lengthy analysis time to obtain the sensitivity and specificity needed to generate an accurate profile of the drug's metabolism have been described. Polarography (1), colorimetry (2), UV spectrophotometry (3), GLC (4), TLC (5), and liquid chromatography (6) have been employed to analyze the benzodiazepine class of compounds in blood and urine. A comprehensive review (7) of all methods for the analysis of benzodiazepines was published recently.



	R_1	R_2
I:	CH_3	H
II:	H	H
III:	CH_3	OH
IV:	H	OH

The methodology presented in this paper for the analysis of triflubazam [1-methyl-5-phenyl-7-trifluoromethyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione], Compound I[1], and its metabolites (II–IV) in blood and urine by high-pressure liquid chromatography (HPLC) exemplifies the advantages of this technique to this area of research.

EXPERIMENTAL

Reagents—All solvents were analytical reagent quality and were used without further purification.

[1] Synthesized by Boehringer-Ingelheim G.m.b.H., Ingelheim, Germany; referred to as ORF-8063.

Table I—Recoveries from Serum (Human)

Sample	I Added, μg	I Found, μg	Recovery, %	II Added, μg	II Found, μg	Recovery, %
1	1.00	0.96	96.0	1.00	1.03	103.0
2	1.00	0.98	98.0	1.00	1.01	101.0
3	1.00	0.99	99.0	1.00	0.99	99.0
4	1.00	1.01	101.0	1.00	0.99	99.0
5	1.00	0.98	98.0	1.00	1.04	104.0
		$\bar{X}$ = 98.0			$\bar{X}$ = 101.2	
1	0.10	0.096	96.0	0.10	0.103	103.0
2	0.10	0.101	101.0	0.10	0.095	95.0
3	0.10	0.101	101.0	0.10	0.094	94.0
4	0.10	0.100	100.0	0.10	0.102	102.0
5	0.10	0.095	95.0	0.10	0.102	102.0
		$\bar{X}$ = 99.0			$\bar{X}$ = 99.2	

Sample	III Added, μg	III Found, μg	Recovery, %
1	1.00	1.01	101.0
2	1.00	0.95	95.0
		$\bar{X}$ = 98.0	

Sample	IV Added, μg	IV Found, μg	Recovery, %
1	1.00	1.06	106.0
2	1.00	1.05	105.0
		$\bar{X}$ = 105.5	

Apparatus—A liquid chromatograph[2] fitted with a standard 254-nm detector[3] was used. The column was a tube (1.0 m × 2.1 mm i.d.) with a stationary phase of pellicular silica gel[4]. The column was operated at 35 kg/cm^2 (500 psi) at 30°, with a mobile phase of 15% dioxane in isooctane at a flow rate of 3 ml/min.

Procedure—Stock solutions (10 μg/ml) of I–IV and 11α-hydroxy-17α-methyltestosterone were prepared in chloroform. A stock standard solution (A) was prepared to contain 1 μg of each of I–IV and 10 μg of internal standard/5 μl of chloroform. Exactly 1.0 ml of the internal standard solution was pipetted into a 10-ml glass-stoppered extraction tube and evaporated to dryness in a 65° water bath under a stream of nitrogen. Exactly 1.0 ml of blood or 5.0 ml of urine was added to the tube. Five milliliters of toluene was added, and the tube was shaken for 5 min.

The tubes were then centrifuged for 2 min and cooled in a dry ice–acetone bath for 20 sec, and the toluene was decanted into a conical centrifuge tube. One milliliter of toluene was added to the extraction tube; the contents were mixed for 30 sec, centrifuged, and cooled in the dry ice–acetone bath; and the toluene was decanted into the respective conical tube. The toluene was evaporated to dryness in a 65° water bath under a stream of nitrogen.

