Improved Method for Determination of Inorganic Sulfate in Biologic Fluids.* (22299)

CHARLES R. KLEEMAN, EVA TABORSKY, AND FRANKLIN H. EPSTEIN.
(Introduced by P. K. Bondy.)
Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.

Numerous adequate technics for the determination of fairly large amounts of inorganic sulfate in biologic material have been available for many years(1-3). However, the low concentration of inorganic sulfate in the plasma (2-3 mg % as $SO_4^=$) has rendered most of these methods inadequate for use with relatively small amounts of plasma (*i.e.* one to 2 cc). The basic technic which has appeared most applicable to the analysis of small quantities of inorganic sulfate in biologic fluids is benzidine precipitation of the sulfate(4). Benzidine, a weak organic base, forms stable salts with strong mineral acids. The sulfate is insoluble in the presence of excess benzidine in acid solution and particularly in the presence of organic solvents such as acetone and alcohol(4). The optimum conditions necessary for the precipitation of benzidine sulfate have been extensively studied(4-6). The benzidine sulfate must be quantitatively determined either titrametrically(7) or colorimetrically(8). In the former, the benzidine sulfate is titrated with NaOH by the following reaction: $C_{12}H_8(NH_2)_2\ H_2SO_4 + 2NaOH = C_{12}H_8(NH_2)_2 + Na_2SO_4 + 2H_2O$. According to Peters (4) the free benzidine is so weak a base that it is not alkaline to phenolphthalin or phenolred. The H_2SO_4 combined with it may be titrated as the free acid. This procedure, although accurate, requires titrating at 100°C. Letonoff and Reinhold(9) made a notable advance in the colorimetric determination of benzidine by using sodium β naphthoquinone-4-sulfonate as a color reagent. With benzidine in alkaline solution this compound develops a red brown color which changes to red on addition of acetone. However, the spectral characteristics and stability of the final color were not adequately studied. The precipitate of benzidine sulfate, once formed, must be separated and washed free of excess benzidine. As emphasized recently by Dodson and Spencer(6), considerable losses of the precipitate may occur during these steps. There is a tendency for the precipitate to break away during decantation regardless of the type of centrifuge tube used; this they prevented by adding small amounts of powdered glass as a rough surface on which the precipitate could be packed down. The benzidine sulfate has usually been precipitated from an acetone or ethanol solution and subsequently washed with either of these solvents. In the present study it was found that small but consistent losses of the benzidine sulfate precipitate occurred when washed with either acetone or ethanol. This could be prevented by washing with a mixture of ⅔ ethanol and ⅓ ethyl ether. Benzidine sulfate is considerably more insoluble in ethyl ether than in either acetone or alcohol.

By utilizing powdered glass(6), washing the precipitate with an ethanol-ether mixture and employing the color reagent, sodium β naphthoquinone-4-sulfonate(9) an improved technic for the measurement of small amounts of inorganic sulfate (10 or more μg) was obtained.

Materials and methods. Reagents. 1. H_2O, redistilled; 2. Ethanol, 95%, redistilled over NaOH pellets; 3. Benzidine, (Fisher Scientific Co., B-259), 1 g in 100 ml redistilled ethanol, preserved in dark bottle; 4. Trichloroacetic acid, 20%, redistilled with 208 mg $BaCL_2$ in 2 ml H_2O/200 g trichloroacetic acid (analytical reagent); 5. Sodium borate, (Fisher Scientific Co. S-248), 1 g in 100 ml of 0.1 *N* NaOH, stored in pyrex bottle. 6. Sodium sulfate, anhydrous, A. R., 1.4788 g/l of water to give a 100 mg % $SO_4^=$ solution. 7. Benzidine hydrochloride, purified according to Letonoff and Reinhold(9), 0.1606 g

* Supported in part by U. S. Public Health Service Grant A-625.

benzidine hydrochloride are placed in 200 ml volumetric flask, dissolved in about 50 ml water at 50°C, cooled to room temperature, diluted to mark and stored in the cold; 8. Sodium 1, 2-naphthoquinone-4-sulfonate (S. N.S.) (Eastman-Kodak 1372), purified: 2 g of crude compound are dissolved in minimum amount of boiling water, filtered through heated funnel, salted out with absolute ethanol, by adding ethanol drop wise to filtrate, filtered by suction on Büchner funnel, washed with absolute ethanol, dried with ether and stored in dark bottle. The solution must be freshly prepared each time by dissolving 150 mg in 100 ml water;† 9. Ether, A.R.; 10. Acetone, A.R.; 11. Powdered glass, washed, (Fisher Scientific Co. S-248) 15 ml heavy wall centrifuge tubes are used. All glassware should be rinsed with dilute nitric acid.

