Application of Ion-Pair Methods to Drug Extraction from Biological Fluids II: Quantitative Determination of Biguanides in Biological Fluids and Comparison of Protein Binding Estimates

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Abstract ☐ Simple methods for the quantitative determination of the biguanides (buformin, metformin, and phenformin) in blood, plasma, bile, and cerebrospinal fluid were based on the extraction of the 1:1 bromthymol blue ion-pair into methylene chloride. The spectrophotometric determination of the biguanide in the aqueous solution obtained by freeing it from the ion-pair by the addition of excess tetrabutylammonium hydroxide to the organic phase and reextracting gave sensitivities of 0.2 mcg./ml. Since direct extraction precipitated proteins and irreversibly coprecipitated biguanides, the proteins were removed from blood plasma either by precipitation with trichloroacetic acid or by centrifugal ultrafiltration. The biguanides remained in solution under these circumstances. It was necessary to do a prior extraction of bile with methylene chloride to remove interferences before applying the ion-pair method with the subsequent assay procedures. Buformin and phenformin were more readily extractable as free bases from highly alkaline aqueous solutions by 1:1 chloroform-*tert*-amyl alcohol than methylene chloride, whereas the highly lyophilic metformin was inextractable. The partition coefficients of buformin and phenformin in such systems were determined as functions of sodium hydroxide concentrations, and the pK'a values in water at 25° were estimated as 13.0. There was no significant difference between the 50% centrifugal ultrafiltration method and the equilibrium dialysis method in determining the extent of protein binding of biguanides in both dog and human plasma, *i.e.*, about 7–10%. Fifty percent ultrafiltration of biguanide-spiked blood or plasma is the preferred method over complete filtration for the determination of protein and erythrocyte binding. The latter tends to coprecipitate soluble biguanides and overestimates the extent of protein binding. There was no significant binding of the biguanides to the erythrocytes of whole human and dog blood.

Keyphrases ☐ Ion-pair drug extraction—biguanides from biological fluids ☐ Biguanides—analysis in biological fluids, ion-pair extraction, comparison of protein binding estimates ☐ Buformin—analysis in biological fluids, ion-pair extraction, protein binding estimates ☐ Metformin—analysis in biological fluids, ion-pair extraction, protein binding estimates ☐ Phenformin—analysis in biological fluids, ion-pair extraction, protein binding estimates ☐ Protein binding estimates—buformin, metformin, phenformin, dog and human plasma ☐ Bromthymol blue ion-pair extraction—biguanides from biological fluids

A simple method for the quantitative determination of the three biguanides (metformin, buformin, and phenformin) in water and human urine was described in the previous paper of this series (1). This method was based on the ion-pair extraction of the biguanide into methylene chloride with bromthymol blue, with the subsequent reextraction into water of the biguanide freed from its ion-pair with bromthymol blue by tetrabutylammonium hydroxide. The biguanide in this aqueous solution was determined spectrophotometrically at 232 nm.

This ion-pair method was not directly applicable to the determination of the three biguanides in plasma because of the presence of plasma proteins. The plasma proteins were too easily denatured by organic solvents such as methylene chloride, chloroform, and benzene. Also, the denatured proteins apparently coprecipitated most large cations, such as the biguanides. Such precipitation was observed, and no ion-pair of bromthymol blue–biguanide could be extracted from biguanide-spiked plasma by the above-mentioned organic solvents.

Since proteins may be removed either by the treatment of plasma with a strong acid such as trichloroacetic acid or by ultrafiltration through a semipermeable membrane, it was thought that the above-mentioned ion-pair procedures could be applied to the determination of biguanides in plasma subsequent to such methods of protein removal.

Since De Waard (2) first used centrifugal force for the process of ultrafiltration, improved apparatus and techniques have been developed and widely used in protein–drug binding studies (3–6). Although it has been argued that this method may be less quantitative than dialysis because of the continually changing protein concentration and the accumulation of proteins on the membrane surface (7–10), it has been shown to estimate the extent of drug–protein interaction in agreement with dialysis methods (11–14). The advantages of this centrifugal ultrafiltration method are that it is simple and rapid. Recently, a high flux, preformed, ultrafiltration membrane formulated into a rigid and strong cone configuration became commercially available. These ultrafiltration cones have been shown to give results in excellent agreement with those determined by other methods for the protein binding of calcium (15).

This paper presents the methods developed for the analyses of biguanides in whole blood and plasma; it also presents and compares the results of protein and erythrocyte binding studies by the ultrafiltration and equilibrium dialysis methods in the dog and man.

EXPERIMENTAL

Materials and Reagents—The materials used and the preparation of 10^{-2} *M* bromthymol blue and pH 7.5 buffer were reported previously (1).

Equipment—Spectral readings were recorded on a spectrophotometer[1]. Polypropylene centrifuge tubes[2] with screw caps and different capacities (30, 50, and 85 ml.) were used. These tubes could be sealed tightly so that there was no solution loss due to the vapor pressure of the organic solvent (methylene chloride) on shaking[3]. The use of these polypropylene tubes prevented the loss of samples which was encountered when glass tubes were used for the rapid

[1] Cary 15 recording spectrophotometer, Cary, Calif.
[2] The Nalge Co., Inc., Rochester, N. Y.
[3] Reciprocating, variable speed shaking machine (S-74070), Sargent-Welch, Birmingham, Ala.

Table I—Partition Coefficients[a] of Biguanides

		Methylene Chloride/Water System									
	[NaOH]:	0.00	0.005	0.01	0.02	0.05	0.10	0.20	0.40	0.80	
Phenformin		0.01	0.03	0.06	0.08	0.17	0.24	0.32	0.43	0.57	
Buformin		—	—	—	—	0.03	0.03	0.04	0.07	0.10	
Metformin		—	—	—	—	0.00	0.00	0.00	0.01	0.01	
		(1:1) Chloroform–*tert*-Amyl Alcohol/Water System									
	[NaOH]:	0.004	0.008	0.016	0.032	0.056	0.08	0.16	0.24	0.32	0.64
Phenformin		0.18	0.32	0.55	0.80	1.11	1.08	1.76	1.92	2.40	2.37
	[NaOH]:	0.004	0.016	0.04	0.08	0.16	0.32	0.48	0.64	0.80	
Buformin		0.14	0.23	0.33	0.46	0.81	1.17	1.48	1.37	1.42	
Metformin		0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.045	0.05	

[a] The oil/water partition coefficient of the biguanides was calculated from $0.2\,[(R_1 - R_{b_2}) - (R_2 - R_{b_2})]/(R_2 - R_{b_2})$, where R_1 was the spectrophotometric absorbance of a biguanide at 232 nm. in the aqueous solution before extraction (about 1.30) and R_2 was the absorbance after extraction of 4.0 ml. of aqueous biguanide solution with 20.0 ml. of methylene chloride or 20.0 ml. of 1:1 chloroform–*tert*-amyl alcohol as read against a water blank. The correction factors against the same blank were: R_{b_1} which was the absorbance of the particular sodium hydroxide solution and ranged from 0.00 to 0.07 with the increase in alkalinity; and R_{b_2} which was the background absorbance of the aqueous phase after the aqueous solutions without biguanide were extracted and included the correction for the saturation of the alkaline aqueous phase with the respective organic solvents. The R_{b_2} correction for the methylene chloride extractions ranged from 0.045 to 0.115 with increasing alkalinity. The R_{b_2} correction for the chloroform–*tert*-amyl alcohol extractions ranged from 0.00 to 0.50.

separation of the extraction solutions by high speed centrifugation[4]. No additional spectral background at 232 nm. was found when these polypropylene tubes replaced the glass tubes used previously (1). The organic phase in the bottom layer was removed conveniently by the use of a glass syringe with an 18-gauge, 15.24-cm. (6-in.) needle. The needle was cut at a 90° angle to facilitate removal of the phase. In one method, the plasma proteins were removed by ultrafiltration through a membrane cone[5].

