The ratios of the peak heights of disopyramide and its metabolite to the peak height of the internal standard were calculated. Statistical analysis of the data (Table I) by linear regression indicated excellent linearity and reproducibility with correlation coefficients of 0.9991 and 0.9955, slopes of 0.3655 and 0.3100, and intercepts of 0.0030 and −0.0038 in the range of 0.5–12.0 μg/ml of plasma for disopyramide and its metabolite, respectively. This range includes the therapeutic range of the drug.

The use of reversed-phase chromatography on a cyanopropylsilane column and a mobile phase of 50% acetonitrile and 50% acetate buffer (pH 4, 0.01 *M*) afforded excellent separation with sharp peaks for disopyramide, mono-*N*-dealkyldisopyramide, and cyclomethycaine (Fig. 1).

The method was applied for the determination of disopyramide and its metabolite in patient plasma before and after administration of disopyramide phosphate. No interference was noted from commonly utilized cardiac drugs. Quinidine, lidocaine, procainamide, and the more recent β-blockers metoprolol and timolol were eluted at different retention times. Although propranolol and its 4-hydroxy metabolite have the same retention times as disopyramide and its mono-*N*-dealkylated metabolite, respectively, the accurate determination of the two drugs is still possible since disopyramide and its metabolite do not fluoresce while propranolol and its metabolite fluoresce but have no appreciable absorption at 254 nm. Cyclomethycaine is an ideal internal standard since it absorbs at 254 nm and fluoresces at 276-nm excitation with a 340-nm cutoff filter (14).

Major advantages of the proposed method are its simplicity and rapidity. Both disopyramide and its metabolite are determined in a single procedure using a standard single wavelength UV detector. Moreover, propranolol can be determined using the same extraction and chromatographic conditions by the incorporation of a spectrofluorometric detector. Other chromatographic methods require separate conditions for the determination of each drug or even for the determination of metabolites. In addition, the method gave excellent separation without the ion-pair technique, which results in limited use of the column and a decrease in the capacity factor (15).

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Plasma Binding of Benzodiazepines in Humans

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Received December 29, 1978, from the *Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine and Veterans Administration Hospital, Nashville, TN 37203.* Accepted for publication April 5, 1979.

Abstract □ Plasma binding of chlordiazepoxide, diazepam, lorazepam, and oxazepam was determined by equilibrium dialysis in 20 male, healthy volunteers, 25–86 years old. A wide range of binding was observed, with the free fraction varying twofold for lorazepam, fourfold for chlordiazepoxide and diazepam, and over 20-fold for oxazepam. Statistically significant linear relationships were not observed between the degree of binding and age, serum albumin, or total protein for any of the drugs. There was, however, a correlation between the extent of binding for the four drugs. Because of the importance of unbound benzodiazepine levels in eliciting any pharmacological response and also in disposition, consideration of the wide interindividual variability in plasma binding must be made in interpreting pharmacodynamic and pharmacokinetic data.

Keyphrases □ Benzodiazepines—plasma binding, equilibrium dialysis, humans, individual variation, age □ Sedatives—benzodiazepines, plasma binding, equilibrium dialysis, humans, individual variation, age □ Plasma binding—benzodiazepines, humans, individual variation, age

During a study of the effects of age on lorazepam disposition in humans, the results obtained from 11 apparently healthy subjects, 15–73 years old, suggested that the extent of plasma binding of this drug decreased with age (1). This result was in contrast to previous findings with the related 1,4-benzodiazepines, diazepam (2), chlordiazepoxide (3), and oxazepam (4). Accordingly, studies of lorazepam binding were performed with plasma from a larger group of subjects to determine whether the observed trend was real or had occurred by chance. The investigation also provided an opportunity to compare directly the relative plasma binding behavior of the various benzodiazepines.