Procedure. All urine and serum samples are diluted to give a concentration of 20-140 μg $SO_4^=$ per cc. To 2 ml of the diluted sample are added 2 ml of water and 4 ml of 20% trichloroacetic acid. Samples are mixed, allowed to stand for 10 minutes and centrifuged for 10 minutes. From the supernatant fluid 2 ml aliquots are withdrawn and introduced into centrifuge tubes. To each of these tubes 5 ml of 1% benzidine solution is added. The mixtures are stirred and stirring rods washed with small amounts of ethanol. Centrifuge tubes are covered with parafilm and placed in ice-bath overnight. On following day‡ 3 to 5 mg of glass powder is added to each tube and precipitated benzidine sulfate is separated by centrifugation at 2250 rpm for 15 minutes. The supernatant fluid is decanted, with care taken not to loosen any of the precipitate. The tubes are drained, inverted on filter paper, 5 to 10 minutes and mouths of tubes are then wiped with tissue paper. The outsides of tubes are washed with acetone by medicine dropper. After draining for 1 minute, the insides of the inverted tubes are rinsed with ethanol and tubes are drained for 5 minutes. The mouths of tubes are again wiped with tissue paper and tubes are placed upright. Ten ml of an alcohol-ether mixture (1:2) is added to each tube and the precipitate suspended by a stirring rod. Care is taken to break the precipitate completely in order to dissolve all excess benzidine. The glass rods are washed with the same alcohol-ether mixture and tubes are centrifuged at moderately high speeds for 15 minutes. The supernatant fluid is decanted and tubes drained for 5 minutes. The tubes are then wiped and washed inside and outside as before. One ml of sodium borate solution is then added, the precipitate is resuspended and the tubes placed into a water-bath with occasionally stirring at about 60°C until the precipitate completely dissolves. To each tube 10 ml of water and 1 ml of the color reagent (sodium 1:2 naphthoquinone-4-sulfonate) are added and allowed to stand for 5 to 6 minutes. Two ml of acetone are then added to each tube and the solutions are mixed, centrifuged and supernatant fluid is poured into colorimeter tubes. Care is exercised that identical numbers of minutes elapse between addition of color reagent and of the acetone to each tube. The optical density of the solutions is determined in the Coleman Junior Colorimeter at 490 mμ. All analyses were done in triplicate or quadruplicate.

Standards. Stock standard benzidine hydrochloride is diluted 30 fold. To 7 centrifuge tubes 1 to 7 ml of this diluted standard solution is added and the volumes made up to 10 ml with water. A blank tube containing 10 ml of water is prepared with each determination. To all tubes sodium borate, color reagent and acetone are added as outlined before.

Results. Characteristics of benzidine-sodium β naphthoquinone-4-sulfonate (S.N.S.) complex. Fig. 1 illustrates the absorption spectrum of final color compound. Maximum optical density occurs at 490 mμ. It is

† In the present study, in contrast to Letonoff (9), a definite fall in optical density of standards was noted if fresh reagent was not prepared with each set of analyses. However, as the optical density of the standards and unknowns are lowered by proportional amount no error is introduced by using S.N.S. previously prepared and stored in the cold (4°C).

‡ Complete precipitation will occur within 3 hours; leaving samples overnight is a matter of convenience.