Determination of Partition Coefficients of Biguanides in Organic Solvent–Aqueous Solution Systems—Aqueous solutions of biguanides (10^{-4} *M*) at different sodium hydroxide concentrations (from 0.005 to 0.80 *N*) were prepared. Aliquots (4.00 ml.) of these solutions were shaken with 20.00 ml. of organic solvent (methylene chloride or chloroform–*tert*-amyl alcohol, 50:50) for 30 min. After centrifugation, the aqueous phase was spectrophotometrically measured at 232 nm. Aqueous solutions of sodium hydroxide similarly extracted by the organic phases were also measured spectrophotometrically. The detailed studies are summarized in Table I.

Determination of Biguanides in Human Plasma—*Method a*—V_1 (1.00 or 2.00) ml. of distilled water and 0.5 ml. of 100% or 1.0 ml. of 50% trichloroacetic acid were added to 5.00 ml. of plasma. After shaking and centrifugation, 5.00 ml. of the clear solution (almost all) was siphoned to another centrifuge tube (30 or 50 ml.). The pH was then adjusted to about 7.5 with concentrated sodium hydroxide and hydrochloric acid. V_2 (2.0, 3.0, or 4.0) ml. of pH 7.5 buffer, V_3 (2.0, 3.0, or 4.0) ml. of 10^{-2} *M* bromthymol blue solution, and V_4 (5.0, 10.0, or 20.0) ml. of methylene chloride were added for ion-pair extraction. After 20 min. of shaking on the mechanical shaker, the solution was centrifuged for 10 min. at 2000 r.p.m. Then (V_4 − 1) ml. of the separated organic phase was transferred to another polypropylene centrifuge tube, to which an excess amount of tetrabutylammonium hydroxide (usually 0.10–0.20 ml. of 25% tetrabutylammonium hydroxide in methanol) and 4.00 ml. of pH 7.5 buffer solution (or distilled water) had been added. The back-extraction of biguanide into water was effected by 20 min. of shaking and 10 min. of centrifugation. The aqueous phase was measured spectrophotometrically at 232 nm. against a water blank. The detailed studies are summarized in Table II, and typical calibration curves are given in Fig. 1.

Method b—An aliquot (4.00 ml.) of biguanide-spiked plasma was centrifuged at 2000 r.p.m. through an ultrafiltration cone[5] placed in an unsealed 50-ml. polypropylene tube for 2 hr. (The filtered proteins were resuspended in the cone after the addition of 1.00 ml. of distilled water with the aid of a Vortex shaker for 1 min. The suspension was recentrifuged for 1.5 hr. at 2000 r.p.m. This washing process was repeated again.) The combined filtrates were transferred to a polypropylene centrifuge tube (30 or 50 ml.) with a screw cap. The original centrifuge tube with the cone removed was washed twice with 0.5 ml. of distilled water, which was then combined with the filtrates. The combined filtrates were extracted by the ion-pair method by adding 0.5 ml. of pH 7.5 buffer, 5.0 of 10^{-2} *M* bromthymol blue solution, and methylene chloride (10.0 ml. for phen-

[4] Size I, model SBR, International Equipment Co., Needham Heights, Mass.

[5] Centriflo CF50 membrane filter cone with CSTL support, Amicon Corp., Lexington, Mass.

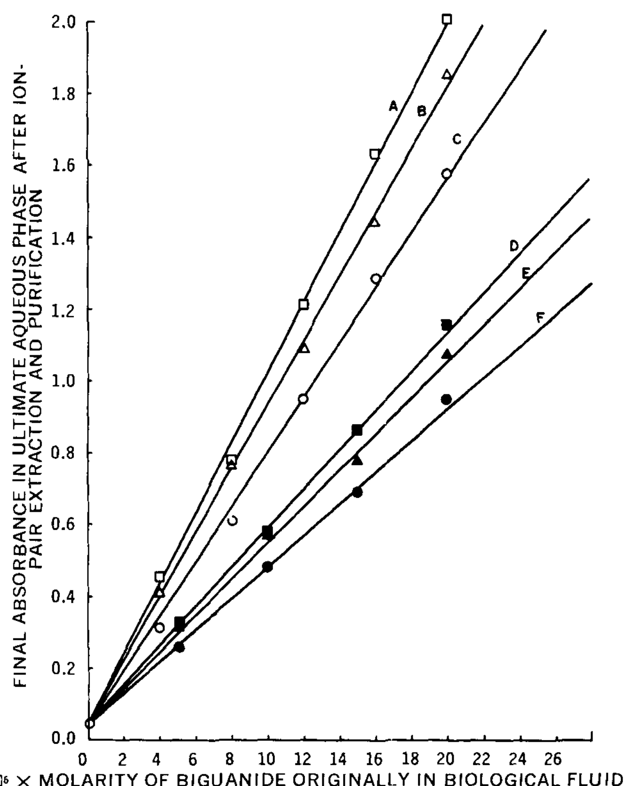


Figure 1—*Spectrophotometric analyses of various concentrations of biguanides in 4.00 ml. of human plasma and 10.00 ml. of whole blood after trichloroacetic acid precipitation of proteins (Method a) and separation from interfering plasma constituents by the ion-pair method. The labeled curves for the compounds and the statistical parameters of their respective linear regressions were [curve, slope* (b) *or regression coefficient in absorbance units/mole/l. ± standard error of slope, and the intercept* (a) *of regression ± standard deviation about regression]: A, buformin in plasma, 9770 ± 77, 0.053 ± 0.013; B, phenformin in plasma, 8871 ± 143, 0.048 ± 0.024; C, metformin in plasma, 7761 ± 148, 0.021 ± 0.025; D, buformin in whole blood, 5418 ± 101, 0.057 ± 0.016; E, phenformin in whole blood, 4982 ± 144, 0.064 ± 0.023; and F, metformin in whole blood, 4454 ± 104, 0.41 ± 0.016.*

Table II—Quantitative Analyses[a] of Biguanide-Spiked Plasma and Whole Blood after Trichloroacetic Acid Precipitation of Proteins

