# Application of Ion-Pair Methods to Drug Extraction from Biological Fluids I: Quantitative Determination of Biguanides in Urine

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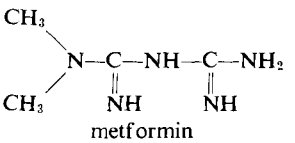
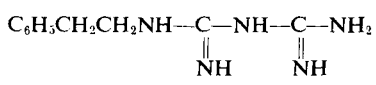
**Abstract** □ A simple method for the quantitative determination of the biguanides (buformin, metformin, and phenformin) in water and urine is based on the extraction of the 1:1 bromthymol blue ion-pair into methylene chloride. Ion-pair extraction constants were determined. The biguanide was freed from the ion-pair by the addition of excess tetrabutylammonium hydroxide and reextracted into water. Spectrophotometric analysis at 630 nm. of the resultant 2:1 ion-pair of the tetrabutylammonium–bromthymol blue in methylene chloride has a sensitivity of less than 0.13 mcg./ml. for metformin in 3 ml. of aqueous solution. However, components of urine are also extracted into the methylene chloride and, in such circumstances, the concentration of the reextracted biguanide in the water can be determined spectrophotometrically at 232 nm. and is sensitive to less than 1.3 mcg./ml. for metformin in 3 ml. of urine. The sensitivity can be greatly increased by using larger amounts of urine. Calibration curves in a concentration range from $10^{-5}$ to $2 \times 10^{-4}$ *M* were obtained for 3 ml. of biguanide-spiked urine samples. No statistically significant effects on buformin calibration curves were found from storage up to 5 days, among different individuals, and with increased numbers of washings of the organic extract with a dilute bromthymol blue solution. Urine without drug demonstrated various backgrounds by these procedures; a principal one was attributable to the nicotine present in the urine of smokers.

**Keyphrases** □ Ion-pair extraction of drugs with bromthymol blue—analysis of biguanides in urine □ Bromthymol blue ion-pair extraction—analysis, biguanides in urine □ Biguanides—analysis, bromthymol blue ion-pair extraction, urine □ Buformin—analysis, bromthymol blue ion-pair extraction, urine □ Metformin—analysis, bromthymol blue ion-pair extraction, urine □ Phenformin—analysis, bromthymol blue ion-pair extraction, urine □ UV spectrophotometry—analysis, biguanides in urine, ion-pair formation

Buformin (1-butylbiguanide), metformin (1,1-dimethylbiguanide), and phenformin (1-phenethylbiguanide) are the three hypoglycemic biguanides clinically used in the treatment of diabetes in Germany, France, and the United States, respectively (1). The development of simple, sensitive, and quantitative methods for the measurement of these drugs and their metabolites in biological fluids is essential to aid in the elucidation of the yet unclear mechanism of action of these compounds



metformin



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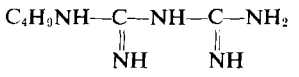

buformin

(2). Present methods of analysis are based on:

1. The reaction with ninhydrin to produce a fluorescent compound (3).
2. The quenching effects of phenformin on the fluorescence exhibited by *n*-propanol in water (4).
3. The color reaction of phenformin with α-naphthol–diacetyl reagent (5).
4. The color reaction of metformin with sodium hypochlorite (6).
5. The large difference in UV absorption between the monovalent and divalent cations of the three biguanides (7).

The sensitivity of 0.025 mcg./ml. claimed for Method 1 (3) was no greater than 0.1 mcg./ml. in our hands. There was a very high background from this method's use of strong potassium hydroxide (analytical reagent)[1] solution, which was not mentioned in the original paper (3). Also, since many other compounds in the biological fluids, such as creatine, arginine, and guanidinium compounds, give much higher fluorescent activity than the biguanides (3, 8), this procedure is not readily applicable to the assay of biguanides from biological fluids without the further development of elegant methods to separate the biguanide from these compounds.

Method 2 has been applied only to phenformin in homogenized tissues and urine. Although the separation procedure of chloroform–methanol extraction from alkalinized solution is comparatively simple and effective since the simultaneously extracted creatine and guanidine do not interfere in the reaction, the method has poor sensitivity (10–30 mcg./ml.).

Method 3 is applicable to biological fluids and tissues with a lower sensitivity of 5 mcg./ml. in urine and a sensitivity no greater than 20 mcg./ml. in blood plasma. However, the separation procedures involved are rather tedious.

Since a sensitivity of $10^{-6}$ g./l. or 1 ng./ml. was reported for Method 4, we systematically checked this method but only obtained a maximum sensitivity of 15 mcg./ml. Also, this color reaction is not suitable for routine metformin assay, since the amount of sodium hypochlorite required has to be comparable to that of metformin and an excess of sodium hypochlorite destroys the color development. In other words, you have to know the amount of metformin in order to assay for it!

Method 5, coupled with column separation, has been used to obtain data from urine in the pharmacokinetic studies of phenformin and buformin (9). The sensitivity of this method was apparently not good enough for

[1] Fisher.

plasma in such studies. Thus, the need for sensitive, specific, and simple methods for the separation and analysis of biguanides still existed.

A widely used method to determine a basic organic compound is to form an ion-pair with an anionic dye (10). This ion-pair is extracted into an organic phase and may be determined spectrophotometrically. Direct application of the ion-pair method to biological fluids is very limited to date because of the presence of large and variable amounts of interfering cations. To our knowledge the only direct application of this method to biological fluids is the recently reported quantitative determination of basic drugs in human urine for special cases where the drugs (*e.g.*, dextropropoxyphene and piribenzil) are stronger ion-pair formation agents than the possibly interfering compounds (*e.g.*, nicotine and guanidinium compounds) (11). In some cases the drugs were extracted and purified by solvent–solvent extractions, and the ion-pair methods were finally used for detection (10, 12–14).