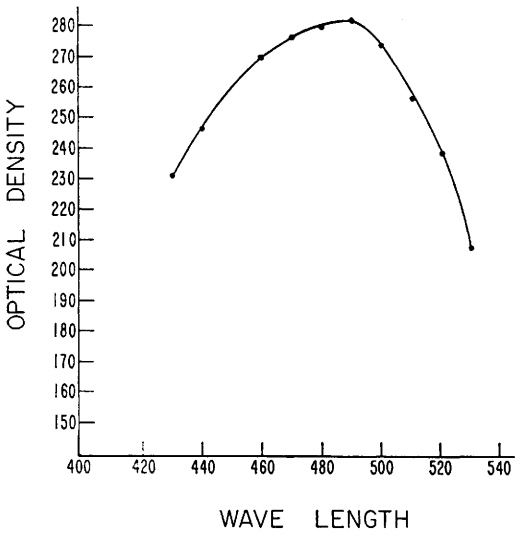

FIG. 1. Absorption spectrum for final benzidine-β naphthoquinone-4-sulfonate compound as determined on Coleman Junior Spectrophotometer.

of interest that this wave length also represents the optimum length for the color developed by interaction of this reagent and amino acids(10). the amine groups of benzidine combining with the S.N.S. to give the observed color. At this wave length, the standard curve follows Beer's law quite closely between zero and 50 μg of sulfate (the benzidine hydrochloride equivalent to these amounts of $SO_4^=$). Rarely at 50 μg, and invariably above this level the curve deviates from a straight line as illustrated in Fig. 2. Therefore for maximum accuracy the precipitate of benzidine sulfate should be equivalent to 50 γ of sulfate or less. Although the slope of this curve is readily reproducible small variations in optical density of the standards from one analysis to the next necessitates that a set of standards be run simultaneously with the unknowns. Maximum color development occurs in 5 to 6 minutes and this is stable for at least 2 hours if room temperature is relatively constant. After 2 hours optical density decreases slowly and proportionally in all samples.

Recovery of benzidine sulfate from standard solutions, urine and serum. In Table I are listed recoveries of sulfate from solutions of known concentration. The mean recovery for this series was 98.8% (range 94-104) however, recoveries below 97% were seen only with the sample containing 10 γ of sulfate. Sulfate added to serum and urine was quantitatively recovered (Table II). The mean recovery from serum was 100.5% (range 97-102) and from urine 99.0% (range 97-104). Duplicate determinations on fresh

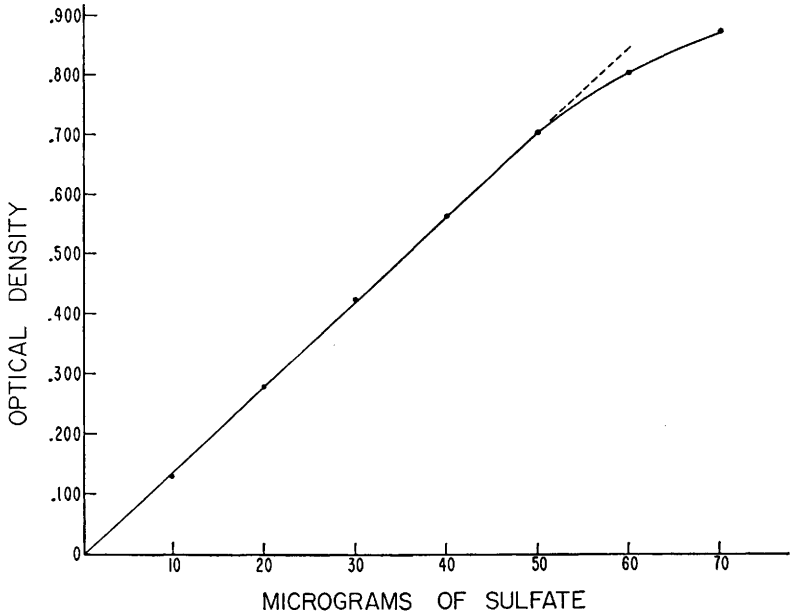

FIG. 2. Calibration curve for benzidine-β naphthoquinone-4-sulfonate compound at 490 mμ.

TABLE I. Analysis of Pure Sulfate Solutions. Five determinations* in each solution.