Medium	Compound	Studied Concentration Range, 10^5 *M*	Range of Absorbance at 232 nm.	V_1, ml.	V_2, ml.	V_3, ml.	V_4, ml.	Background[b] Reading	Slope-(b)[c], Absorbance Units/mole/l.	*SE* (b)	Percent Recovery: Calculated[d]	Percent Recovery: Experimental[e]
Plasma	Metformin	2.86–28.60	0.195–0.975	2.50	0.50	3.00	5.00	0.120	3023	132	43.8	23.3
		1.60–16.60	0.230–1.230	1.50	0.25	2.00	20.00	0.115–0.16	6225	120	100	40.3
		4.00–20.00	0.257–1.570	1.50	0.50	4.00	10.00	0.050[f]	7761	148	98.5	52.9
	Buformin	1.67–16.67	0.215–1.560	1.50	0.25	2.00	20.00	0.112	8805	99	100	55.3
		4.00–20.00	0.412–1.850	1.50	0.50	4.00	10.00	0.050[f]	9770	77	100	64.3
	Phenformin	1.67–16.67	0.260–0.957	1.50	0.25	2.00	20.00	0.160	4905	160	100	29.9
		4.00–20.00	0.455–2.010	1.50	0.50	4.00	10.00	0.050[f]	8871	143	100	54.4
Whole blood[g]	Metformin	4.65–18.60	0.260–0.949	2.00	0.50	4.00	10.00	0.060[f]	4676	104	98.5	41.6
	Buformin	4.65–18.60	0.330–1.160	2.00	0.50	4.00	10.00	0.060[f]	5794	101	100	47.9
	Phenformin	4.65–18.60	0.316–1.080	2.00	0.50	4.00	10.00	0.060[f]	5386	144	100	41.0

[a] Five milliliters of the spiked plasma (or 4 ml. of the plasma obtained from the spiked whole blood) was mixed with V_1 ml. of distilled water and 0.50 ml. of 100% trichloroacetic acid. After shaking and centrifugation, 5 ml. of the clear solution from the spiked plasma (or all of the solution from the spiked whole blood) was transferred to another centrifuge tube. The pH was then adjusted to 7.5 with concentrated sodium hydroxide and hydrochloric acid. V_2 ml. of pH 7.5 buffer, V_3 ml. of 10^{-2} *M* bromthymol blue, and V_4 ml. of methylene chloride were then added for ion-pair extraction. The biguanide was freed from the ion-pair and back-extracted into water from ($V_4 - 1$) ml. of separated organic phase, to which 0.1–0.2 ml. tetrabutylammonium hydroxide solution and either 4.00 ml. of distilled water or 4.00 ml. of pH 7.5 buffer had been added. The spectrophotometric reading at 232 nm. was read in the resultant aqueous phase. [b] The background readings of a calibration curve were obtained by following the same analytical procedures except that no biguanide was spiked in the plasma or whole blood. [c] The slope of the calibration curve for the designated original concentrations of biguanide in plasma or whole blood where the absorbances were determined at 232 nm. in the final aqueous phase. [d] When the concentration of bromthymol blue is much higher than the biguanide concentration and the back-extraction of biguanide is quantitative, the percent recovery may be approximated by:

$$\% \text{ recovery} = \frac{C^{o}_{\text{bromthymol blue}} \cdot E_{1:1}}{(E_{1:1} \cdot C^{o}_{\text{bromthymol blue}} + 1 + Ka'/[\text{H}^+])} \cdot \frac{V_4}{(5 + V_2 + V_3)}$$

where $C^{o}_{\text{bromthymol blue}}$ is the original concentration of bromthymol blue in the aqueous solution, $E_{1:1}$ is the 1:1 ion-pair extraction constant, and *K* = 7.18 (1). [e] The experimental percent recoveries from plasma were calculated from $(b/\epsilon_{232}) \times (4/5) \times (V_4/V_4 - 1) \times 100$, where b is the slope of the calibration curve and the ϵ_{232} values were given previously (1). The experimental percent recoveries from whole blood were calculated from $(b/\epsilon_{232}) \times (4/4) \times (4.3/4) \times (10/9) \times 100$. [f] These studies were conducted by back-extraction of the biguanide into 4.00 ml. of pH 7.5 buffer whereas the others were conducted with 4.00 ml. of distilled water as the aqueous phase. [g] Average amount of plasma obtained from 10 ml. of whole blood was 4.3 ± 0.1 ml.

Table III—Quantitative Determinations[a] of Biguanide-Spiked Plasma and Whole Blood after Separation of Proteins by Ultrafiltration

Compound	V_1	*n*	Concentration, 10^5 *M*	Reading at 232 nm.	Background[b] Reading	Slope (b), Absorbance Units/mole/l.	*SE* (b)	Experimental[c] Recovery, %
				Plasma				
Metformin	20.00	0	4.00–12.00	0.375–0.980	0.050	7500	40	58.6
	20.00	2	4.00–12.00	0.372–1.108	0.080[d]	8633	187	67.5
Buformin	20.00	0	4.00–12.00	0.409–1.110	0.050	8803	66	65.2
	20.00	2	4.00–12.00	0.477–1.310	0.050	10287	525	76.2
Phenformin	10.00	0	4.00–12.00	0.393–0.949	0.110[d]	7028	89	48.5
	10.00	2	4.00–12.00	0.427–1.161	0.050	9293	140	64.1
				Whole Blood[e]				
Metformin	20.00	0	4.76–14.3	0.293–0.800	0.040	5052	44	41.4
Buformin	20.00	0	4.76–14.3	0.370–0.950	0.040	5898.6	7	45.9
Phenformin	10.00	0	4.77–14.3	0.320–0.785	0.040	4850	248	35.1

[a] An amount of 4.00 ml. of plasma was filtered through an ultrafiltration cone for 2 hr. at 2000 r.p.m. The proteins left in the filter were dispersed by 1.0 ml. of distilled water by a Vortex shaker and refiltered for 1.5 hr. This protein washing procedure was repeated *n* times, and the filtrates were combined. Four milliliters of 10^{-2} *M* bromthymol blue, 0.50 ml. of pH 7.5 buffer, and V_1 (10.0 or 20.0) ml. of methylene chloride were added to the combined filtrates for ion-pair extraction. The biguanide was then freed from the ion-pair and back-extracted into water from all of the separated organic phase, to which 0.20 ml. tetrabutylammonium hydroxide solution and 4.00 ml. of pH 7.5 buffer had been added. [b] The background readings were obtained by following the same analytical procedures except that no biguanide was spiked in the plasma and whole blood and the absorbances were determined at 232 nm. in the final aqueous solution. [c] The experimental recoveries from plasma were calculated from $(b/\epsilon_{232}) \times 100$, while those from 10 ml. of whole blood were calculated from $(b/\epsilon_{232}) \times (4.2/4) \times 100$ since only 4.00 ml. of the resultant plasma was used. When filtered plasma was spiked and refiltered and the ion-pair procedure was carried through, the percent recoveries of phenformin, buformin, and metformin were 91, 89, and 79%, respectively. The difference between these values and the experimental recoveries of biguanide (49, 65, and 59%, respectively; see items in table under plasma for $n = 0$) from plasma spiked before filtration gives preliminary estimates of protein binding of 42, 24, and 20%, respectively. However, although all buformin and metformin came through the ultrafiltration cone when 4.00 ml. of 10^{-4} *M* biguanide solution was filtered, 20% of the phenformin was retained by the cone. Thus, the estimated extent of protein binding would be 20% for phenformin, 24% for buformin, and 20% for metformin when the plasma is completely filtered. It has been argued (7, 8) that this procedure tends to overestimate protein binding since complete filtration of plasma protein with precipitation in the cone from a substrate-containing plasma has been claimed to trap and inhibit the free passage of other soluble plasma components. [d] Part of the background reading is attributable to the new ultrafiltration cone used. [e] Average plasma obtained from 10 ml. of whole blood was 4.2 ± 0.1 ml.

