# Spectrophotometric Determination of a Mixture of Secondary and Tertiary Amines in Urine

By BARRIE M. PHILLIPS, PAUL J. KRAUS, and MEL E. STRATMEYER

**A procedure for the determination of a mixture of secondary and tertiary amines in urine is described. The method is based on the formation of dithiocarbamic acids from secondary amines and carbon disulfide, followed by the reaction of this acid with copper to form a yellow salt or complex, and on the production from tertiary amines of a yellow acid salt of the quinoid form of a halogenated sulfonphthalein dye. Data describing the recovery of three secondary and three tertiary amines from urine are presented.**

IN STUDIES of the disposition of organic bases in animals, it is often necessary to determine both secondary and tertiary amines in urine. Many methods have been reported for the determination of such amines separately; this report describes a method for determining both amines in a single chloroform extract. The method was developed initially to investigate the possibility of *in vivo* Mannich hydrolysis of the tertiary amine Mannich base MA1050, a new antiinflammatory-analgetic agent, to, among other neutral products, the secondary amine phenylpiperazine.

Among the various methods described, that of Woods *et al.* (1) possesses adequate sensitivity, reproducibility, and applicability and, with some modification, has been employed in this procedure for the determination of tertiary amines. The reaction of bromophenol blue with organic amines in chloroform occurs with primary, secondary, and tertiary amines (except aromatic amines). In the procedure described, acetic anhydride is employed to remove primary and secondary amines. Hence, the method is specific for tertiary amines. The reaction of carbon disulfide with secondary amines in aqueous solution has been employed in their determination by Umbreit (2), but this procedure could not be adapted to urine (recovery of added secondary amine varied from 50 to 500%). Modification of the method of Umbreit (2) resulted in a satisfactory procedure for the determination of secondary amines in a chloroform extract of urine.

## EXPERIMENTAL

**Reagents.**—Unless otherwise noted, all chemicals are reagent grade.

*Chloroform.*—Technical grade chloroform is purified by passing through a column (18-mm. glass tubing) containing (from bottom to top) a fritted glass disk, 12–15 cm. of alumina,[1] a glass wool plug, and 15–20 cm. of 14–20 mesh silica gel.[2] Such a column can be used to purify 1 L. of chloroform, which is stable for 24 hr. at refrigeration temperature.

*Bromophenol Blue.*—A 0.5-mg./ml. solution was prepared in purified chloroform. The reagent is satisfactory for 2 weeks if kept at refrigeration temperature.

*Sodium Hydroxide Solution.*—0.1 *N* in distilled water.

*Sucrose.*—A commercial grade of dried granular sucrose[3] was found to perform as satisfactorily as that described by Woods *et al.* (1).

*Carbon Disulfide–Pyridine–Isopropanol.*—A mixture of 35 ml. of carbon disulfide, 25 ml. of pyridine, and 65 ml. of isopropanol was utilized. The reagent is stable for 2 months in a glass-stoppered bottle.

*Cupric Chloride Solution.*—0.11 Gm. of $CuCl_2 \cdot 2H_2O$ in 500 ml. of distilled water.

*Acetic Acid.*—Ten per cent v/v in distilled water was employed.

*Isopropanol.*

**Procedure.**—To 0.5 to 2 ml. of urine in a glass-stoppered 50-ml. round-bottom centrifuge tube add sodium hydroxide sufficient to give the pH necessary for complete extraction of the base. All extractions in this study were conducted at pH 8.0. Depending on the pKa of the base and the pH necessary for complete extraction, appropriate changes in the amount of sodium hydroxide added should be made. Adjust the final volume to 4 ml. with distilled water. Add 10 ml. of purified chloroform and extract (tubes at a 20° angle to the horizontal) on a reciprocal shaker[4] at 90 excursions per minute for 15 min.