Sulfate,† μg	Found Mean	Found Range	% variation
10	10.1	9.4-10.4	94-104
20	19.7	19.4-20.0	97-100
30	30.1	29.0-30.4	98-103
40	39.7	39.0-40.0	99-100
50	49.9	49.2-52.0	98-104
60	59.5	58.0-62.0	97-103
70	69.5	68.0-70.7	97-101

* Each determination done in triplicate.
† Sulfate present in initial aliquot of solution.

or frozen serum and urine were reproducible within a range of ± 2.5%. If, however, serum or urine were stored at 4-5°C in the unfrozen state for 24 hours or longer an increase in concentration of inorganic sulfate was almost always noted, probably as a consequence of some degeneration of organic sulfates in these biologic fluids.

The mean concentration of inorganic sulfate in the sera of 15 normal subjects was 2.84 mg % (range 2.23-3.90). These values are comparable to those obtained by other investigators using benzidine or other technic (1,3,11).

It has been reported that inorganic phosphates in concentrations found in human urine are coprecipitated as benzidine salts and must be removed prior to precipitating inorganic sulfate(5,7). With the present technic, however, urinary inorganic phosphates in concentrations up to 150 mg % did not interfere with sulfate precipitation. This was investigated by determining urinary sulfates before and after precipitation of phosphate as magnesium ammonium phosphate(12).§

TABLE II. Recovery of Sulfate Added to Serum and Urine.

Serum	Added sulfate, μg	Total Expected, μg	Total Found, μg	% of expected $SO_4^=$ recovered
μ				
1 (15.4)*	15.0	30.4	31.0	102
"	30.0	45.4	46.4	102
"	30.0	45.4	44.2	97
2 (30.0)	15.0	45.0	46.0	102
"	15.0	45.0	44.0	98
"	30.0	60.0	60.2	100
3 (41.6)	3.3	44.9	45.0	101
"	6.6	48.2	49.4	102
Urine				
1 (11.2)*	30.0	41.2	39.8	97
2 (22.5)	20.0	42.5	41.5	98
"	15.0	37.5	37.3	100
3 (21.6)	22.5	44.1	44.8	102
"	15.0	36.6	38.0	104
4 (16.0)	22.5	38.5	38.0	99
5 (32.0)	22.5	54.5	55.7	102
"	15.0	47.0	47.6	101
6 (9.6)	7.5	17.1	16.7	98

* Sulfate present in initial aliquot of serum and urine.

The reproducibility and accuracy of the present method was equal to or better than other procedures utilizing larger quantities of plasma or serum(1,2,3,7,13). However, it must be stressed that, *like* all procedures requiring quantitative recovery of a small precipitate, utmost care is essential.

Summary. 1) An improved method for the determination of inorganic sulfate in biologic fluid has been presented.

2) The results compare favorably with other methods requiring larger quantities of serum or plasma.

§ Powdered calcium hydroxide(4) was found unsuitable as an agent to precipitate phosphate because varying amounts of sulfate were found to be coprecipitated with calcium phosphate.

1. Kahn, M., and Postmontier, R. S., *J. Lab. and Clin. Med.*, 1925, v10, 317.
2. Loeb, R. F., and Benedict, E. M., *J. Clin. Invest.*, 1927, v4, 33.
3. Denis, W., and Reed, L., *J. Biol. Chem.*, 1926-27, v71, 191.
4. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry Methods*, 1932, Baltimore, Williams and Wilkins Co.
5. Schwartz, B. M., Thesis for degree of Doctor of Medicine, Yale University, 1939.
6. Dodgson, K. S., and Spencer, B., *Biochem. J.*, 1953, v55, 436.
7. Cope, C. L., *ibid.*, 1931, v25, 1183.
8. Wakefield, E. G., *J. Biol. Chem.*, 1929, v81, 713.
9. Letonoff, T. V., and Reinhold, J. G., *ibid.*, 1936, v114, 147.
10. Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *ibid.*, 1943, v149, 255.
11. Hoffman, W. S., and Cardon, R., *ibid.*, 1935, v109, 717.
12. Fiske, C. H., *ibid.*, 1921, v47, 59.
13. Cuthbertson, D. P., and Tompsett, S. L., *Biochem. J.*, 1931, v25, 1937.

Received February 17, 1956. P.S.E.B.M., 1956, v91.