This paper describes a modified ion-pair method, coupled with solvent–solvent extraction and spectrophotometry, which permits the quantitative determination of buformin, metformin, and phenformin. This method can be applied to urine with reasonable sensitivity and by simple procedures. In the absence of contaminants which also form ion-pairs, bromthymol blue can extract the biguanide into an organic phase which can be analyzed spectrophotometrically *in situ*. When interfering compounds are present, as in urine, the biguanide may be freed from the ion-pair by reaction with tetrabutylammonium hydroxide, reextracted into water, and then determined spectrophotometrically. An alternative procedure would be to derivatize the concentrated biguanide and use a sensitive GC detection method for quantitation.

Bromthymol blue was used in these studies as the anionic dye for the ion-pairs. Schill and coworkers (15–18) made extensive quantitative studies on bromthymol blue ion-pairs of quaternary ammonium compounds and amines. Bromthymol blue is a strong ion-pair formation agent with moderate solubility at high pH and with high molar absorptivities of its ion-pairs at 630 nm. (1 bromthymol blue to 2 monovalent cations) and at 410 nm. (1 bromthymol blue to 1 cation). The molar absorptivity of the 1:2 ion-pair is about threefold that of the 1:1 ion-pair. Methylene chloride- or chloroform-extracted 1:1 ion-pairs can be changed to 1:2 ion-pairs by the addition of an excess amount of tetrabutylammonium hydroxide so that the higher sensitivity at 630 nm. can be used in the conventional ion-pair method.

## EXPERIMENTAL

**Materials**—The buformin (1-butylbiguanide)[2], mol. wt. 157.2. phenformin (1-phenethylbiguanide)[2], mol. wt. 205.3, and metformin (1,1-dimethylbiguanide)[3], mol. wt. 129.1, were used. Other materials used were bromthymol blue, certified reagent[4]; tetrabutylammonium hydroxide, 25% in methanol, "highest purity"[4]; and methylene chloride, certified spectroanalyzed ACS grade[4]. Also used were "A grade" creatinine[5], creatine, and arginine hydrochloride[5]. Additional compounds used were guanidine hydrochloride[5] and methyl- and 1,1-dimethylguanidine sulfates[6]. All compounds were used without further purification.

**Instruments**—Spectral readings were made on the recording spectrophotometer[7].

**Reagents**—Bromthymol blue solution ($10^{-2}$ *M*) was prepared by dissolving 0.6244 g. in 20 ml. 0.1 *N* NaOH. The final volume was brought up to 100 ml. with distilled water. This solution was allowed to stand for 3 days with occasional shaking to permit complete dissolution. The solution was then adjusted to pH 7.8 with concentrated sodium hydroxide and hydrochloric acid if necessary. Buffer solutions at pH 7.5 and 7.8 were prepared by dissolving 6.9000 g. $NaH_2PO_4 \cdot H_2O$ in 21 ml. 2 *N* NaOH. After adjusting to the final pH with saturated sodium hydroxide, the final volume was brought to 100 ml. with distilled water.

**Determination that Only 1:1 Ion-Pairs Are Formed in Methylene Chloride**—Aliquots (4.00 ml.) of aqueous solutions containing $10^{-4}$ *M* of each of the three biguanides, $2.00 \times 10^{-3}$ *M* bromthymol blue, and buffer solution were shaken with 5.00 ml. methylene chloride for 20 min. After centrifuging, the organic phase was measured spectrophotometrically at 416 nm.

**Determination of Ion-Pair Extraction Constants of Biguanides in Methylene Chloride Aqueous Solution System**—Buformin, metformin, or phenformin ($10^{-3}$ *M*) stock solution (0.25 or 0.50 ml.) and 0.5 ml. pH 7.8 phosphate buffer were added to a centrifuge tube (heavy duty Pyrex conical centrifuge tubes with ground stoppers, 45-ml. capacity). Various amounts of $10^{-2}$ *M* bromthymol blue and distilled water were added to make a final volume of 5.0 ml. A volume of 5.0 ml. of methylene chloride was then added. After vigorous shaking for 3 min. on a Vortex shaker, the solution was centrifuged and 3 ml. of the lower organic phase was removed by a syringe and transferred to a quartz spectrophotometric cell with a Teflon stopper. Then 30 $\mu$l. of 25% tetrabutylammonium hydroxide in methanol was added, and the solution was mixed well. The absorbance, $A'$, at 630 nm. was immediately taken. The background absorbance, $A_B$, was determined by a similar procedure, except that an equivalent amount of distilled water was substituted for the biguanide solution. The value of the net absorbance, $A$, was obtained by subtracting the background absorbance, $A_B$, from the observed absorbance, $A'$.

**Determination of Biguanides in Urine**—*Procedure A*—$V_1$ (2.00 or 3.00) ml. urine, $V_2$ (0 or 1.00) ml. distilled water, $V_3$ (0.5 or 1) ml. pH 7.8 buffer, and $V_4$ (1.0 or 3.0) ml. of $10^{-2}$ *M* bromthymol blue were added to a centrifuge tube. Then 5.0 ml. of methylene chloride was added. The mixture was shaken on a Vortex shaker for 3 min. and then centrifuged. [All of the organic phase can then be siphoned to another centrifuge tube, $V_5$ ml. (2.0 ml.) of $10^{-3}$ *M* bromthymol blue solution at pH 7.8 can be added, and the mixture can be shaken for 3 min. and centrifuged.] Then 4 ml. of the organic phase was siphoned into another centrifuge tube. An amount of 60 $\mu$l. of 25% tetrabutylammonium hydroxide in methanol and 4 ml. of distilled water were added, and the mixture was shaken again for 3 min. and centrifuged. The aqueous layer was then measured spectrophotometrically at 232 nm. against a water blank.

*Procedure B*—An alternative procedure, which is valid when interfering substances are small, omits the operations in brackets.