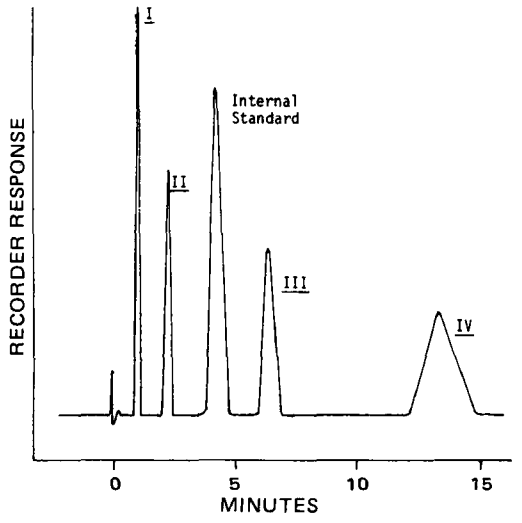


Figure 1—*Chromatogram depicting retention time of internal standard and I–IV.*

The residue was treated with acetone (200-, 50-, and 10-μl portions) and quantitatively transferred to a microflex tube[5]. Five microliters of chloroform was added to the evaporated residue in the microflex tube, mixed[6] for 15 sec, and saved.

Five microliters of Standard Solution A was injected into the liquid chromatograph, and the compounds eluted as shown in Fig. 1. The sample microflex tube contents were injected next. Quantitation was achieved by dividing the ratio of any compound detected in the sample by the ratio of the same compound in the stan-

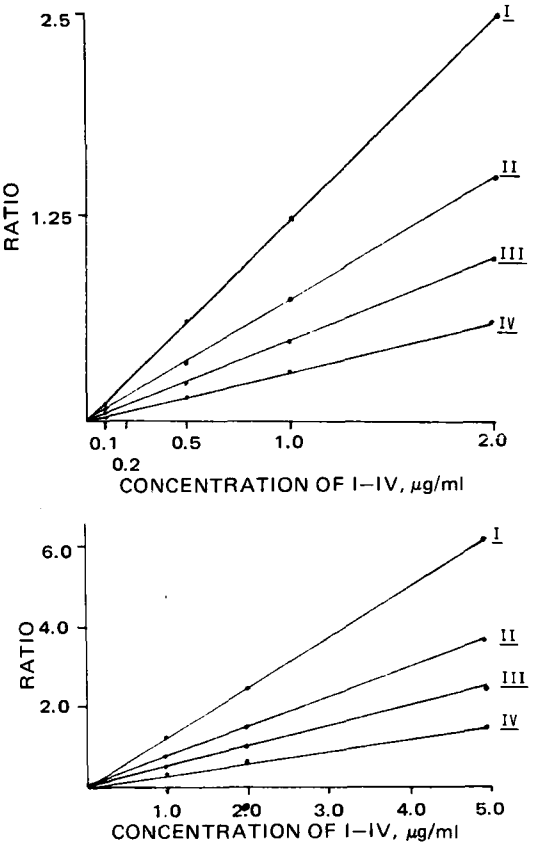


Figure 2—*Plot of concentration* versus *peak height ratio.*

[2] Dupont model 830.
[3] Dupont.
[4] Pelosil HC, Reeve Angel.
[5] Kontes Glass Co.
[6] Vortex Genie.

Table II—Recoveries from Urine (Human)

Sample	I Added, μg	I Found, μg	Recovery, %
1	1.00	1.03	103.0
2	1.00	1.06	106.0
3	1.00	1.05	105.0
4	1.00	1.01	101.0
5	1.00	0.99	99.0
		$\overline{X}$ = 102.8	
Sample	II Added, μg	II Found, μg	Recovery, %
1	1.00	1.09	109.0
2	1.00	0.95	95.0
3	1.00	0.99	99.0
4	1.00	0.99	99.0
5	1.00	1.02	102.0
		$\overline{X}$ = 100.8	
Sample	III Added, μg	III Found, μg	Recovery, %
1	1.00	1.02	102.0
2	1.00	1.05	105.0
		$\overline{X}$ = 103.5	

dard (the ratio is the height of the compound peak divided by the height of the internal standard peak) to give micrograms per milliliter of any compound in blood or micrograms per 5 ml in urine. Typical standard curves demonstrating the detector's response to I–IV over the concentration ranges of 0.1–2.0 and 1.0–5.0 μg/ml are shown in Fig. 2.

Liquid Chromatography–Mass Spectrometry—Compound identification was achieved by evaporating the appropriate aliquot from liquid chromatography and obtaining the mass spectrum[7] of the residue at 70 ev.

RESULTS AND DISCUSSION

The described method using HPLC allows for simultaneous cleanup and analysis and, if necessary, for more rapid identification of sample peaks than is obtained with other methods.