EXPERIMENTAL

^{14}C-Chlordiazepoxide[1] (59.95 mCi/mmole), ^{14}C-diazepam[1] (13.01 mCi/mmole), ^{14}C-lorazepam[2] (10.79 mCi/mmole), and ^{14}C-oxazepam[2] (5.91 mCi/mmole) were examined for radiopurity by TLC. All except lorazepam were >98% pure. Purification of the latter to this level was achieved by preparative TLC using silica GF plates[3] and a mobile phase of ethyl acetate[4]–ethanol[5]–ammonium hydroxide[4] (5:5:1 v/v). Separate drug standard solutions were prepared in pH 7.4 buffer as follows:

[1] Hoffmann-La Roche, Nutley, NJ 07110.
[2] Wyeth Laboratories, Radnor, PA 19087.
[3] I.C.N. Pharmaceuticals, Cleveland, OH 44128.
[4] ACS grade, Fisher Scientific Co., Pittsburgh, PA 15219.
[5] USI absolute ethyl alcohol, USP-NF reagent.

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Table I—Plasma Binding (Percent) of Benzodiazepines

Subject	Age, years	Diazepam	Oxazepam	Lorazepam	Chlordiazepoxide	Serum Albumin	Total Protein
1	25	91.21	96.13	86.30	92.70	5.00	6.90
2	29	96.94	99.23	92.26	96.26	5.60	7.40
3	29	95.85	98.69	91.52	96.45	—	—
4	29	93.39	98.87	91.45	95.09	5.00	7.20
5	29	95.36	98.84	92.47	96.98	5.70	7.60
6	30	97.32	99.31	92.47	96.88	5.40	7.30
7	31	97.39	97.45	93.35	97.13	5.10	7.80
8	34	96.17	93.33	88.00	91.43	5.50	7.10
9	35	97.57	98.62	92.00	93.08	5.20	6.70
10	35	97.58	98.63	91.04	93.08	—	—
11	35	91.04	90.67	86.30	89.07	4.60	7.40
12	35	97.64	99.29	93.33	96.64	—	—
13	42	94.01	98.00	90.49	94.98	5.00	7.60
14	48	95.57	98.80	87.78	95.79	4.60	6.70
15	68	97.27	99.28	91.65	95.53	—	—
16	68	91.73	95.01	93.36	—	4.40	7.60
17	69	97.21	99.43	91.45	95.47	4.10	6.80
18	83	96.52	98.98	91.03	92.21	4.70	6.70
19	83	93.83	98.33	92.79	92.62	—	—
20	86	97.17	99.56	89.92	96.26	4.80	6.90
Mean	46.15	95.54	97.82	90.95	94.66	4.98	7.18
±*SD*	21.23	2.23	2.33	2.21	2.23	0.46	0.38

Table II—Cross-Correlations of Binding Parameters

	Age	Chlordiazepoxide	Diazepam	Lorazepam	Oxazepam	Serum Albumin	Total Protein
Age		0.063[a]	0.248	0.082	0.065	0.656	0.392
		(0.79)[b]	(0.31)	(0.74)	(0.79)	(0.004)	(0.15)
Chlordiazepoxide			0.634	0.636	0.518	0.308	0.243
			(0.004)	(0.003)	(0.023)	(0.23)	(0.382)
Diazepam				0.743	0.810	0.144	0.169
				(<0.001)	(<0.001)	(0.58)	(0.547)
Lorazepam					0.653	0.328	0.326
					(0.002)	(0.25)	(0.25)
Oxazepam						0.237	0.293
						(0.42)	(0.31)
Serum albumin							0.489
							(0.076)

[a] Correlation coefficient. [b] Level of significance.

chlordiazepoxide, 10 μg/ml in 0.05 *M* tromethamine buffer; diazepam, 2.5 μg/ml, and oxazepam, 10 μg/ml, in 0.067 *M* phosphate buffer; and lorazepam, 1 μg/ml in ethanol.

Blood (25 ml) was collected by venipuncture into heparinized glass-stoppered tubes from 20 normal and apparently healthy male subjects, 25–86 years old, who were not receiving any medication. After separation, appropriate drug standards were added to the plasma to produce concentrations typical of those observed in clinical studies: chlordiazepoxide, 0.1, 0.5, and 1.0 μg/ml; diazepam, 25, 50, and 100 ng/ml; lorazepam, 10, 25, and 50 ng/ml; and oxazepam, 50, 100, and 200 ng/ml.