## RESULTS AND DISCUSSION

**Determination of Ion-Pair Extraction Constants**—The symbols used in the following discussion are: $Q$ = ratio of the volume of the organic phase to that of the aqueous phase or $Q = V_{org}/V_{aq}$; $[\ ]_{aq}$, $[\ ]_{org}$ = concentration in aqueous and organic phases, respectively; $A$, $A'$ = absorbance corrected and not corrected for background, respectively; $\epsilon_\lambda$ = molar absorptivity at wavelength $\lambda$; $B^{-2}$, $HB^-$, $H_2B$ = different ionic species of bromthymol blue; $BTB = [B^{-2}] + [HB^-] + [H_2B]$; Bg, $BgH^+$, $BgH_2^{+2}$ = different ionic species of biguanides, $\Sigma B = [Bg] + [BgH^+] + [BgH_2^{+2}]$; $E_{1:1}$ = ion-pair extraction constant for 1:1 ion-pair; $D$ = degree of extraction; $C$ = total concentration of subscripted species; $C^o$ = concentration of original aqueous solution of subscripted species; and $C'$ = concentration of equilibrated organic phase of subscripted species.

[2] USV Pharmaceutical Corp., Tuckahoe, N. Y.
[3] G. D. Searle & Co., Chicago, Ill.
[4] Fisher Scientific Co., Fair Lawn, N. J.
[5] Calbiochem, Los Angeles, Calif.
[6] Eastman Kodak Co., Rochester, N. Y.
[7] Cary 15.

Table I—Initial Bromthymol Blue Concentration, $C^{\circ}_{BTB}$, Needed for $D$% Extraction into Methylene Chloride of Biguanide from Equal Volumes of Aqueous Solution when the Ratio of Concentration Is Bromthymol Blue–Biguanide ~100:1

| | pH 7.8 | | | pH 7.5 | | |
|---|---|---|---|---|---|---|
| | $D$, % | | | $D$, % | | |
| | 99 | 90 | 80 | 99 | 90 | 80 |
| Metformin | $1.20 \times 10^{-1}$ | $1.09 \times 10^{-2}$ | $4.83 \times 10^{-3}$ | $7.15 \times 10^{-2}$ | $6.50 \times 10^{-3}$ | $2.89 \times 10^{-3}$ |
| Buformin | $2.92 \times 10^{-2}$ | $2.66 \times 10^{-3}$ | $1.18 \times 10^{-3}$ | $1.75 \times 10^{-2}$ | $1.59 \times 10^{-3}$ | $7.06 \times 10^{-4}$ |
| Phenformin | $3.88 \times 10^{-3}$ | $3.52 \times 10^{-4}$ | $1.57 \times 10^{-4}$ | $2.39 \times 10^{-3}$ | $2.11 \times 10^{-4}$ | $9.36 \times 10^{-5}$ |

Typical spectra of the bromthymol blue ion-pairs of the three biguanides gave an absorption maximum at 416 nm. [reported as 410 nm. in the literature (15) for the $\lambda_{max}$ of $HB^-$ monoanion of bromthymol blue] with no absorption at 630 nm. (the $\lambda_{max}$ of the $B^{-2}$ anion of bromthymol blue) and suggested that only 1:1 ion-pairs are extracted into methylene chloride. This was confirmed by the fact that the extraction of the ion-pairs decreased sharply with increasing pH. The spectral absorbances at 416 nm., corrected for background, of the 5-ml. methylene chloride solutions of the ion-pairs extracted from 4.00 ml. of buffer solutions that were $10^{-4}$ $M$ in biguanide and $2 \times 10^{-3}$ $M$ in bromthymol blue at pH values of 7.5, 9.4, and 10.9, respectively, were, for metformin: 1.56, 0.338, and 0.038; for buformin: 1.93, 0.610, and 0.094; and for phenformin: 2.15, 1.62, and 0.380.

If $[BgH^+ \cdot HB^-]_{aq}$ is negligibly small or highly dissociated, the apparent 1:1 ion-pair extraction constant for $Q = 1$ is:

$$E_{1:1} = \frac{[BgH^+ \cdot HB^-]_{org}}{[HB^-]\,[BgH^+]} \qquad \text{(Eq. 1)}$$

In the applicable pH range, 6–9, bromthymol blue is present in its monovalent and divalent anionic forms in aqueous solution, while for the three biguanides the monovalent cation, $BgH^+$, predominates. The following relations are valid for $Q = 1$:

$$HB^- \overset{K_a'}{\rightleftharpoons} B^{-2} + H^+ \qquad \text{(Eq. 2)}$$

$$[B^{-2}] + [HB^-] = C^{\circ}_{BTB} - C'_{BTB} \qquad \text{(Eq. 3)}$$

$$[HB^-] = (C^{\circ}_{BTB} - C'_{BTB})/(K_a'/[H^+] + 1) \qquad \text{(Eq. 4)}$$

where $1/(K_a'/[H^+] + 1)$ is the fraction of the bromthymol blue in the aqueous phase as the monoanion (19). Also:

$$[BgH^+] = C^{\circ}_{\Sigma B} - [BgH^+ \cdot HB^-]_{org} \qquad \text{(Eq. 5)}$$

On substitution of Eqs. 3–5 into Eq. 1:

$$E_{1:1} = (1 + K_a'/[H^+])[BgH^+ \cdot HB^-]_{org}/(C^{\circ}_{BTB} - C'_{BTB}) \times (C^{\circ}_{\Sigma B} - [BgH^+ \cdot HB^-]_{org}) \qquad \text{(Eq. 6)}$$

Since, in the organic solution, $C'_{BTB} = A'/\epsilon$, $[BgH^+ \cdot HB^-]_{org} = A/\epsilon$, and $\epsilon_{630} = 4.87 \times 10^4$:

$$E_{1:1} = 4.87 \times 10^4 A_{630} \cdot (1 + K_a'/[H^+])/(4.87 \times 10^4 \times C^{\circ}_{BTB} - A'_{630})(4.87 \times 10^4 \times C^{\circ}_{\Sigma B} - A_{630}) \qquad \text{(Eq. 7)}$$

where pK'a = 7.18. These equations are valid in the concentration ranges where side reactions, such as the formation of dimers and tetramers in the organic phase and the formation of micelles in the aqueous solution (16), are not significant.