The method used to analyze for I and its metabolites, II–IV, was developed after several stationary phases and solvent systems were investigated to give an ideal separation in a short period. Adsorption chromatography employing a stationary phase[4] and a mobile phase of dioxane–isooctane allowed samples to be analyzed within 15 min. Quantitation was accomplished using an internal standard technique to minimize errors caused by the loss of sample during extraction and transfer steps.

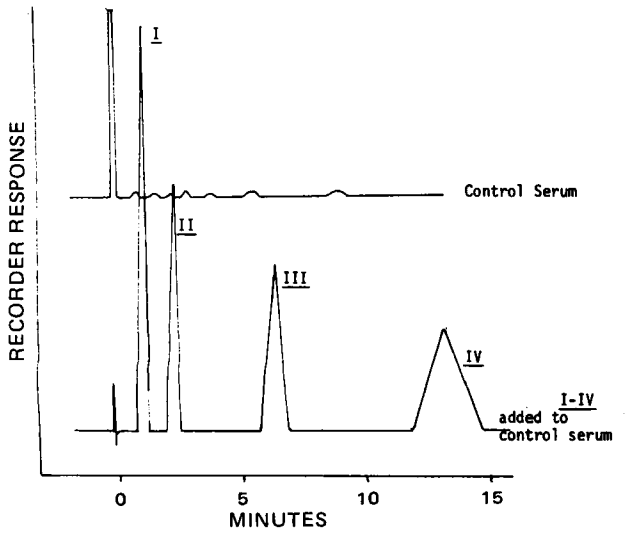


Figure 3—*Liquid chromatogram of I–IV at 1 μg (8×) and a human control serum sample (2×).*

[7] Finnigan model 1015 GC-MS.

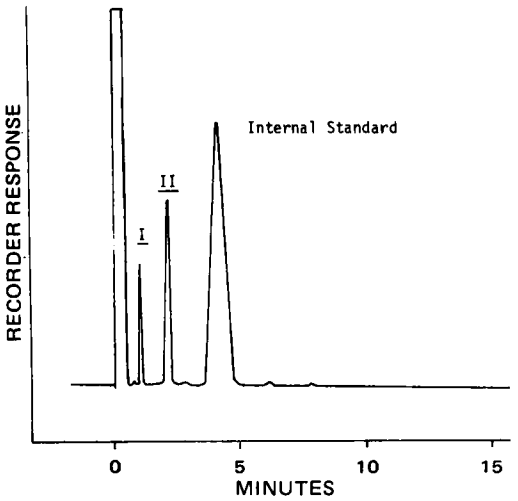


Figure 4—*Chromatogram of a typical human serum sample.*

11α-Hydroxy-17α-methyltestosterone was an ideal internal standard, as demonstrated in Fig. 1. The response of known standard concentrations of I–IV was found to obey Beer's law (Fig. 2). Since studies showed that these slopes varied from day to day due to the quality of the solvent and/or temperature, the daily use of a standard solution is recommended.

The accuracy and precision of the method were determined by the addition of known quantities of I–IV to control human serum and urine samples as well as to rat, rabbit, and dog control serum samples. The samples were then analyzed according to the outlined procedure. Table I summarizes the recoveries from human serum at various levels, Table II summarizes the human urine studies, and Table III summarizes the animal serum studies. All recovery studies were well within acceptable levels. A typical liquid chromatogram obtained by analyzing both spiked and controlled human serums is shown in Fig. 3. No interferences were noted from control serum or urine samples.

To demonstrate the suitability of this methodology for biological materials, blood and urine samples were obtained from patients participating in a clinical trial of I. Table IV depicts the results obtained by both HPLC and TLC densitometry (5) methods for blood samples, while Table V shows the results with urine samples. Both blood and urine samples were obtained 24 hr after dosing. Figure 4 illustrates the chromatogram obtained from typical human serum in the same study.