formin and 20.0 ml. for metformin or buformin). After 20 min. of shaking and 10 min. of centrifugation, the entire organic phase with its ion-pairs was separated by the previously described syringe with its needle. The biguanide was then back-extracted into an aqueous phase by adding 0.2 ml. of tetrabutylammonium hydroxide solution and 4.00 ml. of pH 7.5 buffer to the previously separated organic phase together with 20 min. of shaking and 10 min. of centrifugation at 1000 r.p.m. Spectrophotometric readings were taken of the aqueous phase. The detailed studies are summarized in Table III.

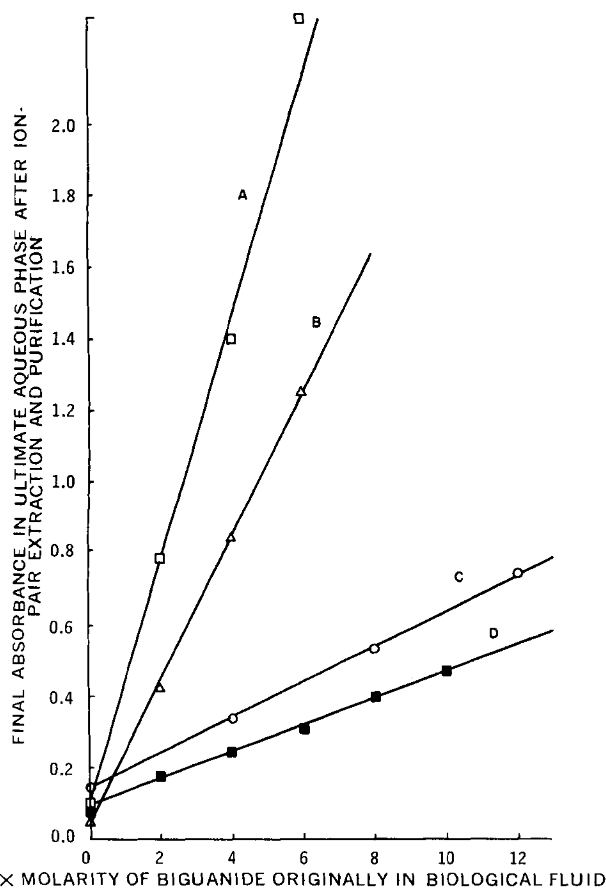


Figure 2—*Spectrophotometric analyses of biguanides in various biological fluids after separation from interferences by the ion-pair method. Curve A* (n = 4) *is for 2.00 ml. of dog cerebrospinal fluid, and curve B* (n = 4) *is for 1.00 ml. of dog bile. The respective slopes in absorbance units/mole/l. ± standard error of slope and the intercepts of the regression ± standard deviation about regression were: A, 3593 ± 203, 0.066 ± 0.091; and B, 2005 ± 40, 0.041 ± 0.018. Curves C and D* (n = 6) *are for more sensitive assays of buformin and phenformin by Methods c and d, respectively, in 20.0 ml. of human plasma.*

Determination of Extremely Low Concentrations of Biguanides in Human Plasma—*Method c*—An amount of 1.4 ml. of 100% trichloroacetic acid was added to 20.00 ml. of phenformin-spiked plasma. After shaking and centrifugation, all of the clear solution was transferred to another polypropylene centrifuge tube (85 ml.). After adjusting the pH to 7.5, 0.5 ml. of pH 7.5 buffer, 15.0 ml. of 10^{-2} *M* bromthymol blue solution, and 40.0 ml. of methylene chloride were added for ion-pair extraction. After shaking and centrifugation, all of the separated organic phase, saturated with water, was transferred to another centrifuge tube and evaporated to ~10 ml. by constantly blowing air across the solution surface. After 2 min. of shaking and 5 min. of centrifugation at 2000 r.p.m., the aqueous phase produced during the evaporation process was removed by the previously described syringe and its needle. The back-extraction into an aqueous phase was effected by the addition of 0.2 ml. tetrabutylammonium hydroxide solution and 4.00 ml. of buffer solution with 20 min. of shaking and 10 min. of centrifugation at 1000 r.p.m. Spectrophotometric readings were then taken of the aqueous phase. The calibration curve is given in Fig. 2.

Method d—An amount of 20.00 ml. of buformin-spiked plasma was filtered through an ultrafiltration cone in the centrifuge at 2000 r.p.m. for 2 hr. The filtrates were divided into equal amounts and transferred to two 85-ml. centrifuge tubes. The ion-pair extraction was made in each centrifuge tube with 10.0 ml. of 10^{-2} *M* bromthymol blue, 0.50 ml. of pH 7.5 buffer, and 30.0 ml. of methylene chloride. After extraction, the organic phases of both tubes were combined in another 85-ml. polypropylene centrifuge tube and evaporated to ~20 ml. The aqueous phase produced by evaporation of this water-saturated organic phase was removed. The back-extraction of the buformin into the aqueous phase was effected by the addition of 0.20 ml. of tetrabutylammonium hydroxide solution and 4.00 ml. of pH 7.5 buffer together with 20 min. of shaking and 10 min. of centrifugation at 1000 r.p.m. The spectrophotometric reading of the aqueous solution was then taken. The calibration curve is given in Fig. 2.

Determination of Biguanides in Whole Blood—Solutions (0.9% saline) that were 10^{-2} *M* in the several biguanides were prepared. Various amounts of these biguanide solutions (0, 20, 40, 60, and 80 μl.) were added to 10.00 ml. of whole blood. After hand-shaking to mix well, the blood stood for 20 min. and was then centrifuged. The separated plasma (a 4.00-ml. aliquot of the obtained 4.1–4.3 ml.) was assayed by Methods a and b. The detailed studies are summarized in Tables II and III, and typical calibration curves are given in Fig. 1.

Determination of Protein Binding up to 3 × 10^{-4} *M* Biguanides in Human Plasma—*Method e*—The filterable biguanide was determined by assaying the filtrates of plasma samples using Method b for the determination of biguanides in plasma, except that the washing procedures stated in the parenthesis were omitted. The fraction of biguanide bound to the cone membrane was determined by the comparison of 232-nm. UV readings of 10^{-4} *M* biguanides with those of the filtrate of 4.00 ml. of 10^{-4} *M* biguanide filtered through the ultrafiltration cone. The background reading was obtained by taking the 232-nm. UV reading of 4.00 ml. distilled water filtered through the ultrafiltration cone. The data obtained from complete filtration of plasma are given in Table III.