The spectrophotometric reading may be made at 630 nm. after the addition of an excess amount of tetrabutylammonium hydroxide to the methylene chloride solution of a 1:1 ion-pair to form the more highly absorbing dianion of bromthymol blue, *i.e.*, $B^{-2}$ in the organic phase.

The degree of extraction for $Q = 1$ is defined as:

$$D = [BgH^+ \cdot HB^-]_{org}/C^{\circ}_{\Sigma B} \qquad \text{(Eq. 8)}$$

when the value of the numerator derived from Eq. 6 is substituted into Eq. 8:

$$D = \frac{(C^{\circ}_{BTB} - C'_{BTB}) \cdot E_{1:1}}{E_{1:1}(C^{\circ}_{BTB} - C'_{BTB}) + 1 + K_a'/[H^+]} \qquad \text{(Eq. 9)}$$

To extract the biguanide effectively from dilute solutions, the bromthymol blue concentration should be as high as possible. For the condition $C^{\circ}_{BTB} \gg C'_{BTB}$, Eq. 9 reduces to:

$$D = \frac{C^{\circ}_{BTB} \cdot E_{1:1}}{E_{1:1} \cdot C^{\circ}_{BTB} + 1 + K_a'/[H^+]} \qquad \text{(Eq. 10)}$$

Knowledge of the ion-pair extraction constant $E_{1:1}$ in Eq. 10 permits the estimation of the concentration of bromthymol blue, $C^{\circ}_{BTB}$, needed to achieve a desired degree of extraction, $D$. In the conventional ion-pair method, where bromthymol blue is used as the anionic dye, the lower limit of the extraction pH is 7.5; below that pH the interference due to the background of bromthymol blue is too high (15, 16). In this study, a pH value of 7.8 was chosen to minimize the background.

The determined average values of the ion-pair extraction constants, $E_{1:1}$, as calculated from Eq. 7 and their standard errors of the mean ($\pm\sigma/\sqrt{n}$) were $1.32 \pm 0.03 \times 10^5$ for phenformin, $1.75 \pm 0.06 \times 10^4$ for buformin, and $4.28 \pm 0.12 \times 10^3$ for metformin. The initial concentrations of bromthymol blue used in these determinations ranged from $4.00 \times 10^{-5}$ to $20.00 \times 10^{-5}$ $M$ for $10 \times 10^{-5}$ $M$ phenformin and from $4.00 \times 10^{-5}$ to $80.0 \times 10^{-5}$ $M$ for $5 \times 10^{-5}$ $M$ buformin and metformin. The calculated initial bromthymol blue concentrations ($C^{\circ}_{BTB}$) needed to achieve 99, 90, and 80% degrees of extraction are given in Table I.

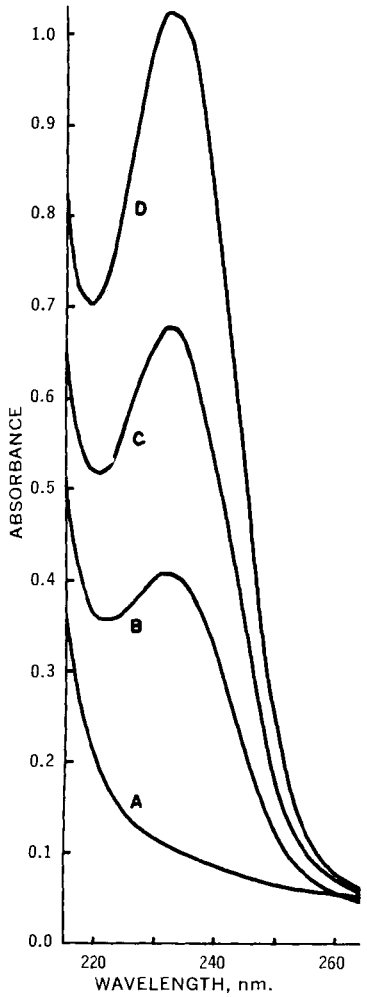


**Figure 1**—*UV spectra of metformin in the final solutions obtained from the urine of dogs administered metformin intravenously. The spectra were similar to those for buformin and phenformin. Curve A is for the background obtained from urine alone. Curves B, C, and D are for calculated 1.10, 2.10, and 3.39 × $10^{-4}$ M concentrations of metformin in the original urine, respectively. The urine was diluted threefold to minimize the absorbances due to coextracted impurities.*

Table II—Results of Quantitative Analyses[a] of Biguanide-Spiked Urine

| Compound | Concentration Range × ($10^5$ M) | Absorbance[b] at 232 nm. | Urine, $V_1$ ml. | Distilled Water, $V_2$ ml. | Buffer, $V_3$ ml. | Bromthymol Blue, $V_4$ ml. | Bromthymol Blue,[c] $V_5$ ml. | Percent Biguanide[d] Recovery Calculated from Eq. 10 | Experimental[e] | Background Absorbance from Urine Alone[f] |
|---|---|---|---|---|---|---|---|---|---|---|
| Metformin | 1.54–15.4 | 1.88–3.25 | 2.00 | 1.00 | 0.5 | 3.0 | 0 | — | — | 1.75 |
| | 2.22–22.2 | 0.493–2.06 | 2.00 | 1.00 | 0.5 | 1.0 | 0 | 71.99 | 67.2 | 0.350 |
| Phenformin | 2.00–10.0 | 0.610–1.72 | 2.00 | 1.00 | 1.0 | 1.0 | 0 | 98.08 | 98.1 | 0.350 |
| Buformin | 2.22–22.2 | 0.675–3.17 | 3.00 | 0.00 | 0.5 | 1.0 | 0 | 98.08 | 98.5 | 0.425–0.460 |
| | 2.22–22.2 | 0.475–2.96 | 3.00 | 0.00 | 0.5 | 1.0 | 2.0 | 98.08 | 98.5 | 0.210–0.372 |

