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Modification of the Colorimetric Assay for Chloramphenicol in the Presence of Bilirubin

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We recently found that bilirubin interferes with the colorimetric chloramphenicol assay of Kakemi et al. (K. T. Kakemi, T. Arita, and S. Ohashi, Yakugaku Zasshi 82:342-345, 1962). Levels of serum bilirubin alone (4 to 6 mg/dl) resulted in apparent concentrations of chloramphenicol which appear to be in the therapeutic range. Concentrations of serum bilirubin greater than 8 mg/dl resulted in levels of apparent chloramphenicol associated with toxicity (>50 μg/ml). Small amounts of activated charcoal added to the isoamyl acetate extraction step of the assay eliminated this interference by bilirubin.

Numerous authors have emphasized the necessity of monitoring serum concentrations of chloramphenicol in newborn and premature infants to achieve effective therapeutic concentrations and avoid concentrations associated with toxicity (1-3). The original chemical assay measures inactive metabolites as well as free chloramphenicol (4). In the modification of the chemical method by Kakemi et al., only the free chloramphenicol extracted into isoamyl acetate is measured, and the assay is unaffected by the presence of other antibiotics (7, 11). We have found that even small concentrations of serum bilirubin interfere with the assay of Kakemi et al. This report describes this problem and a method we developed to overcome it.

MATERIALS AND METHODS

Chloramphenicol standards. Chloramphenicol standards were prepared in pooled normal human serum or distilled water in concentrations of 10, 20, 30, and 50 μg/ml with chloramphenicol standard powder (Warner-Lambert/Parke-Davis).

The assay was performed by adding 250 μl of standard or sample to 500 μl of 0.1 M phosphate buffer, pH 7.0. Isoamyl acetate, 750 μl, was added, mixed, and placed for 10 min in a reciprocal shaker at 24°C. A 500-μl amount of the upper organic layer, which was separated by centrifugation, was transferred directly to a separate tube. If the sample was to be treated with charcoal, the entire organic layer was removed to a tube containing 5 to 35 mg of 8- to 12-mesh activated coconut charcoal. The charcoal-isoamyl acetate mixture was blended by Vortex mixer at 15-s intervals for 2 min, and 500 μl was transferred to a separate tube. The color was developed by adding 500 μl of a 1:1 mixture of 1.5 N NaOH and 3 g of isonicotinic acid hydrazide per dl and incubating at 37°C for 45 min. The optical density of the aqueous layer was read at 430 nm on a spectrophotometer equipped with a microcuvette attachment. Standard curves were generated for each assay, and standards as well as samples were assayed in duplicate.

Mock sample preparation. A concentrated bilirubin solution was prepared and added in different volumes to serum. The final total bilirubin concentration was measured by the method of Jacobson and Wennberg (6). Different amounts of chloramphenicol were then added to serum or distilled water to make samples of known concentration.

Statistical methods. Statistical analysis was performed by using least mean squares linear regression or Student's *t* test. The percent error of the assay was calculated as described by Sabath et al. (14).

RESULTS

Effect of amount of charcoal on concentration of chloramphenicol. After extraction of chloramphenicol from 12 duplicate samples of normal human serum made to contain 35 μg of chloramphenicol per ml, different amounts of charcoal were added and the concentration of chloramphenicol was measured. There was no significant effect on the concentration of chloramphenicol when 5.7 to 37.5 mg of charcoal was added ($P > 0.10$).

Reproducibility. A single sample of distilled water which contained 15 μg of chloramphenicol per ml and a single sample of ventricular cerebrospinal fluid from a child who was receiving chloramphenicol were assayed with and without charcoal treatment on 22 and 18 different occasions, respectively. The mean value recovered from the untreated distilled water samples was 16 μg/ml (±1 μg/ml) and 15 μg/ml (±1 μg/ml) from the charcoal-treated samples. Mean values of chloramphenicol recovered from the un-

treated and charcoal-treated ventricular fluid were 10 μg/ml (±1 μg/ml) and 10 μg/ml (±2 μg/ml), respectively.

Accuracy. The accuracy of this method with and without charcoal treatment was determined on five mock samples which were made to contain 10, 15, 20, 25, and 45 μg of chloramphenicol per ml (Fig. 1). The average error was 6% for the untreated samples and 7% for the charcoal-treated samples. There was excellent correlation between the charcoal-treated and untreated mock samples by linear regression analyses (r = 0.9908).

Effect of bilirubin on apparent concentration of chloramphenicol. Sixteen umbilical cord sera, which did not contain chloramphenicol, were prepared to contain from 1.4 to 20.1 mg of bilirubin per dl and were assayed in duplicate by the Kakemi method without charcoal treatment. The dose response curve (Fig. 2) shows that as bilirubin concentration increases, apparent levels of chloramphenicol increase directly (r = 0.9852).