TABLE I.—CALIBRATION CURVES FOR SECONDARY AND TERTIARY AMINES

| Compd. | Slope | Intercept | S. E. of Slope | Regression Correlation Coefficient |
|---|---|---|---|---|
| MA1050[a] | 0.0856 | −0.0250 | 0.00081 | 0.998 |
| Chlorpromazine[b] | 0.0702 | −0.0187 | 0.00060 | 0.999 |
| Codeine[c] | 0.0759 | −0.0368 | 0.00083 | 0.997 |
| MA191[d] | 0.0088 | −0.0099 | 0.00010 | 0.996 |
| Desmethylimipramine[b] | 0.0056 | −0.0132 | 0.00009 | 0.995 |
| Ephedrine[c] | 0.0076 | −0.0200 | 0.00010 | 0.994 |

[a] 2-(4-Phenyl-1-piperazinylmethyl)cyclohexanone·HCl. [b] As the monohydrochloride. [c] As the sulfate. [d] *N*-Phenylpiperazine·HCl.

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[1] Baker and Adamson.
[2] Fisher Scientific Co.
[3] Bakers' Special Revere.
[4] Eberbach Corporation reciprocating shaker; a horizontal-action shaker is preferable rather than the wrist-action type.

TABLE II.—A. RECOVERY OF SECONDARY AND TERTIARY AMINES FROM URINE

| Compd. | Recovery of Amine from Urine, %[a] 12.5[b] | 25.0[b] | 37.5[b] | 50.0[b] | 125[b] | 250[b] | 375[b] | 500[b] | Mean |
|---|---|---|---|---|---|---|---|---|---|
| MA1050 | 106.6 ± 3.4 | 104.5 ± 2.3 | 103.9 ± 1.7 | 101.6 ± 0.2 | .... | .... | .... | .... | 104.2 ± 1.1 |
| Chlorpromazine | 97.3 ± 1.4 | 101.8 ± 3.9 | 98.9 ± 1.1 | 98.9 ± 1.8 | .... | .... | .... | .... | 99.2 ± 1.1 |
| Codeine | 99.1 ± 1.6 | 99.3 ± 1.8 | 96.3 ± 0.6 | 96.2 ± 0.2 | .... | .... | .... | .... | 97.7 ± 0.2 |
| MA191 | .... | .... | .... | .... | 103.1 ± 2.2 | 101.2 ± 2.5 | 102.6 ± 1.3 | 104.5 ± 0.2 | 102.8 ± 0.3 |
| Desmethylimipramine | .... | .... | .... | .... | 102.7 ± 1.1 | 102.9 ± 1.4 | 97.9 ± 2.3 | 99.7 ± 0.3 | 100.8 ± 1.1 |
| Ephedrine | .... | .... | .... | .... | 93.3 ± 1.2 | 97.0 ± 1.2 | 97.7 ± 0.3 | 97.8 ± 0.3 | 96.5 ± 0.3 |
| Mean | 101.0 ± 0.3 | 101.9 ± 2.0 | 99.7 ± 1.3 | 98.9 ± 0.3 | 99.7 ± 2.1 | 100.4 ± 1.3 | 99.4 ± 0.3 | 100.7 ± 1.6 | 100.2 |

B. RECOVERY OF SECONDARY AND TERTIARY AMINES FROM URINE IN THE PRESENCE OF EACH OTHER

| Compd. | Recovery of Amine from Urine, %[a] 12.5[c] | 25.0[c] | 37.5[c] | 50.0[c] | 125[d] | 250[d] | 375[d] | 500[d] | Mean |
|---|---|---|---|---|---|---|---|---|---|
| MA1050 | 96.1 ± 1.6 | 95.7 ± 0.3 | 95.7 ± 0.2 | 97.7 ± 1.2 | .... | .... | .... | .... | 96.3 ± 0.2 |
| Chlorpromazine | 101.0 ± 2.1 | 104.1 ± 1.5 | 99.7 ± 1.5 | 97.4 ± 1.2 | .... | .... | .... | .... | 100.6 ± 0.3 |
| Codeine | 99.5 ± 3.1 | 98.5 ± 1.0 | 98.6 ± 1.1 | 99.9 ± 2.1 | .... | .... | .... | .... | 99.1 ± 0.3 |
| MA191 | .... | .... | .... | .... | 102.4 ± 0.3 | 100.6 ± 1.4 | 103.5 ± 1.8 | 105.1 ± 0.1 | 102.9 ± 0.2 |
| Desmethylimipramine | .... | .... | .... | .... | 100.1 ± 1.1 | 97.8 ± 2.7 | 100.1 ± 1.2 | 105.0 ± 0.3 | 100.8 ± 0.3 |
| Ephedrine | .... | .... | .... | .... | 94.9 ± 2.0 | 96.4 ± 1.5 | 98.2 ± 1.0 | 96.7 ± 1.1 | 95.8 ± 0.2 |
| Mean | 98.9 ± 1.4 | 99.4 ± 1.4 | 98.0 ± 0.3 | 98.7 ± 0.3 | 99.1 ± 1.3 | 98.8 ± 0.3 | 100.6 ± 1.0 | 102.2 ± 1.4 | 99.4 |