[a] $V_1$ ml. of urine is admixed with $V_2$ ml. of distilled water, $V_3$ ml. of pH 7.8 phosphate buffer, and $V_4$ ml. of $10^{-2}$ M bromthymol blue and extracted with 5 ml. of methylene chloride. [b] After addition of 60 μl. of 25% tetrabutylammonium hydroxide in methanol, the biguanide is reextracted from 4 ml. of the separated methylene chloride into 4 ml. of water and the absorbance of the aqueous phase is measured at 232 nm. [c] Background from urine may be decreased in the spectrophotometric assay by extracting the methylene chloride solution of the ion-pair with $V_5$ ml. of $10^{-3}$ M bromthymol blue prior to adding the tetrabutylammonium hydroxide. [d] The fraction of biguanide recovery anticipated with bromthymol blue ≫ biguanide concentration may be calculated from:

$$D = \frac{(C^0_{BTB} \cdot E_{1:1})\,[5/(V_1 + V_2 + V_3 + V_4)]}{E_{1:1} \cdot C^0_{BTB} + 1 + K_a'/[H^+]}$$

under the assumption that the back-extraction of the biguanide freed by tetrabutylammonium hydroxide into water is quantitative. [e] Based on $(b/\epsilon_{232}) \times [4/(V_1 + V_2 + V_3 + V_4)] \times 5/4$, where $b$ is the slope of the calibration curve, $4/(V_1 + V_2 + V_3 + V_4)$ is the correction factor for the initial → final volume change of the final solution from the initial, and 5/4 is the correction factor for taking only 4 ml. of the methylene chloride phase out of the 5 ml. available. [f] The background absorbance of a calibration curve was determined by a similar procedure except that no biguanide was spiked in the urine.

**Determination of Biguanides in Urine**—Large amounts of various cations are present in urine and may interfere with the direct measurement of the ion-pair concentration of a drug. The modified methods described herein are based on the fact that all ion-pairs in methylene chloride or chloroform solvents can be destroyed by the addition of an excess of tetrabutylammonium hydroxide. Tetrabutylammonium ion is a stronger ion-pair formation agent with bromthymol blue; *i.e.*, $E_{1:1}$ for the tetrabutylammonium cation with the bromthymol blue monoanion is $1.58 \times 10^9$, a greater value than those given previously for the biguanides. The unpaired drug and other cations can then be extracted back into an aqueous phase. Fortunately, the major interfering cations, such as metal and ammonium ions, do not absorb in the UV above 230 nm. Thus, chromophoric organic bases, such as biguanides, can be determined spectrophotometrically after the described treatment. The measurements of the three biguanides were made at their $\lambda_{max}$ of 232 nm. (7). Typical spectra of the biguanide metformin in the final solution after the application of the assay procedure to urine samples are given in Fig. 1.

The results obtained for the analysis of biguanide-spiked urine samples under different experimental conditions are given in Table II. These show that the experimental recoveries agree well with what would be predicted from the approximative calculations in accordance with Eq. 10. The conditions given in Table II yielded almost quantitative extraction for both buformin and phenformin but not for metformin. Although quantitative conditions can be easily established for metformin by increasing the volume of bromthymol blue solution $V_4$, increasing the volume of methylene chloride used in the extraction, and/or lowering the pH of the buffer, the background increases sharply with these new conditions. An example of such increased background with increased $V_4$ is given for metformin in Table II. Thus, a compromise in the choice of conditions must be made to minimize background and optimize recovery in the metformin case.

Calibration curves which are typical for the biguanides are given for water and urine spiked with buformin in Fig. 2. Curve A for the prepared aqueous solutions of buformin was obtained by following the stated analytical procedure, except that it was not necessary to minimize background by the washing of the methylene chloride solution of the ion-pairs with dilute bromthymol blue solution prior to the addition of tetrabutylammonium hydroxide and reextraction of the biguanide into water. Each point on curve A is the mean of four determinations, and the vertical lines represent the extent of the standard errors of these means. Curve B was obtained from the

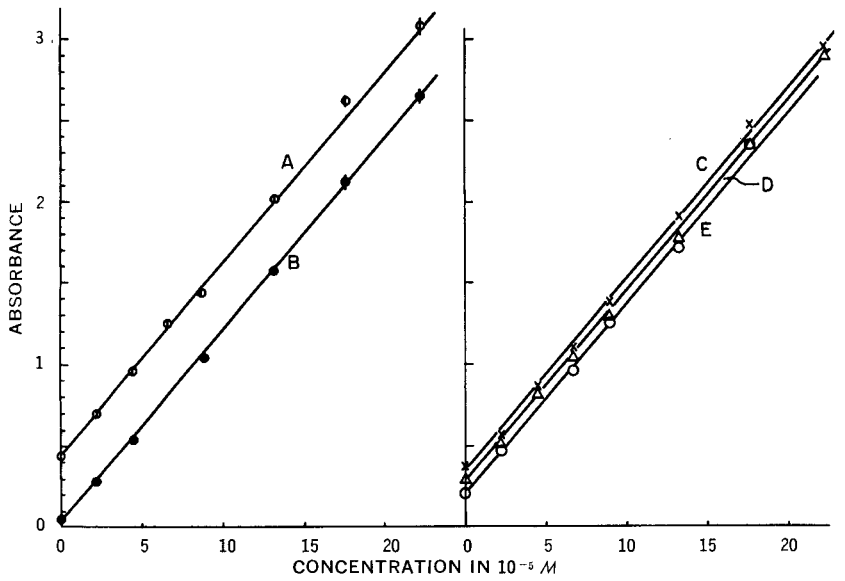


**Figure 2**—*Calibration curves for final spectrophotometric absorbance at 232 nm. of aqueous solution derived from buformin-spiked water (curve A) and urine (curves B, C, D, and E) at various molar concentrations. Curve B is for buformin added to urine to prepare the specified concentrations and assayed on different days to study the effect of storage at 4° for 4 days. There was no significant difference among days, and the vertical lines represent the standard error of the mean of the four determinations. Curves C, D, and E are calibration curves, one analysis at each level, for buformin added to the urines of different individuals. The intercepts of these plots are representative of the backgrounds of urine without added buformin.*