To obtain a better understanding of the absorption characteristics of this drug, a monkey study was initiated. Blood samples were withdrawn over a 12-hr period, and urine samples were collected

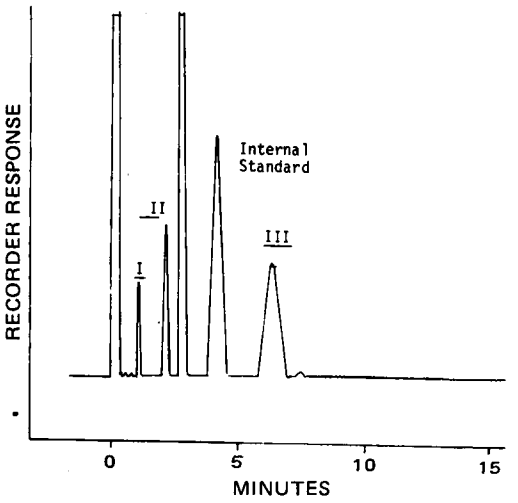


Figure 5—*Chromatogram of a typical monkey urine sample.*

Table III—Recoveries from Serum of Different Species

Species	I			II		
	Added, μg	Found, μg	Recovery, %	Added, μg	Found, μg	Recovery, %
Rat						
1	1.00	1.00	100.0	1.00	0.92	92.0
2	1.00	1.01	101.0	1.00	0.95	95.0
		$\bar{X}$ = 100.5			$\bar{X}$ = 93.5	
Rabbit						
1	1.00	1.05	105.0	1.00	0.98	98.0
2	1.00	1.00	100.0	1.00	1.01	101.0
		$\bar{X}$ = 102.5			$\bar{X}$ = 99.5	
Dog						
1	1.00	1.01	101.0	1.00	1.00	100.0
2	1.00	1.06	106.0	1.00	1.08	108.0
		$\bar{X}$ = 103.5			$\bar{X}$ = 104.0	

Table IV—Serum Levels of I and II in Six Patients by TLC and HPLC

Patient	I, μg/ml		II, μg/ml	
	TLC	HPLC	TLC	HPLC
1	0.94	0.92	0.94	1.00
2	0.84	0.85	0.89	0.90
3	0.57	0.59	1.02	1.05
4	N.D.[a]	N.D.	N.D.	N.D.
5	1.40	1.51	0.86	0.80
6	1.79	1.80	1.73	1.81

[a] N.D. = not detected.

Table V—Urine Levels of I and II in Three Patients by TLC and HPLC

Patient	I, μg/ml		II, μg/ml	
	TLC	HPLC	TLC	HPLC
1	0.15	0.12	0.19	0.19
2	0.20	0.22	0.30	0.35
3	0.68	0.60	1.51	1.63

every 24 hr. Table VI lists the serum results, while Table VII lists the urine results. Figure 5 illustrates the chromatogram of a typical monkey urine extract showing peaks corresponding to I–III. From this study, it appears that I is slowly absorbed and rapidly converted to II in the blood, while urine data suggest slow elimination from the body.

Fractions from the HPLC corresponding to I–IV and any other unknown peaks from any study can be quickly collected, evaporated, and submitted for mass spectrometry to ensure specificity and allow for rapid elucidation of unknown peaks if they appear. The actual cleanup occurs while quantitative analyses are being performed. The HPLC column exhibited good column life even after several weeks of continuous operation.

Table VI—Serum Results of the Monkey Study

Hours	Monkey I		Monkey II	
	I, μg/ml	II, μg/ml	I, μg/ml	II, μg/ml
Predose	N.D.[a]	N.D.	N.D.	N.D.
0.15	N.D.	N.D.	N.D.	N.D.
0.30	N.D.	N.D.	N.D.	N.D.
0.45	N.D.	N.D.	N.D.	N.D.
1	N.D.	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.	N.D.
3	N.D.	N.D.	N.D.	N.D.
4	0.29	0.16	0.60	0.55
5	0.24	0.16	0.56	0.59
6	0.23	0.20	0.57	0.62
12	0.91	2.11	0.35	0.49

[a] N.D. = not detected.

Table VII—Urine Results[a] of the Monkey Study

Monkey	Day	I, μg/2 ml Urine	II, μg/2 ml Urine	III, μg/2 ml Urine
1	1	5.84	19.56	N.D.[b]
1	3	3.88	6.22	1.17
1	4	N.D.	7.83	N.D.
1	5	N.D.	0.72	N.D.
2	1	1.90	5.85	5.17
2	2	0.70	2.83	4.07
2	3	2.00	1.63	—
2	4	2.30	1.06	1.10
2	5	1.61	1.28	1.03

[a] Urine was not hydrolyzed prior to extraction. [b] N.D. = not detected.

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