[a] Mean (of three determinations) ± standard error. [b] Micrograms of amine per milliliter of urine. [c] Micrograms relevant amine per milliliter urine; mixture also contained 500 mcg. MA191/ml. urine. [d] Micrograms relevant amine per milliliter urine; mixture also contained 50 mcg. MA1050/ml. urine.

Centrifuge[5] for 5 min. at 2000 r.p.m. and aspirate off the aqueous material. Add 10 ml. of distilled water and agitate[6] vigorously. Centrifuge at 2000 r.p.m. for 5 min. and aspirate off the aqueous phase. Place a 4-ml. aliquot of the extract into a second 50-ml. round-bottom centrifuge tube for subsequent secondary amine determination and pass the remainder of the extract through a 7 mm. × 5 cm. column of sucrose. Add 0.1 ml. of acetic anhydride to the dried extract and shake vigorously; this portion is then employed for tertiary amine determination.

Transfer 3.0 ml. of the dried acetic anhydride treated extract to a test tube of suitable size, add 0.3 ml. of bromophenol blue reagent, and agitate. Read[7] the resulting absorption in 30 min. at 415 mμ.

To the 4-ml. aliquot of the extract in the 50-ml. round-bottom centrifuge tube add 8 ml. of the carbon disulfide–pyridine–isopropanol reagent and 4 ml. of the cupric chloride reagent and agitate vigorously. Allow the mixture to stand for 15 min. and aspirate off the aqueous layer. Add 5 ml. of 10% acetic acid and agitate vigorously. Centrifuge at 2000 r.p.m. for 5 min. and aspirate off the aqueous layer. Add 8 ml. of the remaining material to 2 ml. of isopropanol in a test tube of suitable size and agitate. Read the resulting absorption in 1 hr. at 440 mμ.

Calibration curves were obtained with pairs of aqueous solutions at concentrations ranging from 1.25 to 25 mcg./ml. at 1.25-mcg. intervals (tertiary amines) and from 12.5 to 250 mcg./ml. at 12.5-mcg. intervals (secondary amines). Final concentrations in the chloroform extract must range from 0.5 to 10.0 mcg./ml. for tertiary amines and from 5 to 100 mcg./ml. for secondary amines. The lines best fitting the points obtained were determined by linear regression analysis.

Recovery values were determined in triplicate on 2.0-ml. urine samples containing 12.5, 25.0, 37.5, and 50 mcg./ml. of tertiary amines or 125, 250, 375, and 500 mcg./ml. of secondary amines.

Recovery of tertiary amines in the presence of a high concentration of secondary amine and vice versa was investigated on triplicate urine samples, each containing one type of amine at the concentrations listed in the preceding paragraph and the highest concentration of an amine from the other category.

## RESULTS AND DISCUSSION

The numerical descriptions of the calibration curves for the determination of several compounds are given in Table I. The standard errors and correlation coefficient values included in this table indicate both the linearity of the calibration curves and the accuracy of the slopes. Recoveries of amines from urine are summarized in Table IIA, and recoveries of one type of amine from urine in the presence of a high concentration of an amine from the other category are summarized in Table IIB. These results demonstrate the applicability of the

[5] International Centrifuge, Universal model U.V., or any centrifuge capable of similar speeds which will accept the 50-ml. centrifuge tubes.
[6] Scientific Industries, Inc., Vortex Jr. mixer, model K-500-J; use of this instrument will reduce markedly the time necessary for agitation.
[7] Beckman DU; a Coleman Junior spectrophotometer has been employed and gave satisfactory results.

method to the accurate determination of these amines in urine, both alone and in the presence of each other.