Method f—An aliquot (6.00 ml.) of biguanide-spiked plasma was filtered through an ultrafiltration cone for 40 min. at 2000 r.p.m. An aliquot (2.00 ml.) of 3.00 ml. of filtrate mixed with 3.00 ml. of 10^{-2} *M* bromthymol blue and 0.5 ml. of pH 7.5 buffer was shaken with 20 ml. of methylene chloride for 20 min. on a mechanical shaker for the ion-pair extraction. After centrifugation, the back-extraction of the biguanide was effected by shaking the separated organic phase with 4.00 ml. of pH 7.5 buffer solution after adding 0.2 ml. tetrabutylammonium hydroxide. The spectrophotometric reading was then taken at 232 nm. An appropriate blank was run by spiking the filtrate of plasma with biguanide. After this procedure

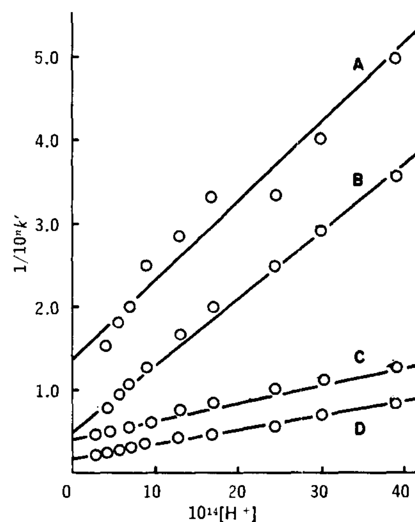
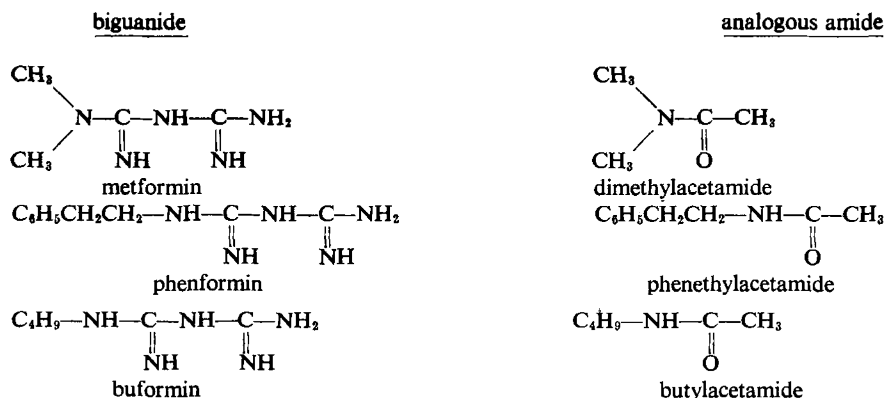


Figure 3—*Plots of the reciprocals, 1/k', of the experimentally determined apparent organic solvent/water partition coefficient, k', against the hydrogen-ion concentrations* $[H^+] = K_W/f[NaOH]$, *where* $K_W = 10^{-14}$ *and f is the activity coefficient of hydroxide-ion concentration in the stated sodium hydroxide concentrations of Table I. The lines are in accordance with* $1/k' = 1/k + [H^+](1/K_a k)$, *where k is the intrinsic partition coefficient of nonprotonated biguanide and* K_a *is the apparent dissociation constant of its conjugate acid. Curves A and D,* n = 1, *are for buformin and phenformin, respectively, in methylene chloride/water; curves B and C,* n = 0, *are for buformin and phenformin, respectively, in 1:1 chloroform-*tert*-amyl alcohol/water. The curves and their respective intercepts and slopes are: A, 13.5, 9.5 × 10^{13}; B, 0.5, 0.81 × 10^{13}; C, 0.4, 0.22 × 10^{13}; and D, 1.8, 1.67 × 10^{13}.*



was effected, the reextracted aqueous phase was read at 232 nm. This procedure differs from Method e mainly in the fact that only one-half of the plasma volume is filtered through the cone.

Comparison of Ultrafiltration and Equilibrium Dialysis Methods in Determination of Protein and Erythrocyte Binding of Metformin in Human and Dog Plasma—*Method g*—Analyses were made of 5×10^{-5} *M* to 1×10^{-2} *M* metformin added to previously refrigerated 4-day-old dog blood and plasma and to 3-week-old human blood and plasma in the manner described in Method f. The major variations were that a 5.00-ml. aliquot of plasma or whole blood was filtered through an ultrafiltration cone for 30 min. at 2000 r.p.m. at 25°. Approximately only 50% of the volume was filtered in this interval to avoid an excessive accumulation of precipitated proteins on the inner surface of the cones and thus to avoid a possible trapping of metformin (3, 6–10, 15). An aliquot of this filtrate (0.5–2.0 ml., which varied with the metformin concentration studied) was carried through the rest of the stated procedure of Method f except that 10 ml. of methylene chloride was used.

Metformin was added to the plasma filtrate in the same concentrations and the same analytical procedure was followed.

The analyses were commenced 5 min. after spiking the plasma with metformin. In the cases of metformin-spiked whole blood, the ultrafiltration step was initiated at 5, 10, 60, and 120 min. and 24 hr. after spiking to check for the possible time-dependent extent of erythrocyte binding. Hematocrits for the determination of the erythrocyte volume of whole blood were carefully determined.

Method h—The protein binding of similar concentrations of metformin in human and dog plasma was determined by equilibrium dialysis. The three-chamber apparatus of Krüger-Thiemer *et al.* (16), separated by two Visking membranes, was used with plasma in the 5-ml. central chamber, metformin in sterile pH 7.4 phosphate buffer solution in one outer chamber, and buffer alone in the other.

Dialysis was permitted for 100 hr. at 4° and subsequently for 6 hr. at 37° to minimize microbial contamination. Equilibrium was ascertained after this interval by the equivalence of metformin concentrations in both outer chambers. The ion-pair extraction procedure with spectral analysis of the back-extracted metformin was used directly on both outer compartments. The ion-pair extraction procedure and analysis of metformin in the middle chamber were conducted subsequent to a 50% ultrafiltration of the plasma.

The pH's of all compartments were within 0.2 unit of each other and were maintained within ±0.4 unit. The dilution of the plasma volume by osmotic water transport did not exceed 10%. When 10^{-4} *M* metformin was placed in all three chambers of the apparatus for 24 hr. at 37°, no change in concentration was observed, indicating no significant binding of metformin to the membranes.

