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### Authors

Goldstein, SA  
Van Vunakis, H

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# Determination of Fluphenazine, Related Phenothiazine Drugs and Metabolites by Combined High-Performance Liquid Chromatography and Radioimmunoassay

STEVEN A. GOLDSTEIN<sup>2</sup> and HELEN VAN VUNAKIS<sup>3</sup>

Department of Biochemistry, Brandeis University, Waltham, Massachusetts

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## ABSTRACT

Goldstein, Steven A. and Helen Van Vunakis: Determination of fluphenazine, related phenothiazine drugs and metabolites by combined high-performance liquid chromatography and radioimmunoassay. *J. Pharmacol. Exp. Ther.* 217: 36-43, 1981. Antibodies have been produced in rabbits immunized with a fluphenazine succinate-human serum albumin conjugate. By radioimmunoassay it is possible to quantify fluphenazine (FPZ), related phenothiazine drugs and several of their metabolites at the femtomole level. As little as 370 fmol (160 pg) of FPZ can be detected and up to 0.4 ml of plasma can be added to the incubation mixture (final volume = 1.1 ml). The phenothiazine heterocyclic nucleus is immunodominant and determines the specificity of the antiserum. When a parent drug cross-reacts significantly with antibody, its 7-hydroxide, N-oxide and N-10 side chain altered metabolites can also be determined by the assay. The 8-hydroxide, sulfoxide and 7-hydroxyglucuronide metabolites are not detectable unless present in large amounts. High-performance liquid chromatography was used to separate phenothiazine drugs and metabolites. Since the antiserum has broad specificity, a combined high-performance liquid chromatography and radioimmunoassay procedure permits the identification and quantification of a phenothiazine drug and its serologically reactive metabolites. Patterns of high-performance liquid chromatographic elution and extent of immunologic cross-reaction are characteristic for metabolites relative to the parent drug. This procedure offers distinct advantages in the analysis of this complex family of compounds. FPZ was quantitatively extracted from plasma samples obtained from patients receiving FPZ per os. Although large amounts of serological activity were present in the samples 2 to 6 hr after FPZ ingestion, only 2 to 23% was extractable. The major contributors to the serological activity at times greater than 6 hr were FPZ metabolites. In a preliminary application of the combined techniques, FPZ and a metabolite identified as N- $\alpha$ -(trifluoro-methylphenothiazinyl-10)propylperazine were quantified in the organic extract of one plasma sample.

Phenothiazine neuroleptics are widely prescribed, yet their fate and mechanism of action in man have not been clearly elucidated. These drugs are rapidly metabolized to numerous products (Usdin, 1971) which possess varying degrees of antipsychotic effectiveness (Perel and Manian, 1977). Both the therapeutic and undesirable effects of these medications may result from the composite action of the parent drug and its metabolites. Attempts to monitor clinical response by measuring drug levels in physiological fluids may fail if levels of active metabolites are not determined (Creese et

al., 1978). Unfortunately, the accurate quantification of drugs and metabolites is a task that has met with limited success.

Fluphenazine (FPZ) is clinically important because it is potent and long-acting when dispensed as an ester in depot formulations (Ayd, 1978). Current techniques with adequate sensitivity to determine physiological levels of FPZ include gas chromatography (GC)-electron capture detection (Larsen and Naestoft, 1973), GC-nitrogen/phosphorus sensitive detection (Franklin et al., 1978), gas-liquid chromatography (GLC)-nitrogen sensitive detection (Whelpton and Curry, 1976), GLC-electron capture detection (Rivera-Calimlin and Siracusa, 1977), ion-pair partition chromatography-UV detection (Johansson et al., 1976), high-performance liquid chromatography (HPLC)-anodic coulometric detection (Tjaden et al., 1976) and the administration of radiolabeled drug (Schreiber and Grozier, 1973; Whelpton and Curry, 1976). High-performance thin-layer chromatography (HPTLC)-UV reflectance detection (Fenimore et al., 1978), GC-mass spectrometry (Alfredsson et al., 1976), formation of fluorescent derivatives followed by TLC (Kaul et al., 1972) and formation of radiolabeled derivatives followed by selective solvent partition (Efron et al., 1971) have also been used to assay phenothiazine drugs in physiological fluids.

For the most part, these methods can detect only a single drug or an undifferentiated total of drug and metabolites. Endogenous biological materials, co-medications and problems associated with derivitization (Usdin, 1971) have impeded quantitative procedures. Although direct radioimmunoassay (RIA) analyses (Shostak, 1974; Kawashima et al., 1975; Jørgensen, 1978; Sakalis et al., 1978; Wiles and Franklin, 1978; Midha et al., 1979) avoid some of these problems, they are incapable of differentiating between a drug and those serologically reactive metabolites which are present in physiological samples.

In this paper, we report the development of an RIA for FPZ, related phenothiazines and several of their metabolites. This assay was used in conjunction with an HPLC system devised to separate phenothiazine drugs and metabolites. The combined HPLC-RIA technique offers many distinct advantages over previous analytical procedures. The technique obviates the need for administration of radiolabeled drugs to man, does not require chemical derivitization reactions to achieve adequate sensitivity, employs the specificity of an antiserum to overcome interference by endogenous compounds that may contribute to measurements carried out by spectral and physiochemical procedures, has greater selectivity than direct RIA analyses and allows simultaneous determination of a parent compound and its serologically reactive metabolites.

