Gas Chromatographic Determination of Quazepam and Two Major Metabolites in Human Plasma

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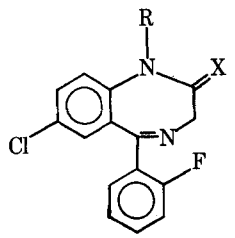
Abstract □ Two rapid, sensitive, and specific gas chromatographic (GC) methods for the quantitative determination of quazepam (I), 7-chloro-5-(2-fluorophenyl-1,3-dihydro-1-(2,2,2-trifluoroethyl)- 2*H* -1,4-benzodiazepine-2-thione, and its major active plasma metabolites, the 2-oxo compound (II) and the dealkylated oxo compound (III), have been developed; the first measures I and II and the second measures III. The compounds are extracted from plasma with toluene and quantitated by electron-capture detection using the internal standard method. The methods are capable of quantitating plasma levels of I and II as low as 0.75 ng/mL and plasma levels of III as low as 1.5 ng/mL. Correlation coefficients of standard curves were >0.9995 for all compounds. Precision of the methods was measured at two different concentrations for each compound; the *CV* values were 3–6% for all three compounds. The recovery of all compounds was >80%, and the ratio of recovery of each compound to that of its internal standard did not vary at different concentrations, indicating appropriate internal standards have been selected for the methods. Quazepam metabolites, other benzodiazepines, and drugs which are potential comedication do not interfere with either method. The methods were shown to be suitable for investigating the bioavailability and pharmacokinetics of quazepam at therapeutic doses.

Keyphrases □ Quazepam—gas chromatography two major metabolites, human plasma □ Gas chromatography—quazepam and two major metabolites in human plasma □ Metabolites—quazepam, human plasma, gas chromatography

Quazepam, 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-1-(2,2,2-trifluoroethyl)-2*H*-1,4-benzodiazepine-2-thione, a novel benzodiazepine which has been reported to have good sedative and hypnotic activity (1, 2), is extensively metabolized in humans. Two major active metabolites have been identified in plasma (Scheme I), the 2-oxo compound II [7-chloro-5-(2-fluorophenyl) -1,3-dihydro-1-(2,2,2-trifluoroethyl)-2*H*-1,4-benzodiazepin-2-one] and III [7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one] (2). To fully evaluate the bioavailability and pharmacokinetics of quazepam, two sensitive and specific gas chromatographic (GC) assays have been developed to measure the unchanged drug and the two major metabolites in plasma; one method measures I and II, and the other measures III.

Polarographic (3) and GC methods (4–6) have been published for III. However, these methods either lack sensitivity or involve derivatization. The methods described in this report require simple extraction procedures, no derivatization, and are more sensitive than previously reported chromatographic assays.



I: X = S, R = —CH_2CF_3
II: X = O, R = —CH_2CF_3
III: X = O, R = H

EXPERIMENTAL

Chemicals and Reagents—Quazepam[1] (I), II[1], 7,9-dibromo-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one (IV)[1] (the internal standard for the assay of III), III[2], and diazepam[2] (the internal standard for the assay for I and II) were used. A stock solution of I and II (0.75 mg/mL) was prepared by accurately weighing 75 mg each of I and II into a 100-mL volumetric flask and dissolving them in toluene. The standard solution of the two compounds (1.5 μg/mL) was prepared by diluting 0.20 mL of the stock solution to 100 mL with toluene. A stock solution of III (1 mg/mL) was prepared by weighing accurately 10 mg of III into a 10-mL volumetric flask and dissolving the compound in toluene. The standard solution of III (1.5 μg/mL) was prepared by diluting 0.15 mL of the stock solution to 100 mL with toluene.

Both standard solutions of the internal standards (diazepam and IV) were prepared by accurately weighing 10 mg of each compound, dissolving it in 10 mL with toluene, and diluting 1 mL of the resulting stock solution to 100 mL of toluene. Toluene[3] and cyclohexane[3] were glass distilled.