Table III—Tests[a] of the Significance of the Difference of Intercept[b] from Zero (20)

| $N$ | $R$ | $10^5\ b \times 2.22$ | $a$ | $10^5\ s \times 2.22$ | $t$ | Significance at 5% Level |
|---|---|---|---|---|---|---|
| | | **(1) Buformin Spiked in Urine, Effect of Storage at 4°** | | | | |
| 8 | 0.99985 | 0.2644 | 0.00406 | 0.01702 | 0.412 | n.s. |
| 8 | 0.99944 | 0.2616 | 0.01119 | 0.03308 | 0.583 | n.s. |
| 8 | 0.99922 | 0.2735 | 0.04850 | 0.04080 | 2.050 | n.s. |
| 7 | 0.99580 | 0.2711 | 0.00215 | 0.04575 | 0.753 | n.s. |
| | | **(2) Buformin Spiked in Water, Result of Four Replications** | | | | |
| 27 | 0.9988 | 0.2634 | 0.02817 | 0.04657 | 1.95 | n.s. |
| | | **(3) Buformin Spiked in Urine of Three Different Persons, Including Reextraction Process[c]** | | | | |
| 6 | 0.99904 | 0.2628 | 0.01371 | 0.03879 | 0.519 | n.s. |
| 8 | 0.99965 | 0.2586 | 0.02007 | 0.02562 | 1.351 | n.s. |
| 8 | 0.99961 | 0.2636 | 0.03610 | 0.02765 | 2.250 | n.s. |

[a] $R$ is the coefficient of correlation; $b$ is the slope; $a$ is the intercept of the regression line; $s$ is the standard error of the variance about regression for $N$ data points. The calculated $t$ values are $a/Sa$ and provide the criterion for deciding whether or not the intercept is significantly different from zero when $Sa$ is the standard deviation of the intercept (20). When the calculated $t$ values are smaller than those in the $t$ tables at the 95% level for $(N - 2)$ degrees of freedom, the conclusions of "no significant difference" or n.s. can be made. [b] The values of intercept have been corrected for background readings. [c] The procedure of extracting the methylene chloride solution of the ion-pair with dilute bromthymol blue solution prior to adding the tetrabutylammonium hydroxide is included.

determination of four series of buformin-spiked fresh urine samples with different storage times (0, 1, 2, and 5 days) at 4°. Again the washing procedure with dilute bromthymol blue solution to reduce background was omitted. Curves C, D, and E were obtained from buformin spiked in the urines of different persons. Here the washing procedure with dilute bromthymol blue was included.

The slopes and intercepts of these calibration curves were obtained after subtracting the background from blank studies on urine or water in accordance with least-squares fitting (20) to the linear regression equation.

Replicate samples of buformin added to water and urine in known concentration were assayed immediately and after various days of refrigerated storage. In addition, buformin added to the urine from various individuals was assayed, and the back-extraction procedure to minimize background was included. The analysis of variance of Tables III and IV demonstrated that there was no significant differences in slopes among all these calibration curves. After subtracting the background correction for absorbance, the intercepts of all the calibration curves were not significantly different from zero.

**Variation in Urine Background**—The composition of urine varies among and within individuals. It has been shown already that the background is very sensitive to variation in experimental conditions in the metformin case (Table II). The variation in background among different persons was studied by collecting from six individuals, of which three were smokers. These urines were spiked with metformin and were studied under the experimental conditions of the second line of Table II. Under these conditions, the background absorbance at 232 nm. varied from 0.205 to 0.805 (Table V).

Borg *et al.* (11) described a method to reduce the background caused by those cations that only form weakly bonded ion-pairs. They demonstrated that it can be reduced by shaking the organic phase containing the various ion-pairs with a dilute aqueous solution of bromthymol blue. This solution should have sufficient bromthymol blue concentration to maintain the ion-pair of the drug in the organic phase but not that of the contaminants. An amount of 2 ml. of $10^{-3}$ $M$ bromthymol blue was chosen to extract these interferences from 5 ml. of the organic phase. The urine of a heavy smoker was used to prepare four solutions without and four solutions with metformin ($1.6 \times 10^{-4}$ $M$). These solutions were analyzed under the conditions given in the second line of Table II. The extraction of the organic phases was carried out zero, one, two, and three times with 2 ml. of $10^{-3}$ $M$ bromthymol blue for the first two successive extractions and with 1.5 ml. for the third successive extraction. The conditions and results are summarized in Table VI and Fig. 3. The first extraction reduced the backgrounds at 232 nm. by about half without any significant reduction in the absorbance due to metformin. However, little advantage was obtained on subsequent extractions. The results of one extraction purification on background absorbance are also given in Table V for the urines from individuals other than those used for the study of Table VI. In all cases, the background was reduced by half or more. Therefore, it can be concluded that one extraction is suitable to reduce background and has been included in our standard experimental procedures.

Only those organic bases that can be extracted as ion-pairs and absorb in the UV will interfere with the assay. The ion-pair extrac-

Table IV—Analysis of Variance by Testing for Equality of Slopes[a] (20)

| Source | Degrees of Freedom | $SS$ | $MS$ | $F$ | Significance at 5% Level |
|---|---|---|---|---|---|
| | | **(1) Buformin in Urine, Effect of Storage at 4°** | | | |
| $b'$ | 1 | 21.721 | 21.721 | 15,515 | — |
| $b_i'/b'$ | 3 | 0.0092 | 0.0031 | 2.21 | n.s. |
| Error | 23 | 0.0316 | 0.0014 | — | — |
| | | **(2) Buformin Spiked in Urine of Different Persons, Reextraction Process Included** | | | |
| $b'$ | 1 | 14.7898 | 14.7898 | 16,226 | — |
| $b_i'/b'$ | 2 | 0.001199 | 0.0005995 | 0.6577 | n.s. |
| Error | 16 | 0.014584 | 0.0009115 | — | — |
| | | **(3) Comparison of Calibration Curves of Buformin Spiked in Water to Those in (1) and (2)** | | | |
| $b'$ | 1 | 60.1578 | 60.1578 | 33,719 | — |
| $b_i'/b'$ | 7 | 0.022 | 0.003143 | 1.762 | n.s. |
| Error | 64 | 0.1142 | 0.001784 | — | — |

[a] Analyses of variances were actually performed on factors proportional to the slope, $b$; *i.e.*, $b' = 2.22 \times 10^5\ b$. When the calculated $F$ values are compared with those given in the tables for the stated degrees of freedom and are smaller, the conclusions of "no significant difference" or n.s. can be made.