## Materials and Methods

Table 1 lists the abbreviations used to represent phenothiazine drugs and their metabolites. Some of these compounds were generous gifts from the following sources: FPZ, FPZ enanthate and FPZ decanoate, Dr. A. Sudilovsky, Squibb Medical Laboratories, Princeton, NJ; FPZ-SO, Dr. L.F. Traficante, New York University Medical Center, New York, NY; CFaPPP, CFaPPMED, CFaPPHED, CFaPPED and CFaPPA, Dr. U. Breyer, Institut für Toxikologie der Universität Tübingen, West Germany; 7-OH-CPZ, 8-OH-CPZ, 7-MeO-CPZ, CPZ-NO, 7-OGlu-CPZ,

TABLE 1

Phenothiazines and related compounds: inhibition of [<sup>3</sup>H]CPZ-antibody reaction and HPLC retention volumes

Abbreviation	Compound	<i>r</i> <sup>a</sup>	HPLC Retention Volume <sup>a</sup> ml	Substitution					
				C-2	C-7	C-8	S-5	N-10	
FPZ	Fluphenazine	1.00	20.3	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -OH	
	Fluphenazine enanthate	0.40	29.1	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -O-CO-(CH <sub>2</sub> ) <sub>5</sub> -CH <sub>3</sub>	
	Fluphenazine decanoate	0.32	35.0	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -O-CO-(CH <sub>2</sub> ) <sub>8</sub> -CH <sub>3</sub>	
FPZ-SO	Fluphenazine acetate	1.00	20.5	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -O-CO-CH <sub>3</sub>	
CF <sub>3</sub> PP-	Fluphenazine sulfoxide	0.006	11.2	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -OH	
	N-[α-(2-trifluoromethylpheno- thiazinyl-10)-propyl]- -perazine	0.91	29.4	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-CH <sub>3</sub>	
CF <sub>3</sub> PPHED	N'-(β-hydroxyethyl)-ethyl- enediamine	0.63	∞	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -NH(CH <sub>2</sub> CH <sub>2</sub> )NH-(CH <sub>2</sub> ) <sub>2</sub> -OH	
CF <sub>3</sub> PPMED	N'-methylethylenediamine	0.19	∞	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -NH(CH <sub>2</sub> CH <sub>2</sub> )NH-CH <sub>3</sub>	
CF <sub>3</sub> PPED	-ethylenediamine	0.50	∞	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -NH(CH <sub>2</sub> CH <sub>2</sub> )NH <sub>2</sub>	
CF <sub>3</sub> PPA	-amine	0.56	24.4	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -NH <sub>2</sub>	
CPZ	Chlorpromazine	0.63	28.4	Cl	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
CPZ-NO	Chlorpromazine N-oxide	3.80	15.5	Cl	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -NO-(CH <sub>3</sub> ) <sub>2</sub>	
7-OH-CPZ	7-Hydroxychlorpromazine	0.15	22.2	Cl	OH	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	7-Methoxychlorpromazine	0.37	29.3	Cl	OCH <sub>3</sub>	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
8-OH-CPZ	8-Hydroxychlorpromazine	0.009	22.4	Cl	H	OH	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	7,8-Dimethoxychlorpromazine	0.024	22.4	Cl	OCH <sub>3</sub>	OCH <sub>3</sub>	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
CPZ-SO	Chlorpromazine sulfoxide <sup>b</sup>	0.001	19.8	Cl	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
7-OGlu-CPZ	Chlorpromazine 7-hydroxy- glucuronide <sup>c</sup>	<0.001	7.5	Cl	O-Glu	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Perphenazine	5.90	19.8	Cl	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -OH	
	7-Hydroxyperphenazine	0.56	13.9	Cl	OH	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-(CH <sub>2</sub> ) <sub>2</sub> -OH	
	Prochlorperazine	3.30	27.2	Cl	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-CH <sub>3</sub>	
	7-Hydroxyprochlorperazine	0.37	20.5	Cl	OH	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-CH <sub>3</sub>	
	Triflupromazine	0.27	27.2	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	7-Hydroxytriflupromazine	0.036	21.5	CF <sub>3</sub>	OH	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Promazine	0.005	27.5	H	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	2-hydroxypromazine	0.005	21.1	OH	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Trifluoperazine	0.83	26.5	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Thioridazine	0.007	34.0	CF <sub>3</sub>	H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	±Flupenthixol	0.019	21.0		H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Imipramine	0.077	27.3		H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Protryptiline	0.013			H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N-(CH <sub>3</sub> ) <sub>2</sub>	
	Haloperidol	<sup>c</sup>			H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-CH <sub>3</sub>	
	Dopamine	<sup>c</sup>			H	H	H	(CH <sub>2</sub> ) <sub>3</sub> -N(CH <sub>2</sub> CH <sub>2</sub> ) <sub>2</sub> N-CH <sub>3</sub>	

<sup>a</sup> The RI and HPLC analyses were carried out as described in "Materials and Methods."<sup>b</sup> In preliminary analyses of cross-reactivity of standard inhibitor solutions with antibody, an *r* value of 0.017 was determined for CPZ-SO. Based on the antibody affinity for CPZ, FPZ and FPZ-SO, the presence of an impurity was suspected. HPLC-RIA analysis revealed that the sample contained CPZ as a minor impurity (approximately 3%). CPZ-SO, separated from impurities, had an *r* value of 0.001. The cross-reactivity of CPZ-SO with antibody was verified in the same manner.<sup>c</sup> No inhibition was observed for haloperidol or dopamine at 15 and 270 nmol per incubation tube, respectively. Twenty-two percent inhibition was obtained with 1 nmol of 7-OGlu-CPZ per incubation tube.