Determination of Metformin in Bile and Cerebrospinal Fluid of Dogs—Aliquots (0–60 μl.) of metformin solution (10^{-2} *M*) were added to 1 ml. of dog bile, which was mixed with 1.00 ml. of water and 0.100 ml. of 100% trichloroacetic acid. The solution was decanted after centrifugation into another centrifuge tube, and 5.00 ml. of methylene chloride was added. The mixture was agitated (5 min.) and centrifuged for 5 min., and the organic phase was discarded. This purification procedure was repeated. The pH of the aqueous phase was adjusted to neutrality with concentrated sodium hydroxide, and 0.5 ml. of pH 7.5 buffer, 3.0 ml. of 10^{-2} *M* bromthymol blue, and 10.0 ml. of methylene chloride were added for ion-pair extraction. The back-extraction of the separated organic solutions of the ion-pair was effected by the addition of 0.15 ml. of 25% tetrabutylammonium hydroxide in methanol and 4.00 ml. of pH 7.5 buffer with subsequent shaking (20 min.) and centrifugation for 10 min. The resultant aqueous phase was spectrally assayed for metformin at 232 nm.

Attempts had been made previously to use the trichloroacetic acid Method a and the cone-filtration Method b given for the determination of biguanides in plasma without further modification. However, colored components in the bile interfered with the assay. They formed gelatinous precipitates in the organic phase. Prior extraction of the pH 7.5 adjusted bile with methylene chloride before ion-pair extraction into this solvent was effective in removing these interferences, but the final yield of the biguanide was signficantly reduced. The procedure that gave the highest yield and effectively removed the interference was prior methylene chloride extraction of acidified bile before the subsequent ion-pair extraction into the same solvent from a pH 7.5 adjusted aqueous solution.

Method a for the determination of biguanide in plasma was applicable to the determination of metformin in cerebrospinal fluid. Trichloroacetic acid (0.3 ml. of 100%) was added to 2.00 ml. of metformin-spiked cerebrospinal fluid, and the mixture was shaken and centrifuged. The decanted clear solution was adjusted to pH 7.5, and 0.5 ml. of pH 7.5 buffer, 3.00 ml. of 10^{-2} *M* bromthymol blue solution, and 15.0 ml. of methylene chloride were added for ion-pair extraction into the organic phase. The addition of 0.15 ml. of 25% tetrabutylammonium hydroxide in methanol and 4.00 ml. of distilled water to the separated organic phase was used to back-extract the metformin for spectrophotometric assay at 232 nm. in the aqueous phase.

The calibration curves for metformin in dog bile and cerebrospinal fluid are given in Fig. 2. These cover the ranges of concentrations encountered in the pharmacokinetic studies which will be reported in a subsequent paper.

RESULTS AND DISCUSSION

Determination of Partition Coefficients of Biguanides—The partition coefficients of the three biguanides for the methylene chloride–water system were determined as a function of sodium hydroxide concentration (Table I). Metformin could not be extracted from water by methylene chloride even in a very strong sodium hydroxide solution (0.8 *N*). The only significant practical extractability of a biguanide was effected for phenformin with this system. It was indicated that buformin might partition slightly at higher alkalinities, although metformin partitioning into methylene chloride would not be significant. Thus, as a general rule it would be best to minimize the volume ratio of the solvents, $Q = V_{org}/V_{aq}$, for the back-extraction of the freed biguanides from their ion-pairs into water from a methylene chloride phase, especially for the case of phenformin. A low pH of the aqueous phase should be maintained to ensure ready partition of phenformin into the aqueous phase. However, under the conditions specified previously (Table II of *Reference 1*), each of these biguanides could be quantitatively reextracted back into water from its ion-pair in methylene chloride solution by the addition of the requisite amounts of tetrabutylammonium hydroxide to the organic solvent.

A more effective solvent for the extraction of buformin and phenformin from aqueous alkali was with a 1:1 mixture of chloro-

Table IV—Estimated Protein Binding of 0-3 × 10^{-4} *M* Biguanide in Human Plasma by Centrifugal Filtration of Half of Potential Filtrate

	—Absorbance Units/mole/l. ± Standard Error— S^a	S'^b	Percent Biguanide Bound to Protein[c] $[(S' - S)/S' - B] \times 100$
Metformin	5040 ± 98	5441 ± 55	7.4
Buformin	6118 ± 63	6626 ± 45	7.7
Phenformin	5060 ± 50	7251 ± 211	12

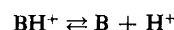
[a] Slope of absorbance units at 232 nm. of final aqueous solution of biguanide (after ion-pair and back-extraction in accordance with Method f) against biguanide concentrations of spiked blood plasmas, which volumes were 50% centrifugally filtered after spiking. [b] Slope of absorbance units at 232 nm. of final aqueous solution of biguanide (after Method f) against biguanide concentrations of the spiked plasma filtrate, which filtrate was obtained from blood plasma that was 50% centrifugally filtered. [c] The *B* correction is the fraction of filtered biguanide bound to the filter cone in filtration of spiked filtrate which filtrate had been obtained from a previous 50% centrifugal filtration of blood plasma. *B* was only significant for phenformin and was 0.20 fraction of the concentration.

form and *tert*-amyl alcohol. The partition coefficients of the biguanides were determined as a function of sodium hydroxide concentration and are given for this organic solvent mixture in Table I.

The fact that metformin was negligibly extractable into organic solvents even at the high alkalinities, when it should be at least partially deprotonated, may be rationalized by its molecular resemblance to dialkylated amides.

The analogous dialkylated amides such as dimethylacetamide are low melting compounds (actually liquids) and are highly miscible with water in all proportions. Dialkylated amides have high aqueous solubilities relative to the corresponding unsubstituted amides. Even high molecular weight compounds such as the *N,N,N',N'*-tetramethylphthalamides and *N,N,N',N'*-tetramethylsuccinamides have solubilities in water of the magnitude of sodium chloride (17). These properties are attributed to the high degree of molecular association which occurs among the unsubstituted amide molecules as a result of hydrogen bonding (18). Thus, unprotonated and neutral monoalkylated compounds such as buformin and phenformin have a relative tendency to "escape" into an organic solvent in contrast to the unextractable dialkylated metformin. Substitution of alkyl groups for the acidic *N*-hydrogens in these amides decreases the possibility of association among the amide molecules and favors solute-solvent interaction or solubilization (1). Thus, the higher molecular weight *N,N,N',N'*-tetramethylphthalamide, even with its greater lipophilic substituents, is soluble in water to the extent of 710 g./l. at 30°, while phthalamide itself has a solubility of only 5.9 g./l. at 30° (17). This same phenomenon readily explains the inability of metformin to be extracted into organic solvents. The phenethyl group of phenformin gives this compound greater lipophilicity than the butyl group gives buformin.

The dissociation constant for the liberation of a protonated biguanide in the aqueous phase (Scheme I):



$$BH^+ \rightleftarrows B + H^+$$

Scheme I

is expressed by:

$$K_a = [B]_w[H^+]/[BH^+]_w \quad \text{(Eq. 1)}$$

The experimental oil/water partition coefficient is:

$$k' = [B]_o/([B]_w + [BH^+]_w) \quad \text{(Eq. 2)}$$

and the pH-independent intrinsic partition coefficient for the distribution of the unprotonated species between the organic solvent and the aqueous phase is:

$$k = [B]_o/[B]_w \quad \text{(Eq. 3)}$$

where the subscripts designate the species in the respective organic and water phases.