Chromatographic Conditions for the Assay of I and II—The chromatograph[4] was equipped with a ^{63}Ni pulse-modulated electron-capture detector, an automatic injector[5], and a data system for measuring peak heights and calculating concentrations[6]. The coiled glass column (1.83 m × 2 mm i.d.) was silanized[7] and then packed with 3% OV-25 on 80–100 mesh Supelcoport[8]. The temperatures of the column, injector, and detector were maintained at 220°C, 280°C, and 350°C, respectively. Ultra-high purity nitrogen was used as the carrier gas with a flow rate of 28 mL/min.

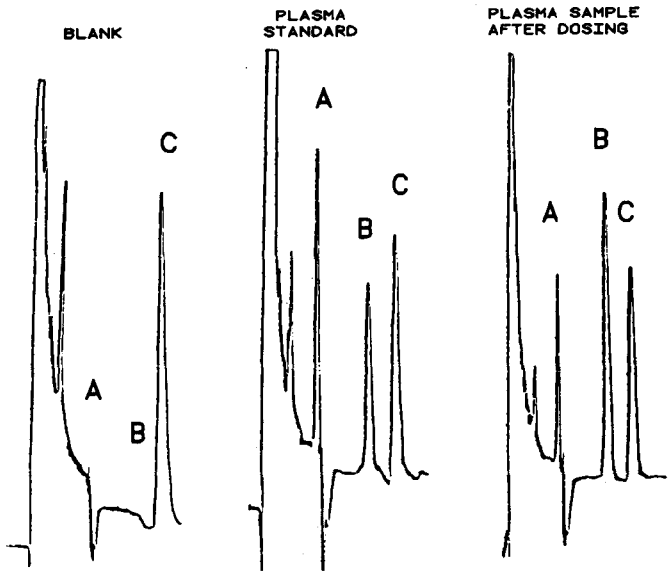


Figure 1—*Typical gas chromatograms of blank plasma, a standard concentration of 12 ng/mL of quazepam and II plasma, and human plasma withdrawn at 1.5 h after an oral dose of 15 mg of quazepam. Chromatography was on OV-25 at 220°C with electron-capture detection. Key: (A) II; (B) quazepam; (C) diazepam.*

[1] Synthesized at Schering Corporation.
[2] Gifts from Hoffmann-La Roche, Nutley, N.J.
[3] Burdick and Jackson, Muskegon, Mich.
[4] Model 3700; Varian Associates, Palo Alto, Calif.
[5] Model 8000; Varian Associates, Palo Alto, Calif.
[6] HP 3353A Data System; Hewlett Packard, Co., Paramus, N.J.
[7] Silyl-8; Pierce Chemical Co., Rockford, Ill.
[8] Supelco, Inc., Bellefonte, Pa.

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Table I—Standard Curves for Quazepam and Its Two Plasma Metabolites

Amount Added to Plasma, ng/mL	Amount Found in Plasma, ng/mL[a]	Linear Regression Equation
	Quazepam	
0.75	0.881	
1.50	1.636	
3.00	3.461	
6.00	6.365	$y = 1.043(0.003)x$
12.00	12.667	$+ 0.193(0.093)$
24.00	25.533	$r^2 = 0.9999$
48.00	50.636	
96.00	100.123	
	Compound II	
0.75	0.853	
1.50	1.389	
3.00	3.074	
6.00	5.537	$y = 0.979(0.007)x$
12.00	12.373	$- 0.001(0.265)$
24.00	23.895	$r^2 = 0.9996$
48.00	45.600	
96.00	94.542	
	Compound III	
1.50	1.531	
3.00	3.008	
6.00	6.095	$y = 1.004(0.004)x$
12.00	12.737	$+ 0.109(0.140)$
24.00	23.829	$r^2 = 0.9999$
48.00	48.602	
96.00	96.455	

[a] Values are the average of duplicate determinations.

Sample Preparation for the Assay of I and II—Aliquots (0.5 mL) of plasma samples were pipetted in a glass centrifuge tube, mixed with 6 μL of the 10-μg/mL solution of diazepam, and extracted with 0.5 mL of toluene. Following centrifugation[9] at 1500 rpm, 1.0 μL of the toluene extract was injected into the gas chromatograph.