7-OH-perphenazine, 7-OH-prochlorperazine and 7-OH-triflupromazine, Dr. A. A. Manian, National Institute of Mental Health, Rockville, MD; trifluoperazine, prochlorperazine, promazine, triflupromazine and thioridazine, Dr. A. Mathe, Boston University School of Medicine, Boston, MA; CPZ, CPZ-SO and  $\pm$ flupenthixol, Smith Kline and French Laboratories, Philadelphia, PA; perphenazine, Schering Corporation, Kenilworth, NJ; protryptiline, Merck Sharp and Dohme, Rahway, NJ; imipramine, Ciba Pharmaceutical Company, Summit, NJ; haloperidol, McNeil Laboratories, Inc., Fort Washington, PA, [ $^3$ H]CPZ (49Ci/ mmol), with methyl groups labeled, was purchased from Schw11Z Mann, Orangeburg, NY. [ $^3$ H]FPZ (15.2Ci/mmol), generally labeled, was obtained from the Israeli Atomic Energy Commission, Negev, Israel. Fluphenazine acetate was synthesized by the method of Whelpton and Curry (1976).

1-Ethyl-3-(dimethylaminopropyl)carbodiimide-HCl (CDI) was purchased from the Ott Chemical Company, Muskegon, MI, human serum albumin (HSA) from Miles Laboratories, Inc., Elkhart, IN, horse serum from Grand Island Biological Company, Grand Island, NY, HPLC grade MeOH from Fisher Scientific Company, Medford, MA. All other chemicals were reagent grade and were used without further purification. All procedures reported in this study were carried out under muted gold fluorescent lights purchased from Sylvania Inc., Danvers, MA. Phenothiazines were stored in the dark at 0°C.

Preparation of the conjugate for immunization. FPZ was succinylated at the terminal ,8-hydroxyethyl function of the piperazine side chain and covalently linked to free primary amino groups of HSA by formation of an amide bond. To FPZ,2HCl (102 mg, 0.2 mmol) dissolved in 2.0 ml of anhydrous pyridine was added succinic anhydride (20 mg, 0.2 mmol) and the reaction mixture allowed to stir overnight at 25°C. An additional 25 mg of succinic anhydride was added and stirring continued for 60 min. The reaction mixture was applied directly to preparative silica gel plates and separated using EtOAc/MeOH/ NILOH, 65:35:11 by volume, as solvent; (FPZ,  $r_r$ = 0.70; FPZ succinate,  $r_r$ - 0.39).

To prepare the conjugate, FPZ succinate (6.5 mg) dissolved in 1 ml of 10% aqueous pyridine was added to 25 mg of HSA in 0.1 ml of water (pH adjusted to 7.0). CDI (25 mg) was added and the reaction mixture stirred overnight at 25°C. The conjugate was separated from unreacted materials by gel filtration on a Sephadex G-50M column (35 X 1.5 cm), using saline-phosphate buffer (0.15 M NaCl and 0.005 M sodium-phosphate, pH 7.4) as the solvent. Fractions (3 ml) were collected and the contents of tubes 12 to 15 were pooled based on optical density measurements. The combined fractions were exhaustively dialyzed against the eluting solvent at 4°C. The extent of substitution was 20 mol of hapten per mole of HSA as measured by the difference in absorbance between solutions of unsubstituted HSA and the conjugate at 256nm.

Immunization. Aliquots of the conjugate were emulsified in two volumes of complete Freund's adjuvant by a Sorvall Omnimixer at 0°C. Four New Zealand albino rabbits were injected i.m. and in toe pads on day 1. All subsequent injections were administered i.m. Animals received 1 mg of the conjugate on days 1, 8, 15 and 50 and 2.5 mg on day 114. Blood drawn on day 122 from one rabbit was used in this study. All rabbits produced antibodies that bound the radiolabeled hapten.

RIA procedure. The antibodies produced to the FPZ succinate-HSA conjugate bound [<sup>3</sup>H]CPZ and [<sup>3</sup>H]FPZ. When these studies commenced only [<sup>3</sup>H]CPZ of high specific activity was available and was used in all experiments unless otherwise noted. Separation of free radiolabeled antigen from antibody-bound labeled antigen was accomplished by a double antibody technique similar to that used for other assays (Van Vunakis and Levine, 1974). All dilutions were made with a 10% horse serum isotris buffer (10 ml of horse serum: 90 ml of isotonic Tris buffer-0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4 at 20°C).

Performed in duplicate, each assay mixture contained 1.0 ml of buffer, 0.1 ml of a standard dilution of inhibitor or of a sample to be analyzed, 0.1 ml of radiolabeled antigen dilution (30,000 dpm/0.1 ml of [<sup>3</sup>H]CPZ) and 0.1 ml of the immune serum (diluted 1:500). After incubation for 60 min at 37°C, 0.1 ml of a normal rabbit serum (diluted 1:25) and 0.1 ml of a goat antirabbit  $\gamma$ -globulin (diluted to be in the equivalence zone with respect to rabbit  $\gamma$ -globulin) were added to each tube. After standing at 4°C for 16 to 18 hr, precipitates were collected by centrifugation. The supernatant fluid was decanted and the walls of the tubes dried of excess liquid. The precipitates were dissolved in 0.2 ml of 0.1 N NaOH and 2.8 ml of scintillation fluid {2,5-diphenyloxazole, 5 gm/l, p-bis-[2-(5-phenyloxazolyl)]-benzene, 0.2 gm/l, BBS-3 (Beckman Instruments, Inc., Fullerton, CA) (12% v/v), toluene} was added. The samples were counted in a Packard Tricarb model 3320 liquid scintillation counter. When no inhibitor was present, 12,000 dpm were precipitated with the immune serum, whereas 300 dpm were precipitated nonspecifically in the presence of normal rabbit serum. It is also possible to separate free and antibody-bound [<sup>3</sup>H]CPZ by precipitation of the protein with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Chard, 1980).