The procedure described for secondary amine determination is simple and is essentially trouble free. Although considerably less sensitive than the tertiary amine procedure, it can be made more sensitive by reading the final absorption in longer cells. The procedure for tertiary amine determination, on the other hand, is quite demanding. A high pH during the extraction or the introduction of small quantities of water after the extract is dried may result in a high blank or aberrant color development.

It should be pointed out that the specificity of the method largely depends on the pH during the initial extraction. If phenolic metabolites can be anticipated and if separation is desired, the pH should be high enough to prevent their extraction, although the high pH may introduce blank difficulties. Nonphenolic metabolites which retain the original amine character can also be expected to yield comparable color values. As pointed out by Umbreit (2), the presence of primary amines will result in approximately 1% error in the secondary amine determination, but such amines will not interfere in the tertiary amine determination if acetic anhydride is employed.

REFERENCES

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# Recorded Amperometric Tetraphenylborate Titrations of Amines, Quaternary Nitrogen Compounds, and Potassium Salts

By JOSEPH E. SINSHEIMER and DONALD HONG

**Improved efficiency of tetraphenylborate amperometric titrations by constant addition of titrant and continuous recording of current is reported.**

Amos and Sympson (1) reported the first direct amperometric titration of potassium salts with sodium tetraphenylborate (TPB) using a dropping mercury electrode. Smith *et al.* (2) reported the electrochemical oxidation of TPB at the graphite electrode and the application of this reaction to the amperometric titrations of potassium salts. These procedures have been adapted (3) for the titration of amines.

It was found in such titrations of amines that it was not necessary to wait for a constant current value after each addition of titrant. Moreover, with the graphite electrode, stirring could be continuous during the addition of titrant. While current values are higher under these conditions, equivalence points could be obtained easily, providing equal time intervals were used between the addition of titrant and determination of current value.

At this time, we would like to report that the efficiency of TPB amperometric titrations can be significantly improved by the constant addition of titrant and continuous recording of current.

## EXPERIMENTAL

**Apparatus.**—The titration vessel and graphite electrode were as previously described (3). A Leeds and Northrop 1199-31 calomel electrode, but with the potassium chloride solution replaced with a saturated sodium chloride solution, was used as the reference electrode. Standard potassium chloride solution was delivered to the titration vessel by a Sargent model C 10-ml. constant rate buret. Potential was applied and current recorded with a Leeds and Northrop model 62200 Electro-Chemograph.

**Reagents.**—The 0.1 *M* TPB, 0.1 *M* potassium chloride, and pH 4.6 buffer solutions were prepared as previously described (3). Standard and test solutions were prepared by direct weighings of reagent grade chemicals and amines dried to constant weight. Benzalkonium chloride was used as the commercially available solution.[1] At the suggestion of the supplier, hexadecyltrimethylammonium bromide[2] was dried to constant weight under vacuum without heat.

**Procedure.**—To standardize the sodium TPB solution, 4 ml. of this solution was added to 50 ml. of acetate buffer, followed by 2 ml. of 0.1 *M* standard potassium chloride solution. With the electrodes immersed, the solution was stirred for 10 min. A potential of 0.55 v. was established with the graphite being the positive electrode and the polarograph recorder started with a current sensitivity of 100 $\mu$amp. The excess TPB ion was titrated at 25 $\pm$ 0.1° with the standard potassium chloride solution being delivered from the constant rate buret. The titration was continued until a linear change in current with time was obtained. End points were determined by the intersection of the extrapolated linear portions of the current-time curves.

Titration of samples was accomplished by using the dried weighed compound or an aliquot of a known solution equivalent to $1 \times 10^{-4}$ to $6 \times 10^{-4}$ mole of compound. Samples were added to 50 ml. of buffer solution in the titration vessel at 25 $\pm$ 0.1°. While the solution was stirred, an excess of about 4 ml. of standardized sodium TPB

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[1] Marketed as Zephiran Chloride by Winthrop Laboratories.
[2] Marketed as Quatresin by The Upjohn Co.