It follows (19) from Scheme I and Eqs. 1–3 that:

$$1/k' = [B]_w/[B]_o + [BH^+]_w/[B]_o = 1/k + ([BH^+]_w/[B]_w) \times ([B]_w/[B]_o) = 1/k + [H^+]/K_a k \quad \text{(Eq. 4)}$$

so that plots of the reciprocals ($1/k'$) of the experimentally determined apparent partition coefficients, k', against the hydrogen-ion concentrations should give straight lines with intercepts of $1/k$ and slopes of $1/K_a k$. It follows that the reciprocal of the intercept is the intrinsic partition coefficient, k, and the quotient of the intercept and the slope is the apparent dissociation constant, K_a, of the protonated biguanide.

The hydrogen-ion concentrations of the systems in which the partition coefficients were determined may be estimated from:

$$[H^+] = K_w/[OH^-] = K_w/f[\text{NaOH}] \quad \text{(Eq. 5)}$$

where the K_w is taken as 10^{-14} at 25°, and the activity coefficients, f, at 25° of the sodium hydroxide solutions of Table I were obtained from the literature (20). Plots of $1/k'$ against $[H^+]$ in accordance with Eq. 4 are given in Fig. 3. The apparent pKa values are about 13 as estimated from the quotient of the intercepts and slopes. The specific estimates were 12.9 (methylene chloride/water, $k = 0.074$) and 13.2 (1:1 chloroform-*tert*-amyl alcohol/water, $k = 2.0$) for buformin and 13.0 (methylene chloride/water, $k = 0.55$) and 12.8 (1:1 chloroform-*tert*-amyl alcohol/water, $k = 2.5$) for phenformin.

Determination of Biguanides in Plasma and Whole Blood—The results obtained for the trichloroacetic acid precipitation of proteins (Method a of *Experimental*) in the determination of biguanides in plasma under different conditions are summarized in Table II. In contrast to biguanide assays from urine samples (1), the assay from plasma gave very low and reasonably constant spectrophotometric background readings. In the back-extraction of biguanide from the ion-pair into the aqueous phase, the use of 4.00 ml. of pH 7.5 buffer solution (items superscripted *d* in Table II) instead of

Table V—Estimated Protein and Erythrocyte Binding of 5 × 10^{-5} *M* to 1 × 10^{-2} *M* Metformin in Human and Dog Plasma and Blood by Centrifugal Filtration of Half of Potential Filtrate

	Absorbance Units/mole/l. ± Standard Error S^a	S'^b	S''^c	—Percent Metformin Bound[d]— To Protein $[(S' - S)/S'] \times 100$	To Protein and Erythrocyte $[(S' - S'')/S'] \times 100$
Dog	9457 ± 94	10,165 ± 130	9,352 ± 105	7.0	8.0
Human	9887 ± 45	11,001 ± 97	10,253 ± 86	10.1	6.8

[a] Slope of absorbance units at 232 nm. of final aqueous solutions of metformin (after ion-pair and back-extraction in accordance with Method g) against metformin concentrations of spiked blood plasma, which volumes were 50% centrifugally filtered after spiking. [b] Slope of absorbance units at 232 nm. of final aqueous solutions of metformin (after Method g) against metformin concentrations of the spiked plasma filtrate, which filtrate was obtained from blood plasma that was 50% centrifugally filtered. [c] Slope of absorbance units at 232 nm. of final aqueous solutions of metformin (after Method g) against metformin concentration of spiked whole blood corrected by the hematocrit for erythrocyte volumes (generally 0.35 of total blood volume). The volumes of plasma were 50% centrifugally filtered after spiking. [d] There was no significant binding of metformin to the filter cone since $S_B = 9847 \pm 103$ was the slope of absorbance units at 232 nm. of final aqueous solution (after Method g) against biguanide concentrations of spiked pH 7.4 phosphate buffer solutions which were not filtered, and $S_B' = 10{,}400 \pm 140$ was the slope for such plots of spiked pH 7.4 phosphate buffer solutions which were centrifugally filtered through the cones. The difference of these slopes, $S_B - S_B'$, is negligible.

Table VI—Typical Data for Determination of Protein Binding by Equilibrium Dialysis

$10^4 \times M$ of Metformin in Each Chamber after Presumed Equilibrium[a] — Left, C_L	Right, C_R	Middle, C_M	Range of Estimated Percent Bound, $100(C_M - C_L)/C_M$ to $100(C_M - C_R)/C_M$
Human Blood Plasma			
9.80	9.40	10.0	2.0–6.0
1.80	1.70	2.0	10–15
0.80	0.72	0.96	17–25
Dog Blood Plasma			
90.9	90.4	95.6	4.9–5.4
9.71	9.90	10.60	6.6–8.4
9.01	8.60	10.40	13–17
8.90	9.20	10.40	12–14
1.64	1.84	1.96	6.1–16
1.64	1.76	1.96	10–16

[a] The equipment consisting of three chambers separated by two Visking membranes (16) was used. Equilibration was affected for 100 hr. at 4° and a subsequent 6 hr. at 37°. No better equivalence between the C_L values for the left and the C_R values for the right chamber was achieved. The normal volumes of left and right chambers were 2.5 ml. with 5.0 ml. in the middle chamber.

an equivalent amount of distilled water produced significantly less background. As anticipated from the partition studies in a methylene chloride/water system, a relatively large ratio of organic to aqueous phase, when the latter is nonbuffered, gave a low recovery of phenformin (line 6 of Table II). When less volume of methylene chloride and 4.00 ml. of pH 7.5 buffer solution were used for the back-extraction, the recovery of phenformin was greatly improved (line 7 of Table II). The use of 4.00 ml. of pH 7.5 buffer for the back-extraction resulted in a final pH of the aqueous phase of 7.8 ± 0.2, when 0.2 ml. of tetrabutylammonium hydroxide solution was used.

In all the cases given in Table II, the experimental recoveries were significantly lower than the theoretical and could be attributed to some residual degree of coprecipitation of the biguanide with the acid-denatured proteins.

Typical calibration curves by Method a for metformin, buformin, and phenformin spiked in plasma (for the experimental conditions specified in lines 3, 5, and 7 of Table II, respectively) and in whole blood (for the experimental conditions specified in Table II) are given in Fig. 1.

The results obtained for the ultrafiltration method for removal of proteins (Method b of *Experimental*) in the determination of biguanides in plasma under different conditions are given in Table III. The spectral background readings were low and apparently were not a function of different blood from different individuals. This finding was in decided contrast to the previous studies (1) on urine where there was a wide variation in background among urines from different individuals. The sensitivity of Method b is similar to that of Method a, *i.e.*, down to 5×10^{-6} *M* for 4 ml. of plasma. Method b is preferred in the case of biguanides since the acid treatment and the time-consuming pH adjustment procedures can be excluded. However, in the general case, if the protein binding of a drug to a nondenatured protein were important, the acid treatment of Method a may have to be used to free the drug from the proteins.

Table III shows that the washing procedures given in the parentheses under Method b do increase recoveries. However, the increase in sensitivity so gained may not warrant the extra 3 hr. of time and labor.