HPLC. A Waters Associates model 404 LC equipped with a model 440 UV detector set at 254 nm, a model 660 solvent programmer and a model U6K pressureless injection system were used. A Waters Associates C<sub>18</sub>- $\mu$ Bondapak column (0.25 inch outer diameter x 1.0 ft) was used for separation of FPZ, related phenothiazines and several of their metabolites. A precolumn (0.25 inch outer diameter X 1.6 inch) packed with C<sub>18</sub>-Corasil was used to protect column integrity. Aqueous solvents for HPLC were degassed and then filtered through 0.45 Millipore filters before use. The eluting solvents were 0.01 M sodium phosphate, pH 7.4 at 25°C and methanol. The program of elution was linear with respect to increasing methanol, 65 to 90% over 20 min. Flow rate was constant at 1.0 ml/min. The ethylenediamine metabolites of FPZ are retained on the column under these conditions. These compounds elute from C<sub>18</sub>- $\mu$ Bondapak columns when other solvent systems are used: 70% tetrahydrofuran/30% 0.01 M sodium phosphate buffer, pH 7.4 at 25°C; or 70% methanol/30% magnesium acetate buffer (9%), pH 3.5 at 25°C.

HPLC-RIA procedure. Fractions collected during chromatographic separation were diluted in buffer and analyzed directly by RIA when the concentration of serologically reactive material in a sample was high, i.e., greater than 100 pmol of FPZ equivalents/ml of HPLC eluent. Otherwise, samples were dried in a Virtis lyophilizer and reconstituted in buffer for assay and storage.

Plasma samples. Blood from control subjects was donated by workers in this laboratory. The patient plasma was from the source previously described (Sakalis et al.,

1978); treatment histories are described in table 2. Plasma (1 ml) was diluted with 3 ml of H<sub>2</sub>O in 16 x 150 mm screw cap culture tubes and the pH adjusted to 9.0 with NaOH. n-Heptane (8 ml) containing 2% isoamyl alcohol was added, and the tubes shaken vertically on a mechanical shaker at 200 cycles/min for 20 min at 4°C. After centrifugation for 10 min at 1500 x g, the organic phase was transferred to a second tube. The extraction of the aqueous solution was repeated, and the combined organic extracts were dried under nitrogen at 25°C. The residue was resuspended in 3 ml of MeOH (containing 0.1 ml of 0.01 N HCl) or horse serum buffer. Twenty-five control human plasma samples were supplemented with [<sup>3</sup>H]FPZ and/or unlabeled FPZ. The efficiency of the extraction procedure was 91% for 10 pmol and greater than 95% for 20 to 66,000 pmol as measured by radioactivity or RIA. Approximately 5% of the activity was detected in the aqueous phase.

## Results

Sensitivity and accuracy of the RIA. Immunized rabbits produced antibodies with sufficiently high affinities to perform the RIA in a buffer containing 10% horse serum. This medium served to protect labile phenothiazines from decomposition and reduce their adsorption to vessel surfaces. Competitive inhibition of the [<sup>3</sup>H]CPZ- antibody interaction by unlabeled FPZ is described by a standard inhibition curve (fig. 1) that is approximately linear from 20 to 80% inhibition, i.e., 0.16 ng (0.37 pmol) to 3.3 ng (7.5 pmol) of FPZ per incubation tube (logarithmic curve fit correlation  $r = 0.999$ ). FPZ (0.16 ng), FPZ enanthate (0.50 ng), FPZ decanoate (0.67 ng), perphenazine (0.025 ng), trifluoperazine (0.18 ng), prochlorperazine (0.04 ng), triflupromazine (0.48 ng) and CPZ (0.19 ng) were reproducibly detected.

The intra- and interassay coefficients of variance did not exceed 7.2 and 9.7%, respectively, over a concentration range of FPZ which gave 20 to 80% inhibition (fig. 1). Within the same concentration range, the amount of FPZ added and the amounts measured by RIA correlated quantitatively.

Identical standard inhibition curves were obtained when FPZ was assayed in the presence of 10  $\mu$ .l of methanol, 10  $\mu$ .l of heptane, 0.1% ascorbic acid as an antioxidant or horse serum buffer. Neither endogenous materials in physiological samples nor low concentrations of solvents from chromatographic or extraction procedures interfered with the antigen-antibody reaction. Assays carried out in the presence of up to 0.4 ml of human plasma or 0.1 ml of red blood cell lysate were shown to be independent of both FPZ concentration and the amount of sample analyzed. When plasma was assayed directly, the limit of detection was 0.93 pmol of FPZ per ml.

Antibody specificity: drugs. The amount of FPZ required to inhibit the antibody binding of [<sup>3</sup>H]CPZ by 50% ( $I_{50}$ ) was  $1.65 \pm 0.10$  pmol ( $0.72 \pm 0.04$  ng). The ratio  $r$  defined as

$$r = \frac{I_{50} \text{ fluphenazine}}{I_{50} \text{ test compound}}$$

is reported for each test compound to facilitate comparison of antibody affinity for that compound relative to FPZ and other test compounds, table 1. An  $r$  value of 0.1 indicates that, on a molar basis, the test compound competes only 10% as effectively as FPZ for the antibody sites.

All eight C-2-CF<sub>3</sub> and C-2-Cl substituted phenothiazine drugs studied can be sensitively quantified by RIA, despite differences in their N-10 side chain structures (table 1). Effective inhibition of the immune reaction is also exhibited by esters of FPZ (i.e., FPZ decanoate,  $r = 0.32$ ) and by those drugs with N-10 side chain groups less complex than that of FPZ (i.e., triflupromazine,  $r = 0.27$ ) or perphenazine (i.e., CPZ,  $r = 0.63$ ).