Chromatographic Conditions for the Assay of III—The chromatograph[4] was equipped with a ^{63}Ni pulse-modulated electron-capture detector, an automatic injector[5], and a data system for measuring peak heights and calculating concentrations[6]. The coiled glass column (1.83 m × 2 mm i.d.) was silanized[7] and then packed with 3% SP-2250 DB on 100–120 mesh Supelcoport[8]. The temperature of the column, injector, and detector were maintained at 235°C, 250°C, and 350°C, respectively. Ultra-high purity nitrogen was used as the carrier gas with a flow rate of 43 mL/min.

Sample Preparation for the Assay of III—Aliquots (0.25 mL) of plasma samples were prepared in a glass centrifuge tube and extracted twice with 0.25 mL of cyclohexane. The cyclohexane layer was discarded; 0.2 mL of the extracted plasma was transferred to a centrifuge tube and mixed with 0.25 mL of toluene and 6 μL of the 10-μg/mL toluene solution of IV. Following centrifugation at 1500 rpm for 5 min, 3 μL of the toluene extract was injected into the gas chromatograph.

Table II—Accuracy and Precision of the Assays for Quazepam and Its Two Metabolites

Compound	Plasma Concentration, ng/mL[a]	Mean Concentration Found, ng/mL	*CV*, %
Quazepam	1.5	1.50	2.94
	12.0	12.27	5.80
Compound II	1.5	1.51	6.25
	12.0	11.65	5.34
Compound III	3.0	2.97	6.19
	24.0	23.86	2.54

[a] Five replicate determinations were performed for each compound at each plasma concentration.

[9] Size 2 Model K Centrifuge; International Equipment Co., Needham Heights, Mass.

Table III—Retention Times of Quazepam Metabolites, Benzodiazepines, and Other Drugs on the GC Columns Used in the Assays

	Retention Time, min	
	3% OV-25[a]	3% SP-2250 DB[b]
Quazepam	6.8	2.0
Compound II	3.5	1.9
Compound III	13.2	5.9
Compound IV	NP[c]	10.3
Diazepam	8.7	4.3
3-Hydroxy derivative of III	4.4	2.6
3-Hydroxy derivative of II	6.0	8.4
Flurazepam	18.2	10.8
Halazepam	3.4	2.0
N-Demethyldiazepam	14.0	7.4
Oxazepam	5.4	7.6
Nitrazepam	NP	19.7
Chlorpromazine	6.4	3.4
Perphenazine	4.0	2.5
Chlorimipramine[d]	NP	NP
Chlorpheniramine	NP	NP

[a] Used for the assay of I and II. [b] Used for the assay of III. [c] No peak observed for the compound. [d] 3-Chloro-10,11-dihydro- *N*,*N*-dimethyl-5*H*-dibenz[6,*F*]azepine-5-propanamine.

Standard Curves, Reproducibility, and Recovery Studies—A standard curve was prepared for each compound by adding aliquots of each stock solution to a series of control human plasma samples. A set of standard plasma concentrations were made for each of these compounds ranging from 0.75 to 96 ng/mL for I and II and from 1.5 to 96 ng/mL for III. The samples were carried through the respective assay procedures, and the concentration values obtained from the data system were plotted against the actual concentrations.

To demonstrate the precision and accuracy of I–II assay, 10 plasma samples were spiked with I and II, five samples at 1.5 ng/mL and five samples at 12 ng/mL. Similarly five plasma samples were spiked with III at 3 ng/mL and five samples at 24 ng/mL. Then, the plasma samples were carried through the respective assay procedures, and the means, standard deviations, and coefficients of variation calculated.