**Table V**—Background Absorbance at 232 nm. of Urine from Various Individuals Treated in the Biguanide Ion-Pair Separation

| | Smokers | | | Nonsmokers | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| Before reextraction | 0.402 | 0.525 | 0.801 | 0.523 | 0.805 | 0.205 |
| After reextraction | 0.190 | 0.250 | 0.280 | 0.140 | 0.320 | 0.105 |

tion constants and UV molar absorptivities of such possible interfering substances were determined and compared with those of biguanides (Table VII). It is apparent that the ion-pair extraction of an organic base is very selective. Creatinine has a UV absorption maximum of 232 nm. and is present in urine in large amounts. Fortunately, the $E_{1:1}$ value for this compound is so small that it is not readily extractable from the aqueous phase to the organic phase as an ion-pair under the experimental conditions. Creatine and arginine absorb only weakly at 232 nm. In addition, their $E_{1:1}$ values are so small that their interference should be readily removed by the washing with dilute bromthymol blue solution. Guanidine does not absorb in the UV at 232 nm. and thus should give no interference. Its methyl derivatives do absorb weakly at 232 nm., however, and may interfere slightly. Some other possible interferences in urine were investigated by Borg *et al.* (11). They measured the bromthymol blue ion-pair extraction constants for choline, nicotine, tryptamine, and tyramine, which are also listed in Table VII for comparison. Although interferences from nicotine, tryptamine, and tyramine are expected, they should not be very serious since the 232-nm. absorbance of the biguanide happens to be near the absorption minimum of these three compounds (21, 22).

Under the experimental conditions of the second line of Table II, the UV spectra of four out of five urine samples from nonsmokers gave only weak background absorption above 232 nm. Only one of them gave an absorption band with a maximum at about 272 nm. The intensity of this band was greatly reduced by one washing with dilute bromthymol blue solution. The background UV spectra derived from the urine of the three smokers gave an absorption band with an absorption maximum at 258 nm. and two shoulders at 264 and 252 nm. which persisted even with the washing step (Fig. 3). It is probable that the removable interference in the case of the nonsmoker was due to tyramine (11, 21); those interferences from the smokers were due to nicotine (11, 22). The spectrum of nicotine tartrate is also given in Fig. 3 to compare with the background derived from the urine of smokers.

## CONCLUSIONS

The described ion-pair extraction method with spectrophotometric measurement can determine quantitatively the three biguanides, metformin, buformin, and phenformin, in water and in human urine.

The water assay involves the extraction of 1:1 ion-pair of biguanide–bromthymol blue into methylene chloride. The 1:1 ion-pair can be changed to a 2:1 ion-pair of tetrabutylammonium–bromthymol blue by the addition of excess amounts of tetrabutylammonium hydroxide. The concentration of this 2:1 ion-pair can then be measured spectrophotometrically at 630 nm. The lower sensitivity limit of this method is $10^{-6}$ *M* (or 0.13 mcg./ml. of metformin) for 3 ml. aqueous solution. The apparent molar absorptivity is $4.87 \times 10^4/10^{-6} \sim 0.05$ (15).

To assay biguanide in urine, in order to eliminate most of the interferences, the biguanide in methylene chloride freed from the 1:1 ion-pair by tetrabutylammonium hydroxide is extracted back into water and its concentration is determined spectrophotometrically at 232 nm. The spectrophotometric reading, $A_{232}$, from the assay of 4 ml. urine containing $10^{-5}$ *M* biguanide is ~0.15. The spectrophotometric background reading of the urines of different individuals after one washing of the methylene chloride solution of ion-pairs with dilute bromthymol blue solution was statistically evaluated to be $0.249 \pm 0.173$ (mean $\pm$ $t\sigma$, $n = 10$). However, the background from various urines of a single individual has much less variation. The concentration of $10^{-5}$ *M* biguanide (or 1.29 mcg./ml. of metformin) in 4 ml. urine can be estimated within an error of 20% from a calibration curve established for the urine of a nonsmoker.

Fortunately, the concentrations of biguanide in the urine that result from drug administration are much higher. Thus, the necessary dilution of the urine for use of this analytical method drastically diminishes the background interference in the spectrophotometric assay. For example, studies now in process with dogs administered 60 mg./kg. of metformin with a half-life of about 2 hr. demonstrated metformin concentrations in the urine of $300 \times 10^{-4}$ *M* at 4 hr., $70 \times 10^{-4}$ *M* at 10 hr., and $20 \times 10^{-4}$ *M* at 24 hr. Thus, at a sensitivity of $10^{-5}$ *M* biguanide, the average urine background for the 24-hr. sample is reduced from an absorbance of 0.25 to a negligible 0.005. Figure 1 demonstrates the assay of metformin in the urine extracted by these procedures after intravenous administration of the biguanide to dogs.