Differences in specificity are observed for drugs containing identical alkylamino side chains but different substituents at position 2 of the aromatic nucleus. Antibody binding of Cl rather than CF<sub>3</sub> substituted drugs is slightly favored, whereas diminished binding of an unsubstituted analog is observed, i.e., CPZ ( $r = 0.63$ ), triflupromazine ( $r = 0.27$ ) and promazine ( $r = 0.036$ ).

Among those compounds which differ from FPZ in the structure of their tricyclic nucleus,  $\pm$ flupenthixol is bound only 5-fold less effectively than FPZ, although olefinic carbon replaces the N-10 nitrogen. Imipramine ( $r = 0.077$ ) and protryptiline ( $r = 0.013$ ), each with a seven-membered central ring, are also bound with marked avidity when compared to promazine ( $r = 0.036$ ). Haloperidol, dopamine and *o*-trifluoromethylaniline exhibit no inhibition at concentrations greater than 15 nmol ( $r < 0.0002$ ).

Antibody specificity: metabolites. The cross-reactivities of metabolites follow consistent trends. Biotransformation of N-10 side chain structures does not significantly alter antibody affinities (table 1). Metabolites of FPZ (Breyer et al., 1974a,b) which result from dealkylation of its N-10 P-hydroxyethylpi-perazine side chain are effective inhibitors of the immune reaction: CFaPPP ( $r = 0.91$ ), CFaPPHED ( $r = 0.63$ ), CFaPPED ( $r = 0.50$ ), CFaPPA ( $r = 0.56$ ) and CFaPPMED ( $r = 0.19$ ). Oxidation of nitrogen in the N-10 dimethylamino side chain of CPZ yields another effective inhibitor, CPZ-NO ( $r = 3.8$ ).

Metabolites which result from oxidation of the heterocyclic nucleus show decreased antibody affinities, as exemplified by CPZ and its metabolites (fig. 1): CPZ ( $r = 0.63$ ), 7-OH-CPZ ( $r = 0.15$ ) > 8-OH-CPZ ( $r = 0.009$ ), CPZ-SO ( $r = 0.001$ ) and 7-OGlu-CPZ ( $r < 0.0001$ ). Hydroxylation of position 7 yields metabolites which inhibit 4- to 11-fold less effectively than their unoxidized congeners. The 7-hydroxyglucuronide and the sulfoxide metabolites, considered to be pharmacologically inactive (Creese et al., 1978; Dutton et al., 1977), are bound weakly by the antibodies.

Interaction of the [<sup>3</sup>H]CPZ preparation with a subset of antibodies in the antiserum can explain the observation of greater avidity for C-2-Cl rather than C-2-CF<sub>3</sub> substituted compounds. However, when [<sup>3</sup>H]FPZ became available and was used for RIA, only minor changes in specificity and sensitivity resulted. Determined  $r$  values were: FPZ (1.0), perphenazine (3.5), trifluoperazine (0.91), triflupromazine (0.78) and CPZ (0.63).



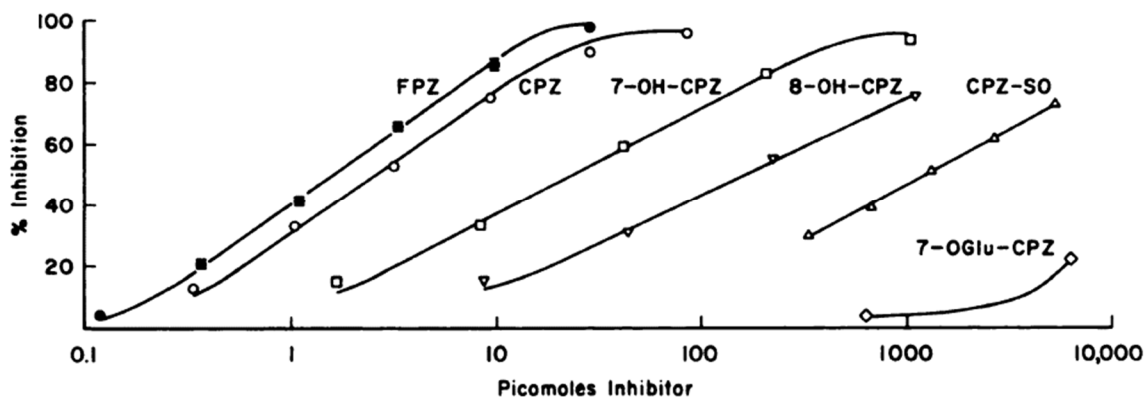


Fig. 1. Inhibition of the [3H]CPZ and FPZ succinate-HSA antibody reaction by FPZ, CPZ and CPZ metabolites. The FPZ inhibition curve represents the  $11 \text{ mean} \pm \text{S.D.}$  for five independent determinations. The RIA was performed as described under "Materials and Methods."

Separation of phenothiazines by HPLC. Reverse phase HPLC effectively separates a phenothiazine drug and its derivatives (table 1). Elution order is primarily a function of compound polarity. Compounds with identical C-2-CFJ tricyclic nuclei show the effect of N-10 side chain moieties on elution volumes (milliliters): FPZ (20.3), CFJPPA (24.4), trifluoperazine (26.5), triflupromazine (27.2), FPZ enanthate (29.1), CFJPPP (29.4) and FPZ decanoate (35.0).

Patterns of elution for metabolites which result from oxidation are shown by the CPZ series: 7-OGlu-CPZ (7.5), CPZ-NO (15.5), CPZ-SO (19.8), 7-OH-CPZ (22.2), 8-OH-CPZ (22.4) and CPZ (28.4). All derivatives resulting from hydroxylation of positions 2, 7 or 8 elute in volumes 70 to 80% as great as their unoxidized parent compound (table 1).

Carbon 2 substituents had little effect on the retention of those compounds available for study. This is shown by the N-10-propyldimethylamino side chain series, C-2-CF3 (triflupromazine, 27.2), C-2-H (promazine, 27.5) and C-2-Cl (CPZ, 28.4).