For determination of recoveries from plasma, the peak heights of extracted standards of each compound at 3 and 48 ng/mL were compared with those of the respective unextracted standards. The unextracted standards were made

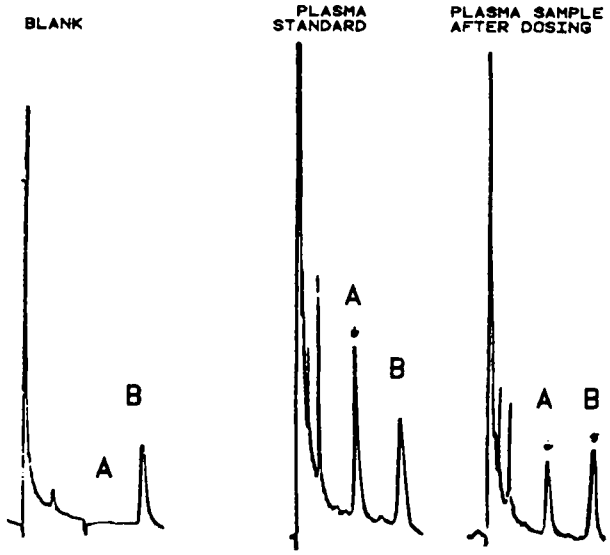

Figure 2—*Typical gas chromatograms of blank plasma, a standard concentration of 24 ng/mL of III in human plasma, and human plasma withdrawn 48 h after an oral dose of 15 mg of quazepam. Chromatography was on SP-2250 DB at 235°C with electron-capture detection. Key: (A) III; (B) IV.*

Table IV—Recoveries of I, II, III, and the Internal Standards From Plasma [a]

Amount Added to Plasma, ng/mL	Assay for I and II: Recovery, % Quazepam	Compound II	Diazepam	Ratio of Recovery: Quazepam/ Diazepam	Compound II Diazepam
3	96.5 ± 7.9	94.7 ± 3.4	99.3 ± 3.6	0.898 ± 0.04	0.954 ± 0.01
48	88.8 ± 8.3	99.2 ± 8.5	102.0 ± 8.6	0.869 ± 0.05	0.971 ± 0.05

Amount Added to Plasma ng/mL	Assay for III: Recovery, % Compound III	Compound IV	Ratio of Recovery
3	78.3 ± 1.1	85.8 ± 5.1	0.915 ± 0.05
48	84.0 ± 5.0	93.5 ± 3.2	0.898 ± 0.04

[a] Values are the mean of five determinations ±*SD*.

by adding the compounds to toluene which had been previously extracted with an equal volume of plasma.

RESULTS AND DISCUSSION

As shown in Fig. 1, I, II, and the internal standard (diazepam) were well separated from one another on the OV-25 stationary phase. The respective retention times of I, II, and diazepam were 6.8, 3.5, and 8.7 min.

Compound III could not be analyzed under the conditions used for I and II due to poor sensitivity and excessive tailing of its peaks on OV-25 and similar stationary phases. It was found that III could be analyzed on SP-2250 DB, a stationary phase deactivated for bases. This packing gave excellent symmetrical peak shapes (Fig. 2) and much higher sensitivity. Compound III and the internal standard IV had retention times of 5.9 and 10.3 min, respectively, on the SP-2250 DB column.

Table I shows that the standard curves were linear for quazepam and both metabolites ($r^2 > 0.9995$) throughout the entire range investigated. The smallest concentration of each compound used in the standard curves, 0.75 ng/mL for I and II and 1.5 ng/mL for III, exhibited acceptable chromatographic peaks which were clearly within the linear range of the GC response. Therefore, the assays are sensitive enough to detect and quantitate concentrations of 0.75 ng/mL for I and II and 1.5 ng/mL for III.

The results of replicate analyses indicate that the methods have a high degree of accuracy, since the mean concentration values determined were very close to known concentrations. The coefficient of variation was 3–6% for the three compounds at each concentration, indicating the assays are very precise (Table II).

To determine the specificity of the methods, possible interferences from two other quazepam metabolites, the 3-hydroxy derivative of II, and the 3-hydroxy derivative of III (7), other benzodiazepines, and drugs that might be used as comedication were checked by chromatographing solutions of pure standards. None of these compounds (with the exception of halazepam, which was not completely resolved from II) had a retention time similar to those compounds being analyzed in either system (Table III). Thus, the assays are specific for quazepam and its two metabolites, II and III, in the presence of these compounds. Furthermore, the assays should be specific for quazepam and its metabolites in the presence of compounds which do not contain a nitro or halogen moiety, since these compounds do not respond to electron-capture detection.