Plasma binding was determined at room temperature by equilibrium dialysis (5) using semimicrocells[6] and a semipermeable membrane with a molecular cutoff of 12,000–14,000[7]. Plasma (1 ml) was dialyzed against an equal volume of 0.067 *M* phosphate buffer, pH 7.4, for 2 hr (chlordiazepoxide and diazepam) and 4 hr (lorazepam and oxazepam). These times had been shown to be adequate for the attainment of equilibrium. After dialysis was completed, 0.5-ml aliquots of plasma and buffer were analyzed for radioactivity by adding 10 ml of scintillant solution[8] and 20 μl of acetic acid[4] and counting in a liquid scintillation counter[9], using the automatic external standardization procedure to correct for quenching. The extent of plasma binding was then calculated from:

$$\% \text{ bound} = \frac{\text{plasma dpm} - \text{buffer dpm}}{\text{plasma dpm}} \times 100 \qquad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

As previously noted (1, 3, 6), plasma binding of the drugs was apparently not affected by drug concentration over the dose and age ranges studied. Accordingly, the three binding values for each subject were averaged. With all four drugs, considerable interindividual variability in the extent of binding was present (Table I). When expressed in terms of free drug, lorazepam exhibited an almost twofold, chlordiazepoxide and diazepam a fourfold, and oxazepam a 20-fold range between the highest and lowest values. Preliminary studies indicated that the coefficient of variation for binding was <0.7% for all of the drugs, and, therefore, the observed variability did not represent a methodological artifact.

No statistically significant linear correlations were seen betweeen the extent of drug binding with serum albumin, total protein, or subject age. However, there were significant relationships between the binding of the four drugs (Table II). Previous binding studies with human serum albumin indicated that the benzodiazepines are mainly bound to a single class of binding sites (7). Thus, it is not surprising that the latter correlation was observed, although the coefficients of determination are quite small. This result, plus the lack of correlation of binding with serum albumin, suggests that other factors probably are involved in the overall binding phenomenon. The absence of any age-related changes in binding, despite the decrease in serum albumin with aging as noted previously (8) and observed in the present study, is consistent with this hypothesis. Therefore, the earlier observation with regard to lorazepam binding and age (1) was fortuitous.

The unbound benzodiazepine concentration is probably more important than the total drug level with regard to pharmacological response. Moreover, for the investigated drugs, clearance from the circulation and extravascular distribution are determined by the unbound fraction (1–4, 9). Therefore, the wide variability in observed binding suggests that this factor must be considered in the interpretation of pharmacodynamic and pharmacokinetic studies with the benzodiazepines, as exemplified by recent studies on sex differences in chlordiazepoxide disposition (10).

[6] Dianorm equilibrium dialyzing system, Spectrum Medical Industries, Los Angeles, CA 90054.

[7] Spectrapore membrane No. 2, Spectrum Medical Industries, Los Angeles, CA 90054.

[8] Aqueous counting scintillant, Amersham Corp., Arlington Heights, IL 60005.

[9] ISOCAP 300, Searle Analytic Inc., Des Plaines, IL 60018.

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Identification of an Imidazolinium Salt, the Major Product from Reaction of Benzathine with Iodine

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Received February 9, 1979, from the *Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Canada K1A 0L2.* Accepted for publication April 4, 1979.

Abstract □ The isolation and identification of an imidazolinium salt are described. Unambiguous determination of structure was accomplished by independent synthesis. The isolated product interferes in the iodometric assay of the benzathine salts of penicillin.

Keyphrases □ Imidazolinium salts—analysis, product from reaction of benzathine with iodine □ Penicillin V benzathine—analysis, iodometric assay, interference by imidazolinium salt reaction product □ Iodometric assays—penicillin V benzathine, interference by imidazolinium salt

A recent report (1) described the interference of benzathine (I) in the official iodometric assay of penicillin V benzathine as presently described in the "Code of Federal Regulations" (CFR) (2). A high-performance liquid chromatographic (HPLC) method (3) of analysis for formulations containing this drug was suggested subsequently.