HPLC-RIA procedure. Figure 2 shows the separation of FPZ, FPZ-SO, FPZ enanthate, CFJPPA and CFJPPP by HPLC and their quantification by RIA. The results are expressed as FPZ equivalents, since the percent inhibition of the [3H]CPZ-antibody reaction by serologically reactive materials was related to the standard inhibition curve obtained for FPZ. To obtain the absolute quantity of a test compound, the percent inhibition can be related to a standard inhibition curve constructed with known amounts of test compound, or the appropriate  $r$  value at Iso can be used (table 1). The  $r$  values can also be used to obtain estimates of absolute quantities of test compounds at levels of inhibition other than 50%, since standard inhibition curves for FPZ and test compounds are approximately parallel from 20 to 80% inhibition.

When control human plasma samples were extracted and analyzed by the HPLC-RIA procedure, no serological activity was observed, indicating that neither endogenous materials nor solvent residues interfered with the assay. When FPZ was applied to the column (2.5-14,200 pmol), greater than 95% was recovered, and serological activity emerged only at the position expected of the FPZ peak. In the overall procedure (i.e.,

extraction, HPLC and RIA), recoveries of FPZ were greater than 90%. When these samples were analyzed in an unrelated immune system (i.e., cotinine-anticotinine; Langone et al., 1973), no serological activity was observed.

Disposition of FPZ in plasma. The organic and aqueous phases resulting from extraction of plasma samples by the procedure shown to remove essentially all FPZ were analyzed by RIA. Although large amounts of serological activity were present in all plasma samples from patients who had ingested FPZ  $\cdot 2\text{HCl}$  2 to 6 hr earlier, only 2 to 23% was extractable and could have been unaltered FPZ (table 2).

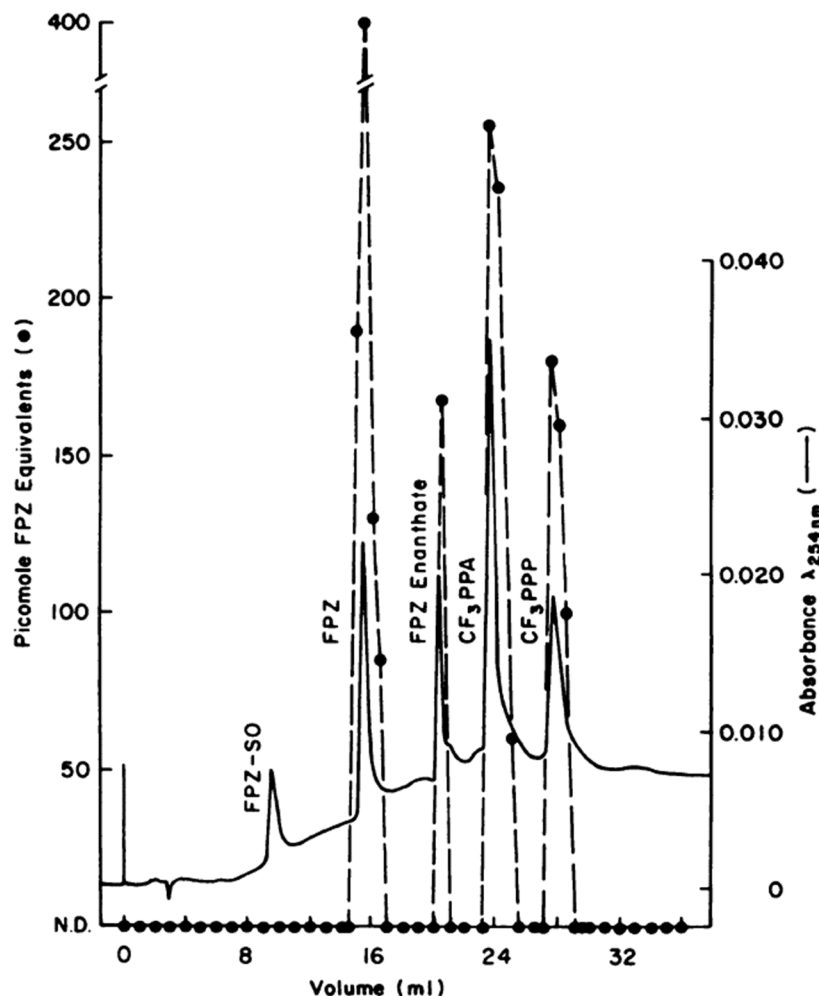


Fig. 2. HPLC-RIA analysis  $\langle \bullet \rangle$  with spectral trace  $(-)$ , of FPZ and four of its derivatives. A solution (50  $\mu\text{l}$ ) containing FPZ (0.82 nmol), FPZ-SO (0.80 nmol), FPZ enanthate (0.45 nmol), CF<sub>3</sub>PPA (1.20 nmol) and CF<sub>3</sub>PPP (0.52 nmol) was applied to the column. Fractions of eluent were collected, lyophilized, reconstituted in horse serum buffer and analyzed by the RIA, as described under "Materials and Methods." N.D. signifies less than 0.37 pmol of FPZ equivalents. Elution volumes are not identical to those reported in table 1 because a different C<sub>18</sub>- $\mu$ Bondapak column and a linear gradient (70 to 100% methanol/0.01 M sodium phosphate, pH 7.4 at 25°C) was used. Although absolute elution volumes vary with different C<sub>18</sub>- $\mu$ Bondapak columns, the relative pattern of elution of a parent compound and its derivatives remains unaltered.