This method provides almost quantitative extraction from an aqueous medium of charged compounds which are normally difficultly soluble in an organic solvent. It serves to give a facile separation of such

**Table VII**—Molar Absorptivities at 232 nm. ($\epsilon_{232}$) and Ion-Pair Extraction Constants ($E_{1:1}$) of Some Biguanides and Organic Bases

| Compound | pH | $\epsilon_{232}$ | $E_{1:1}$ |
|---|---|---|---|
| Metformin | 6.5 | $1.29 \times 10^4$ | $4.28 \times 10^3$ |
| | 10.4 | $1.25 \times 10^4$ | |
| | 11.5 | $1.30 \times 10^4$ | |
| | 12.0 | $1.31 \times 10^4$ | |
| | 12.5 | $1.36 \times 10^4$ | |
| Buformin | 7.0 | $1.37 \times 10^4$ | $1.75 \times 10^4$ |
| | 10.4–12.6 | $1.34 \times 10^4$ | |
| Phenformin | 6.0 | $1.47 \times 10^4$ | $1.32 \times 10^5$ |
| | 10.4 | $1.39 \times 10^4$ | |
| | 11.9 | $1.37 \times 10^4$ | |
| | 12.3 | $1.35 \times 10^4$ | |
| | 12.7 | $1.30 \times 10^4$ | |
| Arginine | 5.5 | 30 | ~58 |
| | 10.9 | 10 | |
| Creatine | 5.7 | 480 | ~3 |
| | 10.8 | 595 | |
| Creatinine | 7.1 | $6.78 \times 10^3$ | ~0 |
| | 9.7–11.1 | $7.02 \times 10^3$ | |
| | 12.1 | $7.2 \times 10^3$ | |
| Guanidine | 6,10,8,12 | 0 | 400 |
| Methylguanidine | 6.0 | 30 | $3.20 \times 10^3$ |
| | 10.8 | 5 | |
| Dimethylguanidine | 5.4 | 100 | $3.70 \times 10^4$ |
| | 10.8 | 90 | |
| Choline | — | — | $1.26 \times 10^3$ (16) |
| Nicotine | — | — | $6.61 \times 10^5$ (11) |
| Tryptamine | — | — | $2.45 \times 10^4$ (11) |
| Tyramine | — | — | $3.24 \times 10^2$ (11) |

**Table VI**—Spectral Analyses at 232 nm. of Metformin Derived from a Urine after Successive Extractions of the Organic Solution of Ion-Pairs with $10^{-3}$ *M* Bromthymol Blue before Reextraction of Drug into an Aqueous Phase on Addition of Tetrabutylammonium Hydroxide

| Number of Extractions | Milliliters of Organic Phase | Milliliters $10^{-3}$ *M* Bromthymol Blue Solution Used | Absorbance ($A_b$) of Urine without Metformin | Absorbance ($A'$) of Urine with Metformin | $\Delta A$ ($A' - A_b$) |
|---|---|---|---|---|---|
| 0 | 5.0 | — | 0.831 | 2.03 | 1.20 |
| 1 | 4.7 | 2.0 | 0.417 | 1.56 | 1.14 |
| 2 | 4.3 | 2.0 | 0.335 | 1.37 | 1.04 |
| 3 | 4.0 | 1.5 | 0.290 | 1.27 | 0.980 |

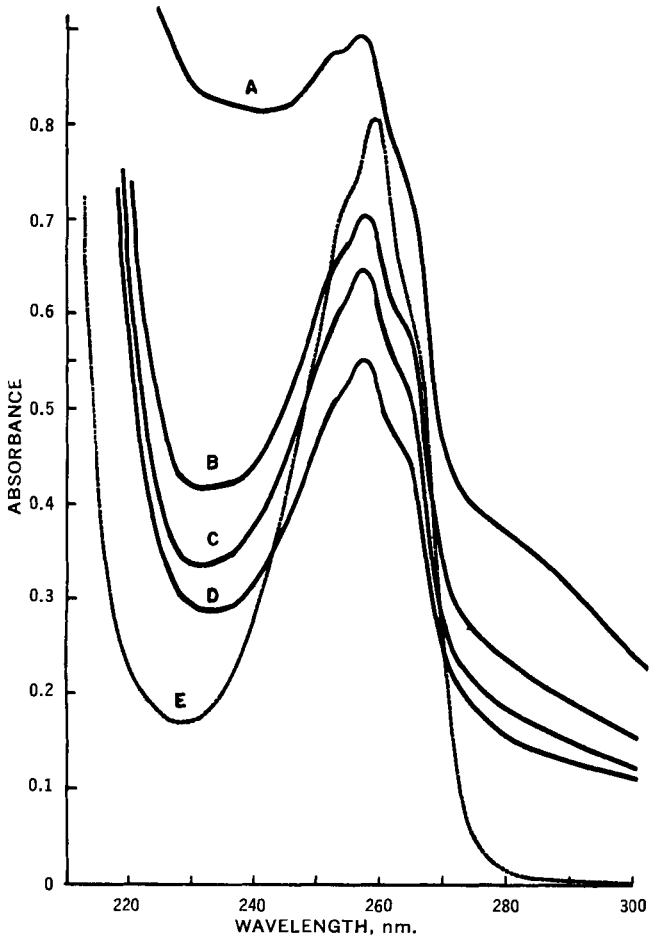


**Figure 3**—*Background UV spectra of final solution obtained from the urine (2 ml.) of a heavy smoker after the treatment given under the experimental conditions of line 2 of Table II. Prior to reading the absorbance at 232 nm. of its aqueous reextract (4.00 ml.), the methylene chloride phase (5 ml.) containing the ion-pairs extracted from urine was washed zero (curve A), one (curve B), two (curve C), and three (curve D) times with 2.0 ml. of $10^{-3}$ M bromthymol blue solution. The dotted line (curve E) is the spectrum of $2.72 \times 10^{-4}$ M nicotine tartrate.*

compounds from many of the normal salts present in biological fluids such as urine and provides a method of concentration. The ready reextraction into aqueous solution provides a simple separation from nonpolar compounds that resist reextraction into water from the organic solvent. Although there are possible interferences from biologically endogenous compounds (examples are given in Table VII) that may also undergo ion-pair extraction and reextraction into an aqueous phase, certain extraction conditions, as delineated in this paper, can minimize such interferences when their ion-pair extraction constants are less than that of the compound to be monitored.

Although the spectrophotometric measurements of the biguanides, concentrated and separated from all but minimal materials, by the cited procedures were adequate to monitor these compounds in animal urine, more sensitive physical chemical methods could be developed and applied to quantify the biguanide removed from its biological matrix.

Such additional methods would be needed when biguanide metabolites are formed that are extracted by the same procedures and would have similar spectra. Fortunately, metformin has no observable metabolites.

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