The present article describes the isolation and identification of an imidazolinium salt (II) as the product from the reaction of iodine with benzathine.

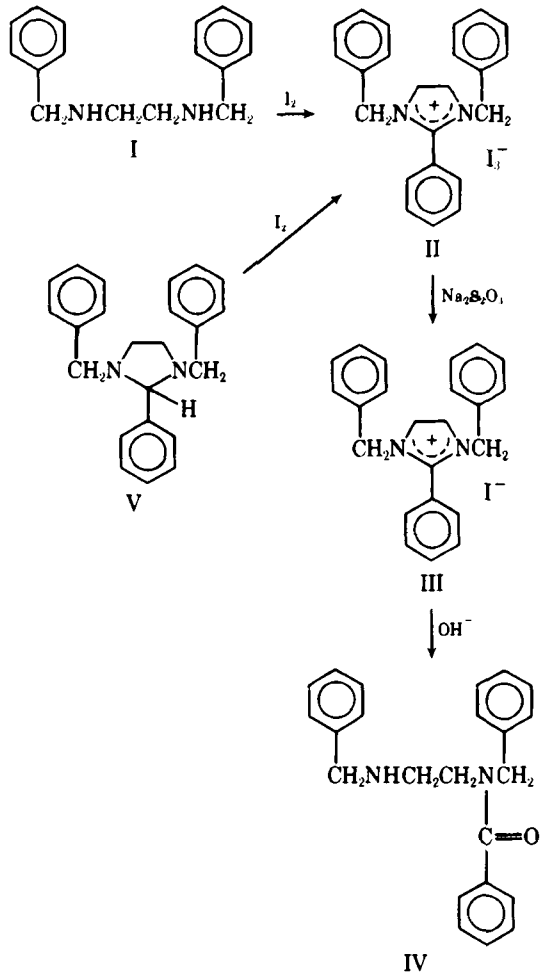
EXPERIMENTAL[1]

1,3-Dibenzyl-2-phenylimidazolinium Triiodide (II)—To 4.0 g of I in 5 ml of methanol at room temperature were added 5-ml aliquots of a methanolic iodine solution (2.5 g in 125 ml); the color was allowed to discharge after each addition. When a reddish color persisted, addition of methanolic iodine was continued until the precipitation of a red product was complete. The red solid was removed by filtration, and recrystallization from methanol–acetone yielded 2.1 g of II as deep-red needles, mp 163.5–165° [lit. (4) mp 165–166°]; NMR (acetone-d_6): δ 4.22 (s, 4, H_2C-4, H_2C-5), 4.68 (s, 4, H_2C-phenyl), 7.42 (s, 10, C-phenyl), and 7.85 (s, 5, phenyl-C-2).

Anal.—Calc. for $C_{23}H_{23}I_3N_2$ (mol. wt. 708.15): C, 39.01; H, 3.27; I, 53.76; N, 3.96. Found: C, 39.18; H, 3.36; I, 53.69; N, 4.05.

Conversion of II to *N*-Benzoyl-*N,N*-dibenzylethylenediamine (IV)—To a slurry of 1.303 g of II in 20 ml of methanol was added a saturated methanolic solution of sodium thiosulfate until a colorless solution was obtained. The solution was evaporated to dryness, and the residue was dissolved in chloroform and evaporated to dryness to give 934 mg of III as a pale-yellow foam; IR (film): 3030, 2915 (C–H), 1590 (C=N⁺<), 1250 (C–N), 1580, 1450, 750, and 700 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 4.17 (s, 4, H_2C-4, H_2C-5), 4.62 (s, 4, H_2C-phenyl), 7.37 (s, 10, C-phenyl), and 7.60–8.13 (m, 5, phenyl-C-2).

To 200 mg of III in 3 ml of methanol was added 3 ml of 3 *N* NaOH, and the solution was heated at 40° for 1.5 hr. Extraction with chloroform



Scheme I

[1] NMR spectra were obtained using Bruker WP-80 and Varian A-60A instruments. IR spectra were obtained on a Beckman IR-20 spectrophotometer. All melting points are uncorrected.

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