The organic phase from one plasma extract was subjected to HPLC-RIA analysis (Patient F', t = 2-6 hr, fig. 3). The complexity of the absorbance profile obtained was characteristic of extracts of plasma samples from patients and control subjects. Contribution by unrelated compounds to absorbance measurements precluded accurate quantification of FPZ or its metabolites by spectral analyses. However, RIA analysis of eluent fractions showed that at least 96% of the serological activity present in the organic phase could be accounted for by materials which eluted in three regions of the chromatogram (i.e., 78 pmol of FPZ equivalents were recovered of 81 pmol of FPZ equivalents applied to the column).

Region A (3-8 ml) was heterogeneous (fig. 3). Peak B had an elution volume identical to FPZ, and the identity of this compound was confirmed by co-elution with labeled and unlabeled drug. Peak C had an elution volume identical to CFJPPP. Peak C was also found to accumulate as a metabolic product of in vitro metabolism of [3H]FPZ by rat liver microsomes. In the latter experiments, sufficient quantities of this material were isolated by chromatography to study its absorbance characteristics, cross-reactivity with antibody and radioactivity content. An r value of 0.91 was determined for the isolated material by comparing the FPZ equivalents assessed by RIA and levels calculated from absorbance measurements. This supports the tentative identification of peak C as CFaPPP (CF3PPP, r = 0.91, table 1). Similar metabolic profiles were observed in analyses of plasma samples from two other high dose patients. The chromatogram from one patient contained additional serologically active peaks which eluted separately in 24, 34 and 60 ml of solvent, respectively.

Equilibrium dialysis of the aqueous phase (Patient F') against normal human plasma showed that 96% of the serologically active materials were dialyzable and, therefore, FPZ metabolites do not appear to be covalently bound to plasma constituents. HPLC-RIA analysis of materials isolated from the aqueous phase by equilibrium dialysis against water showed serological activity. only in region A of the chromatogram. Preliminary studies suggest that a portion of these isolated materials are conjugates of FPZ and its metabolites.

Plasma samples from patients receiving both low and high doses of FPZ-2HC1 were studied in order to facilitate development of the HPLC-RIA methodology. The parameters outlined above indicate that determinations of phenothiazine drugs and their known metabolites, present in samples from patients receiving commonly administered doses of the drugs, should be readily accomplished.

TABLE 2

FPZ equivalents (Eq) In plasma samples of patients receiving the drug •

Patient <sup>a</sup>	Plasma (pmol of FPZ Eq/ml)		Organic Phase (pmol of FPZ Eq)		Aqueous Phase (pmol of FPZ Eq)	
	t = 0 hr	t = 2-6 hr	t = 2-6 hr	%	t = 2-6 hr	%
C	N.D. <sup>c</sup>	832	41	(4.9)	774	(93.0)
D	N.D.	1,210	98	(8.0)	1,010	(83.5)
E	N.D.	502	117	(23.3)	359	(71.5)
F	N.D.	1,610	39	(2.4)	1,550	(96.3)
F'	22,400	39,900	1,950	(4.9)	36,900	(92.5)

- Plasma samples were extracted, dried, reconstituted and analyzed by RIA, as described under "Materials and Methods."
- Alter one week of placebo, samples were drawn from all patients before (I - 0hr) and alter (I - 2-6 hr) 25 mg of FPZ·2HCl had been administered orally (Sakalls et al., 1978). Plasma samples from Patient F were also drawn on day 28 of therapy (F'), before and alter 250 mg of FPZ·2HCl had been administered orally. Patient F received 25 mg t.i.d., increased progressively in 2 weeks to a maintenance dose of 250 mg t.i.d.

c N.D., <0.37 pmol of FPZ Eq/0.4 ml of plasma.