Removal of bilirubin with charcoal. Eight samples of serum without chloramphenicol to which various amounts of bilirubin had been added were assayed with and without charcoal treatment. Apparent levels of chloramphenicol ranged from 9.0 to 48 μg/ml in the untreated sera. Treatment of the samples with charcoal reduced the apparent concentrations of chloramphenicol to less than 10 μg/ml (the limit of sensitivity of our assay).

Chloramphenicol was added to normal human serum, with and without 10 mg of bilirubin per dl, to give concentrations of 15, 25, and 45 μg/ml. Measurement of chloramphenicol without charcoal treatment resulted in concentrations which were 2.5 to 4.5 times the actual concentrations. Assay with charcoal treatment removed the bilirubin and measured chloramphenicol with an error range of 0.4 to 11.8% (Table 1).

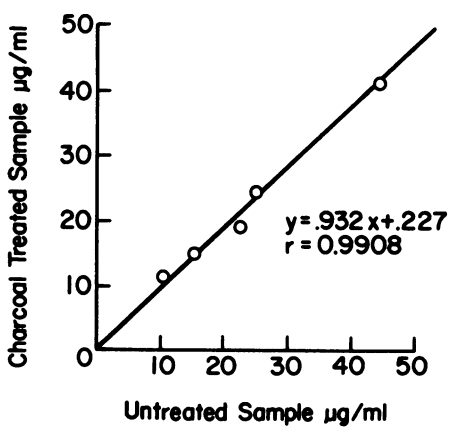

FIG. 1. *Linear regression analysis of concentrations of chloramphenicol in mock samples made to contain 10, 15, 20, 25, and 50 μg of chloramphenicol per ml which were assayed with and without charcoal treatment. r, Correlation coefficient.*

DISCUSSION

Bilirubin in serum interferes with the colorimetric chloramphenicol assay of Kakemi et al., and the apparent chloramphenicol concentration is directly proportional to the bilirubin concentration (Fig. 2). Bilirubin is extracted into isoamyl acetate and diffuses immediately into

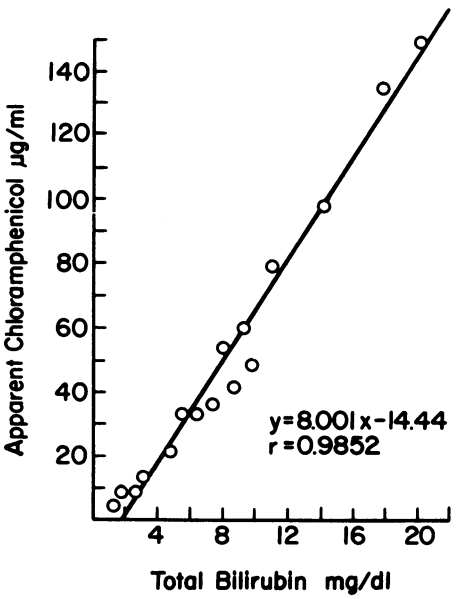

FIG. 2. *Linear regression analysis of the relationship between the concentrations of total bilirubin and apparent chloramphenicol, r, Correlation coefficient.*

TABLE 1. *Results of the chloramphenicol assay in the presence of bilirubin with and without charcoal treatment*

Stated concn (μg/ml)	Charcoal treatment	Bilirubin concn (mg/dl)	Chloramphenicol assayed (μg/ml)	% Error
15	No	10	67	77.6
15	Yes	10	17	11.8
15	No	0	15	0.0
25	No	10	74	66.2
25	Yes	10	24	4.2
25	No	0	25	0.0
45	No	10	113	60.2
45	Yes	10	42	7.1
45	No	0	45	0.0

the aqueous layer when isonicotinic acid hydrazide is added. Levels of serum bilirubin alone as low as 4 to 6 mg/dl result in apparent chloramphenicol concentrations that might be interpreted as being in the therapeutic range (17 to 33 μg/ml) (5). Concentrations of bilirubin greater than 8 mg/dl result in false chloramphenicol concentrations above those found to be associated with the gray baby syndrome (50 μg/ml) (15).

We found that a small amount of activated charcoal (5 to 35 mg) adsorbs the bilirubin, which has been extracted along with chloramphenicol into the isoamyl acetate in the Kakemi assay. The bilirubin which has adsorbed to the charcoal then can be removed, which leaves chloramphenicol alone to be developed by the isonicotinic acid hydrazide in the subsequent steps. The treatment of serum, which contained a known amount of chloramphenicol with charcoal, did not alter the concentration of chloramphenicol, which might be important clinically.