Analytical Methods a and b for biguanide levels in human plasma and in whole blood (10.00 ml.) give similar results, and the data given in Fig. 1 are typical of either method. Also, metformin-spiked plasma samples were stored in the refrigerator for 4 and 9 days and calibration curves were constructed for these times of storage for comparison with those plasmas studied immediately after spiking. There was no significant effect of storage on the plasma assay method by Method b.

Normally, lower doses of the phenformin and buformin biguanides are given than are given for metformin (21, 22). Previous radiotracer-labeled biguanide studies (23) indicated that the plasma level was 0.1 mcg./ml. at 8 hr. (>2 half-lives) after an intravenous injection of 1.2 mg./kg. for buformin-^{14}C or 100 mg. of tritium-labeled phenformin. Since an intravenous dose up to 1000 mg. of metformin can be administered, plasma levels of 3–8 mcg./ml. have been reported (24).

Thus, although the sensitivity of the ion-pair separation procedure combined with spectrophotometric assay, *i.e.*, 1 mcg./ml. for 4 ml. of plasma, may be reasonable for pharmacokinetic studies on plasma metformin in animals, higher sensitivity would be needed for such studies with buformin and phenformin. The more sensitive Methods c and d could be applied to 20 ml. of plasma where larger amounts of organic phase for ion-pair extraction with subsequent concentration by solvent evaporation increased the sensitivity to 0.2 mcg./ml. (Fig. 2). The spectral background (compare intercepts of curves in Fig. 1 to curves in Fig. 2) was increased only slightly when 20.0 ml. rather than 4.0 ml. of plasma was used if the concentrated water-saturated organic phase was centrifuged and the precipitated aqueous phase was removed prior to the back-extraction of the biguanide into pH 7.4 buffer for spectrophotometric measurement. It follows that higher sensitivities of biguanide assay in plasma could result at the expense of using larger volumes of blood.

In summary, the ion-pair method for isolation and concentration of biguanides from blood, plasma, bile, and cerebrospinal fluid is an effective procedure. Both the trichloroacetic acid precipitation of plasma proteins and their centrifugal filtration serve as appropriate methods for the protein removal necessary for the application of the ion-pair techniques. The major limitations are the detection and quantification of the concentrated biguanide in the resultant organic or aqueous solution. A method of detection more sensitive than spectrophotometry, spectrophotofluorometry, or the others in use is still needed for the most detailed pharmacokinetics.

Protein and Erythrocyte Binding of Biguanides—When centrifugally filtered human plasma samples were spiked and refiltered, the percent recoveries of phenformin, buformin, and metformin were 91, 89, and 79%, respectively. The difference between these values and the experimental recoveries of biguanide [*i.e.*, 49, 65, and 57%, respectively (see items under plasma for $n = 0$ in Table III)] from plasma spiked before 100% filtration gave estimates of protein binding of 20, 24, and 20%, respectively, after correction was made for approximately 20% binding by the filter cone of phenformin.

When this centrifugal filtration procedure was repeated with the modification that only *half* the plasma was centrifugally filtered, the estimated protein binding in human plasma (Table IV) by metformin, buformin, and phenformin was 7.4, 7.7, and 12%, respectively. These lower values confirmed the argument (7, 8) that complete filtration of drug-spiked plasma tends to overestimate the extent of protein binding because the complete protein precipitation in the filter cone traps and inhibits the free passage of other soluble plasma components.

The binding of metformin to protein and to protein plus erythrocytes (Table V) determined by the half-filtration method under centrifugation shows no significant differences and indicates no significant binding or adsorption to erythrocytes. It also cannot be concluded that the 7% extent of protein binding of metformin differs between dog and human plasmas.

Typical data for the determination of protein binding by equilibrium dialysis studies are given in Table VI. The apparent variability in the data exceeds that of the centrifugal filtration method (compare the independent studies of Tables IV and V). However, it cannot be concluded that there is any significant difference between protein binding estimates by the two methods. It was previously reported (25) that buformin at one concentration did not demonstrate any protein binding by the gel-filtration method to bovine albumin. Phenformin binding to albumin also was studied previously at various concentrations by equilibrium dialysis through collodion membranes (26). No significant binding could be concluded.

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Preformulation Investigation I: Relation of Salt Forms and Biological Activity of an Experimental Antihypertensive

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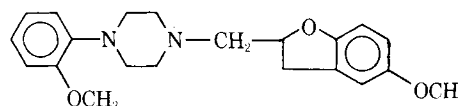
Abstract □ The intrinsic dissolution rates of monohydrochloride, dihydrochloride, and disulfate salts and free base of an antihypertensive were studied. The comparative hypotensive activity of these compounds was studied in anesthetized dogs. The hypotensive responses were also compared to a large number of renal hypertensive dogs who had received a placebo treatment. The results from the anesthetized dog study indicate that the hypotensive potencies of the three salts do not differ from each other; however, the intrinsic dissolution rates of the dihydrochloride and disulfate salts are 54 and 42 times greater, respectively, than that observed with the monohydrochloride salt. These findings seem to indicate the oversensitivity of the *in vitro* dissolution method in reflecting the *in vivo* hypotensive activity of the compound. The free base failed to produce a significant dose–response curve in the anesthetized dog study as well as in the renal hypertensive dog study. This is attributed and correlated to the fact that the *in vitro* dissolution rate of the free base is very low in magnitude as compared to its corresponding salts. The results point out that in searching for a potential candidate of a drug substance, it is advisable to study routinely the effect of the salt form on the biological response.

Keyphrases □ 1-(2,3-Dihydro-5-methoxybenzo[*b*]furan-2-ylmethyl)-4-(*o*-methoxyphenyl)piperazine and salts—dissolution properties, comparative antihypertensive activity in dogs □ Antihypertensive activity—1-(2,3-dihydro-5-methoxybenzo[*b*]furan-2-ylmethyl)-4-(*o*-methoxyphenyl)piperazine and salts, dogs □ Dissolution profiles—free base and salts of an experimental antihypertensive □ Structure–activity relationships—free base and salts of an experimental antihypertensive

The dissolution rates, with respect to pH, of several weak acids and their sodium salts in media representing GI fluids were first investigated by Nelson (1). The salts were found to dissolve much more rapidly than the corresponding free acids. Similar but further elaborated studies on the dissolution kinetics in reactive media were reported by Higuchi and his coworkers (2–4). Salt formation of benzphetamine and etryptamine as a potential means of obtaining timed release and/or prolonged action was studied (5) by measuring the median lethal time in mice and its relationship to *in vitro* dissolution rates and solubilities. It was demonstrated (6) that the dissolution rate of the weak acid, theophylline, was markedly enhanced by employing its amine or alkanolamine salts. The differences in theophylline blood levels after oral administration of the salts in a clinical study were suggested to be related to differences in dissolution rate.

It has been documented that the therapeutic and absorption efficacy of various drugs can be affected by various factors. Consequently, with the emphasis on drug bioavailability in the design of a dosage form, it is pertinent to investigate the various physical–chemical properties of the compound prior to preliminary formulation work. The scope of the preformulation work was aptly outlined by Simons (7). The present study deals with the physical–chemical evaluation of an



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