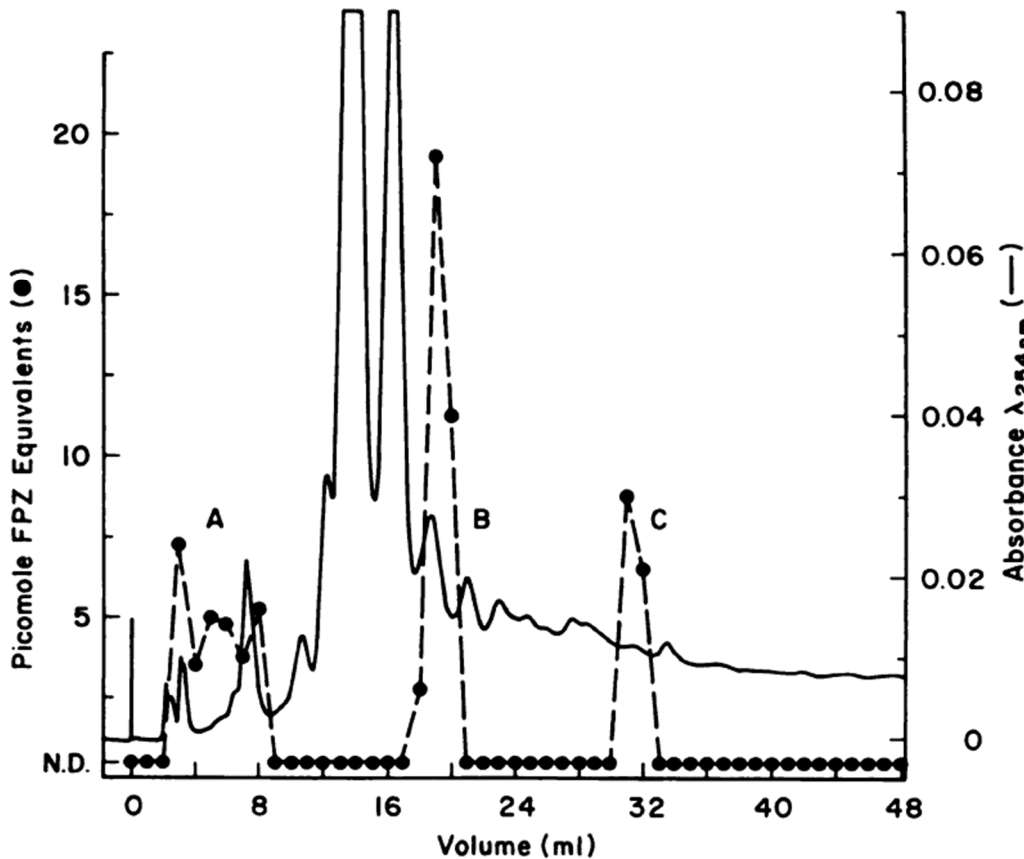


Fig. 3. HPLC-RIA analysis (•) with spectral trace (---), of an extracted plasma sample (organic phase). An aliquot (125  $\mu$ I, 81.25 pmol of FPZ equivalents) of the plasma extract (Patient F', t - 2-6 hr), reconstituted in MeOH/0.01 N HCl, was separated by HPLC and analyzed by RIA, as described under "Materials and Methods." For identification of serologically active materials quantified, see text. N.D. signifies less than 0.37 pmol of FPZ equivalents.

## Discussion

Rabbits immunized with the FPZ succinate-HSA conjugate produced antibodies with specificities directed primarily toward the heterocyclic nucleus of the hapten. RIA allowed sensitive quantification of eight phenothiazine drugs and many of their metabolites after they had been separated by HPLC. The combined HPLC and RIA procedure can separate, identify and quantify a single compound, or simultaneously monitor several, in extracts of physiological samples. It was first used to determine individual compounds in complex mixtures of closely related metabolites by Langone et al. (1975). Such a procedure is needed for clinical and experimental studies of the phenothiazine neuroleptics. Thirty metabolites of CPZ have been identified (Usdin, 1971), and derivatives of FPZ may be more numerous (Breyer et al., 1974b).

Attempts to accurately quantify individual phenothiazine compounds in physiological samples by direct RIA have achieved only limited success. Broadly reactive antisera have been produced despite the use of several different immunizing conjugates. The complex metabolism and lability of these drugs have compounded the problem. When direct RIA determinations have been conducted to assess parent drug levels, immunoreactivity was found in plasma samples for days after drug administration. However, no independent method was used to confirm the contention that the measured activity was due solely to the parent drug (Kawashima et al., 1975; Jørgensen, 1978; Wiles and Franklin, 1978; Midha et al., 1979; Wiles and Gelder, 1980).

While peak concentrations of serological activity are present in plasma samples obtained from patients 2 to 6 hr after FPZ ingestion (Sakalis et al., 1978), we have shown that only 2 to 23% of the activity can be attributed to unaltered FPZ. Unidentified FPZ metabolites that are not extractable from plasma samples at pH 9.0 are the major contributors to the serological activity.

In a previous report (Sakalis et al., 1978), our RIA was used to assess possible correlations between total immunoreactivity in plasma samples and clinical response in patients ingesting FPZ. A direct relationship between dose and plasma level was observed, but no relationship between plasma level and clinical response or extrapyramidal side effects could be established. Recently, a positive correlation between plasma immunoreactivity (as detected by another direct RIA for FPZ) and prolactin levels was obtained in patients receiving FPZ (Wiles and Gelder, 1980). It was suggested, therefore, that circulating FPZ and its active metabolites had been estimated. However, unless a fortuitous relationship exists between the physiological activities of drugs and metabolites and their antibody affinities, direct RIA analysis cannot provide an accurate estimate of pharmacologically active compounds or yield a value which is necessarily relevant to a measurable effect. Without the benefit of an independent method to establish the identity of assayed materials, the significance of serological activity measured by analyzing physiological samples directly by RIA remains unclear.

It has been postulated that clinical response to neuroleptic drugs may be predicted by detection of those compounds which compete with [<sup>3</sup>H]haloperidol in binding to dopamine receptor preparations (Creese et al., 1978). To measure effective dose levels by any method and study their correlation with patient response, it will be necessary to identify physiological samples which reflect levels of parent drug and active metabolites at their active sites (cf., Perel and Manian, 1977).

Whelpton and Curry (1976) identified FPZ, FPZ-SO and 7-OH-FPZ primarily as conjugates in urine, free FPZ and 7-OH-FPZ in feces, but only unmetabolized FPZ and the "virtual absence" of conjugates in the plasma of a patient receiving [14C]-FPZ. These compounds have also been identified as excretion products in urine, feces or bile of dog and rhesus monkey, after [14C]FPZ or [14C]FPZ enanthate was administered (Dreyfuss et al., 1971). The excretion of CFaPPA-80 in the urine of human, rat and dog (Breyer et al., 1974a) and tissue accumulation of CFaPPP, CFaPPMED, CFaPPED and CFaPPHED in rats receiving FPZ (Breyer et al., 1974b) have also been reported.

In preliminary analyses by the HPLC-RIA procedure of plasma samples from patients receiving high doses of FPZ•2HCl, we have found evidence that FPZ and CFaPPP constitute a minor extractable fraction of the total serologically active materials present. The major portion of the serological activity in plasma samples from patients receiving either high or low doses of FPZ remains in the aqueous phase. The fact that such unidentified metabolites are immunologically reactive can facilitate their detection. Once isolated and purified, their structures can be elucidated by classical chemical and enzymatic techniques.

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Send reprint requests to: Dr. Helen Van Vunakis, Department of Biochemistry , Brandeis University, Waltham, MA 0215-c.

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<sup>2</sup> Recipient of the Bachelor's-Master's Honor Degree from the Department of Biochemistry, Brandeis University, 1978. Present address: Harvard Medical School, Boston, MA 02115.

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