Both assays are rapid, since, unlike the extraction procedures in previous assays of III and benzodiazepines similar to I and II (3–6, 8–12), no buffering of the plasma is needed to extract all the compounds with recoveries >80% (Table IV). However, in the assay of III, it is necessary to first wash the plasma with cyclohexane to selectively remove an unidentified plasma constituent which gives a chromatographic peak that interferes with the peak of III.

In addition to good recoveries, the data in Table IV show that the ratio of recoveries of I, II, and III to the respective internal standards were very similar at each concentration examined, indicating that appropriate internal standards have been selected for the methods.

The application of these methods is shown in the mean plasma level–time profile of quazepam and its two metabolites in 12 subjects following administration of a 15-mg quazepam tablet (Fig. 3). Clearly, the described methods are suitable for investigating the bioavailability and pharmacokinetics of quazepam at therapeutic doses.

In summary, rapid, sensitive, specific, and reproducible methods for quazepam and its two metabolites have been developed. The methods are capable of quantitating plasma levels of I and II as low as 0.75 ng/mL and plasma levels of III as low as 1.5 ng/mL. Pharmacokinetic studies which have been conducted utilizing these methods will be reported elsewhere.

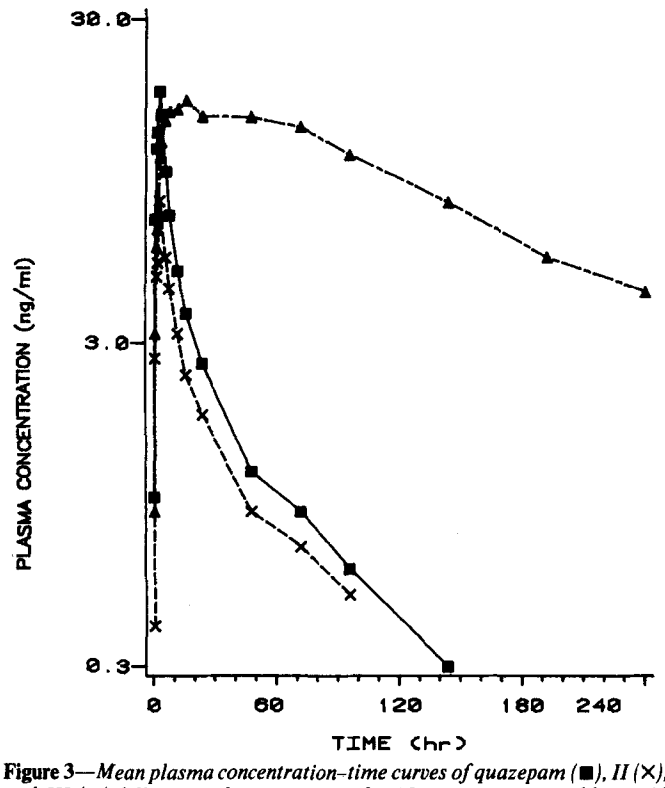

Figure 3—*Mean plasma concentration–time curves of quazepam (■), II (×), and III (▲) following administration of a 15-mg quazepam tablet to 12 subjects.*

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Use of the Fluorescence Probe 1-Anilino-8-naphthalenesulfonate in Predicting Interindividual Differences in the Plasma Protein Binding of Acidic Drugs in Rats

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Abstract □ The possibility that the fluorescence probe, 1-anilino-8-naphthalenesulfonate (I), might be used for predicting the interindividual differences in the plasma protein binding of acidic drugs was examined. The interindividual differences in the free fraction of I (f_I) were found not to be due to corresponding differences in plasma albumin concentration, but to those differences in binding constant. The binding constant of I to the plasma of 18 individual rats ranged from 1.75×10^6 M^{-1} to 2.3×10^7 M^{-1}. The free fraction of I had a highly significant statistical correlation with plasma concentration of free fatty acids, but had no significant correlation with the infinite fluorescence of I or the degree of the polarization of I. Each free fraction of five acidic drugs (warfarin, phenylbutazone, salicylic acid, indomethacin, and sulfaphenazole) was correlated with f_I with high statistical significance. Therefore, the simple and convenient method using I may predict the interindividual differences in the plasma protein binding of acidic drugs in rats.