Dunkle and others recommend that the serum concentration of chloramphenicol should be monitored when a neonate is receiving chloramphenicol (1, 2). Elevated or subtherapeutic serum concentrations of chloramphenicol may require modification of the dose. Our observations emphasize the magnitude of apparent concentrations of chloramphenicol caused by elevated levels of bilirubin as determined by the Kakemi method. The concentrations of chloramphenicol in neonates with unconjugated hyperbilirubinemia are elevated falsely, and the dose of chloramphenicol thus may be reduced inappropriately.

Microbiological assays of chloramphenicol are slow and difficult, if not impossible, when two antibiotics are used in treatment simultaneously (10). There are several rapid noncolorimetric methods which measure concentrations of chloramphenicol and avoid the problem of bilirubin interference as described in this report. The radioenzymatic assay requires an enzyme which is not generally available (9, 13). Gas-liquid and high-pressure liquid chromatography, both rapid, accurate, and specific methods of measuring concentrations of chloramphenicol, require expensive laboratory equipment (8, 12). The advantage of the Kakemi method is that it can be performed in any hospital laboratory with existing equipment. However, the interference by bilirubin in the measurement of chloramphenicol by this method must be recognized. We recommend the addition of charcoal to the isoamyl acetate layer if any yellow color is present before the addition of isonicotinic acid hydrazide. By this simple modification we have found that the serum concentration of chloramphenicol can be measured reliably by the Kakemi method in the presence of bilirubin.

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LITERATURE CITED

1. **Black, S. B., P. Levine, and H. R. Shinefield.** 1978. The necessity for monitoring chloramphenicol levels when treating neonatal meningitis. J. Pediatr. **92**:235–236.
2. **Dunkle, L. M.** 1978. Central nervous system chloramphenicol concentrations in premature infants. Antimicrob. Agents Chemother. **13**:427–429.
3. **Eichenwald, H. F., and G. H. McCracken, Jr.** 1978. Antimicrobial therapy in infants and children. Part I. Review of antimicrobial agents. J. Pediatr. **93**:337–377.
4. **Glazko, A. J., L. M. Wolf, and W. A. Dill.** 1949. Biochemical studies on chloramphenicol (Chloromycetin). I. Colorimetric methods for the determination of chloramphenicol and related nitro compounds. Arch. Biochem. **25**:411–418.
5. **Hodgeman, G. E., and L. E. Bains.** 1961. Safe and effective chloramphenicol dosages for premature infants. Am. J. Dis. Child. **101**:140–148.
6. **Jacobson, J., and R. P. Wennberg.** 1974. Determination of unbound bilirubin in the serum of newborns. Clin. Chem. **20**:783–789.
7. **Kakemi, K. T., T. Arita, and S. Ohashi.** 1962. Absorption and excretion of drugs. IV. Determination of chloramphenicol in blood. Yakugaku Zasshi **82**:342–345.
8. **Koup, J. R., R. Brodsky, A. Lau, and T. R. Beam, Jr.** 1978. High-performance liquid chromatographic assay of chloramphenicol in serum. Antimicrob. Agents Chemother. **14**:439–443.
9. **Lietman, P. S., T. J. White, and W. V. Shaw.** 1976. Chloramphenicol: an enzymological microassay. Antimicrob. Agents Chemother. **10**:347–353.
10. **Louie, R. J., F. P. Tally, J. G. Bartlett, and S. L. Gorbach.** 1976. Rapid microbiological assay for chloramphenicol and tetracyclines. Antimicrob. Agents Chemother. **9**:874–878.
11. **O'Gorman Hughes, D. W., and L. K. Diamond.** 1964. Chloramphenicol in blood: simple chemical estimations in patients receiving multiple antibiotics. Science **144**: 296–297.
12. **Resnick, G. L., D. Corbin, and H. Sandberg.** 1966. Determination of serum chloramphenicol utilizing gas-liquid chromatography and electron capture spectrometry. Anal. Chem. **38**:582–585.
13. **Robison, L. R., R. Seligson, and S. A. Leiner.** 1978. Simplified radioenzymatic assay for chloramphenicol. Antimicrob. Agents Chemother. **13**:25–29.
14. **Sabath, L., J. I. Casey, P. A. Ruch, L. L. Stumpf, and M. Findland.** 1971. Rapid microassay of gentamicin, kanamycin, neomycin, streptomycin and vancomycin in serum or plasma. J. Lab. Clin. Med. **78**:457–463.
15. **Weiss, C. F., A. J. Glazko, and J. K. Weston.** 1960. Chloramphenicol in the newborn infant. A physiologic explanation of its toxicity when given in excess doses. N. Engl. J. Med. **262**:787–794.