Keyphrases □ 1-Anilino-8-naphthalenesulfonate—fluorescence probe, prediction of interindividual differences in plasma protein binding, acidic drugs, rats □ Fluorescence probe—1-anilino-8-naphthalenesulfonate, prediction of interindividual differences in plasma protein binding, acidic drugs, rats □ Binding, plasma protein—1-anilino-8-naphthalenesulfonate, prediction of interindividual differences, acidic drugs, rats

Serum (or plasma) protein binding can have pronounced effects on the pharmacodynamic and toxicological actions of drugs, as well as on their elimination kinetics. This effect is particularly striking with respect to the elimination of the extensively serum protein-bound drugs such as anticoagulants (warfarin, dicoumarol) by rats and humans, since there are pronounced interindividual differences in the free fraction of these drugs (1, 2). Minor interindividual changes in the degree of binding of such highly protein-bound drugs can produce significant changes in the amount of unbound drug. Differences in the structure of albumin or in the plasma concentration of endogenous inhibitory agents were suggested to be alternative reasons for such pronounced interindividual differences in the free fraction of these drugs (1); however, the details are, as yet, unresolved. Based on these facts, Levy and Yacobi (3) suggested that *in vitro* plasma protein binding tests with blood samples prior to drug administration might be useful for predicting quantitatively unusual distribution, elimination, and pharmacological effect characteristics of certain highly plasma protein-bound drugs.

It was previously reported that there are pronounced interindividual differences in binding of the anionic fluorescence probe 1-anilino-8-naphthalenesulfonate (I) to rat plasma (4). Free fatty acids were found to be one of the endogenous inhibitors, and there was a highly significant correlation between the free fractions of I and phenylbutazone (4).

In the present study, we investigated further the correlation between the free fraction of I and those of various acidic drugs extensively bound to plasma protein, and suggested the possibility that I might be used clinically for predicting the interindividual differences in the plasma protein binding of acidic drugs. Furthermore, we tried to examine whether or not differences in the structure of albumin are associated with differences in the free fraction of I.

EXPERIMENTAL

Materials—The following analytical-grade materials were used: 1-anilino-8-naphthalenesulfonate (I) as the sodium salt[1], warfarin[2], indomethacin[3], sulfaphenazole[4], sodium salicylate[5], sodium laurate[6], sodium palmitate[6], sodium stearate[6], sodium oleate[6], and rat serum albumin (fraction V)[7]. The rat serum albumin was defatted by the method of Chen (5).

Plasma Preparation—Adult male Wistar rats, weighing 290–340 g, were used. Approximately 10 min after heparinizing, blood was collected from the carotid artery under light ether anesthesia, and the plasma was obtained by centrifugation (3000 rpm for 10 min). Prepared plasma (2.5 mL) was used on the same day for measuring the bindings of drugs, and the remaining plasma (2 mL) was divided into the three aliquots, which were stored at −40°C and used within 1 week for the measurements of the concentrations of albumin, free fatty acids, and total bilirubin.

Equilibrium Dialysis—The 2-mL fresh plasma sample was diluted to 25% by the addition of 6 mL of Tris-HCl buffer (50 mM, pH 7.4). The bindings of four acidic drugs (sulfaphenazole, salicylic acid, indomethacin, and phenylbutazone) to the dilute plasma were measured by equilibrium dialysis (25°C). The details of the equilibrium dialysis techniques were described previously (4, 6).

[1] Tokyo Chemical Industries, Co., Tokyo, Japan.
[2] Eizai Co., Tokyo, Japan.
[3] Merck Banyu Co., Tokyo, Japan.
[4] Dainippon Seiyaku Co., Tokyo, Japan.
[5] Koso Chemical Co., Tokyo, Japan.
[6] Tokyo Kasei Industries, Co., Tokyo, Japan.
[7] Sigma Chemical Co., St. Louis, Mo.

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