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A SIMPLE METHOD FOR DETERMINATION OF SERUM METHOTREXATE *

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ABSTRACT

A simple bacteriological method for determination of methotrexate concentration in biological fluids is described. The principle of the technic is similar to the bacterial sensitivity test. The sterile filter paper discs with known concentration of methotrexate solutions and the test sera are placed on the test plates containing suspension of Lactobacillus casei in the suitable agar medium. The definite zones of inhibition of bacterial growth after 18 - 24 hours of the incubation at 37 °C are recorded. The MTX calibration curve is obtained when the inhibition zones are plotted against the MTX concentration on the log paper. This calibration should be done each time of the determination. The better results obtained from the tomato juice agar medium, the home - made filter paper disc and a test volume of 0.01 ml. This method has proved satisfactory with the specimens obtained from animal experiment. It is recommended for clinical use due to its feasibility (can be carried out in any routine clinical laboratory), accuracy and inexpensive.

INTRODUCTION

Methotrexate (Amethopterin, 4-amino N^{10}-methyl-pteroylglutamic acid sodium) is of great value in the treatment of acute leukemia, trophoblastic tumors of the uterus (1, 2) and also in primary and

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secondary tumor (3,4). In the more recent reports methotrexate (MTX) has been shown to have enhanced biological activity when it is given by continuous 24 hour intra-arterial infusion rather than a single daily oral dose. Thus, it is of greater value in the treatment of various types of cancer, particularly in solid tumors. (5) The efficiency of MTX is limited partly due to its toxicity to the bone marrow and G.I. tract with rather narrow range of safety. Blood level of MTX is sometime essential for adjusting the suitable dose schedule.

The methods currently in used for the determination of MTX in various biological fluids are the bacteriological method (6), fluorometric technic (7) and radioenzymatic method (8). We are reporting our modification of the bacteriological technic which is more simple, much cheaper, can be carried out in any routine laboratory and accurate enough for clinical purpose.

MATERIALS

I. METHOTREXATE STOCK SOLUTION

Stock solution tubes containing 5 ml each of a MTX at a concentration of 1,000 ng/ml are prepared and then kept frozen at -40°C.* By diluting this stock solution with distilled water (or preferably the pooled normal serum or plasma) standards containing 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 200.0, and 500.0 ng/ml. are prepared. These standard dilution are stored in the refrigerator at 4°C when not in use.

II. LACTOBACILLUS CASEI

Lyophilized Lactobacillus casei** was resuspended in sterile water then inoculated in the transfer medium and incubated for 18 hours at 37°C.

III. FILTER PAPER DISCS

The special filter paper discs, 6.5 mm. diameter are prapared by cutting the Whatman filter paper No. 2 with a standard office paper puncher (but the commercially available ink blotcher is preferable). They are sterilized by autoclaving 15 minutes at 15 pounds pressure (121°C).

IV. THE ASSAY MEDIA

Two types of medium were experimented.

A. FOLIC ACID ASSAY PGA BROTH

Seventy five grams of Folic Acid Assay PGA Broth,*** 30 grams agar, and 20 ug of crystalline pteroylglutamic acid are added to 2,000 ml. of distilled water. This is heated to dissolve the agar then sterilized and aliquoted as described below.

B. TOMATO JUICE AGAR MEDIUM

Mix 51.0 gms. of tomato Juice Agar

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* From Lederle Lab., Division of the American Cyanamid Co.,

** From Difco Laboratories, Detroit, Mich., U.S.A.

*** From BBL, Division of Bioquest, Cockeysville, Md., U.S.A.

Medium * to 1,000 ml. of cold distilled water and heat to boiling to dissolve the medium completely.

The media are sterilized by autoclaving 15 minutes at 15 pounds pressure (121°C), cooled to 45°C in a water bath, and inoculated with 1 ml. of a 18 hour-old broth culture of L. casei per 1,000 ml. of media. After shaking thoroughly to mix the organisms throughout the media, 10 ml. aliquots are pipetted into the specially pressed, flat bottom Petri dishes (Pyrex dishes or disposable plastic dishes) 100 mm. diameter. Allow the media to solidify then kept in the refrigerator at 4°C. These plates are used within 1 to 96 hours.

METHODS

A standard calibration plate is prepared by placing 4 filter paper discs on the agar plate and delivering 0.01 ml. of one of the various standard solutions of MTX to each disc in duplicate. Since the disc absorbs moisture rapidly from the agar, it is essential that the solution be delivered to the disc immediately after it touchs the agar. The plates are then incubated for 18-24 hour. The diameters of the zones of inhibition are measured and plotted against the concentration of MTX on the log paper to give a standard calibration curve.

An anesthetized dog was given 0.5ml. pentobarbital intravenously every hour. Five milligrams of freshly prepared MTX was given intravenous push within one minute period. Blood samples were clotted at intervals. One ml. of blood sample is allowed to clot, rimmed with a wooden applicator stick, centrifuged, and the serum removed. Full strength serum or 1/3 and 1/10 dilutions of these serum sampled in pooled normal sera are used for the study.

RESULTS

I. The comparison between 2 types of agar media.

The Tomato Juice Agar medium is better than the Folic Acid Assay PGA Broth because :

a. It needs shorter incubation time, 18 hours rather than 24 hours or more as required when the latter is used.

b. Although the calibration curve obtained from the former medium is not as steep as obtained from the latter (Fig. I) but the zones of inhibition readings are correlated to the concentrations better.

c. Tomato Juice Agar medium is much cheaper.

II. Application of the method to the animal study.

After intravenous administration of

* From Difco Laboratories, Detroit, Mich., U.S.A.

MTX in dog, the highest blood level is obtained at 15 minutes. The disappearance of MTX occured in 2 phases (Fig. II).

DISCUSSION

Methotrexate is the methylated analogue of aminopterin and exerts its primary effect by inhibiting the action of the enzyme, dihydrofolic reductase (DHFR). Therefore, it prevents synthesis of DNA and thus interferes with cell mitosis. Methotrexate has at least 20,000 times more affinity for DHFR than folic acid. (9)

The bacteriological method of determination of MTX in the biological fluids was first described by Burchenal et al (6) in 1951 and has proved its usefulness in clinical use. Our modification can be carried out in any routine clinical laboratory with limited equipments and budget. When the Tomato Juice Agar is used the calculated cost of the material required is approximately 0.25 Bht per specimen (in duplicate)

The optimum volume of specimen required for each disc is 0.01 ml. since the larger volume tends to overflow the filter paper disc and may cause distortion of the inhibition zone. The accuracy of 0.01 ml. and 0.02 ml. specimen are otherwise comparable (Fig III). It is also noted that when the standard calibrations of MTX are made in pooled normal plasma or serum they will give the same size of inhibition zones but with more distinct diameters.

The Tomato Juice Agar medium is preferable due to its accuracy, better calibration curve, shorter incubation time and much cheaper cost. The results of the attempt to study the blood level of MTX in animal were comparable to those reported earlier. (6, 10)

SUMMARY

A new modification of the bacteriological method of determination of Methotrexate, the anticancer drug, was described. The better medium for L. casei is Tomato Juice Agar Medium. The test can be carried out in any routine clinical laboratory with accuracy.

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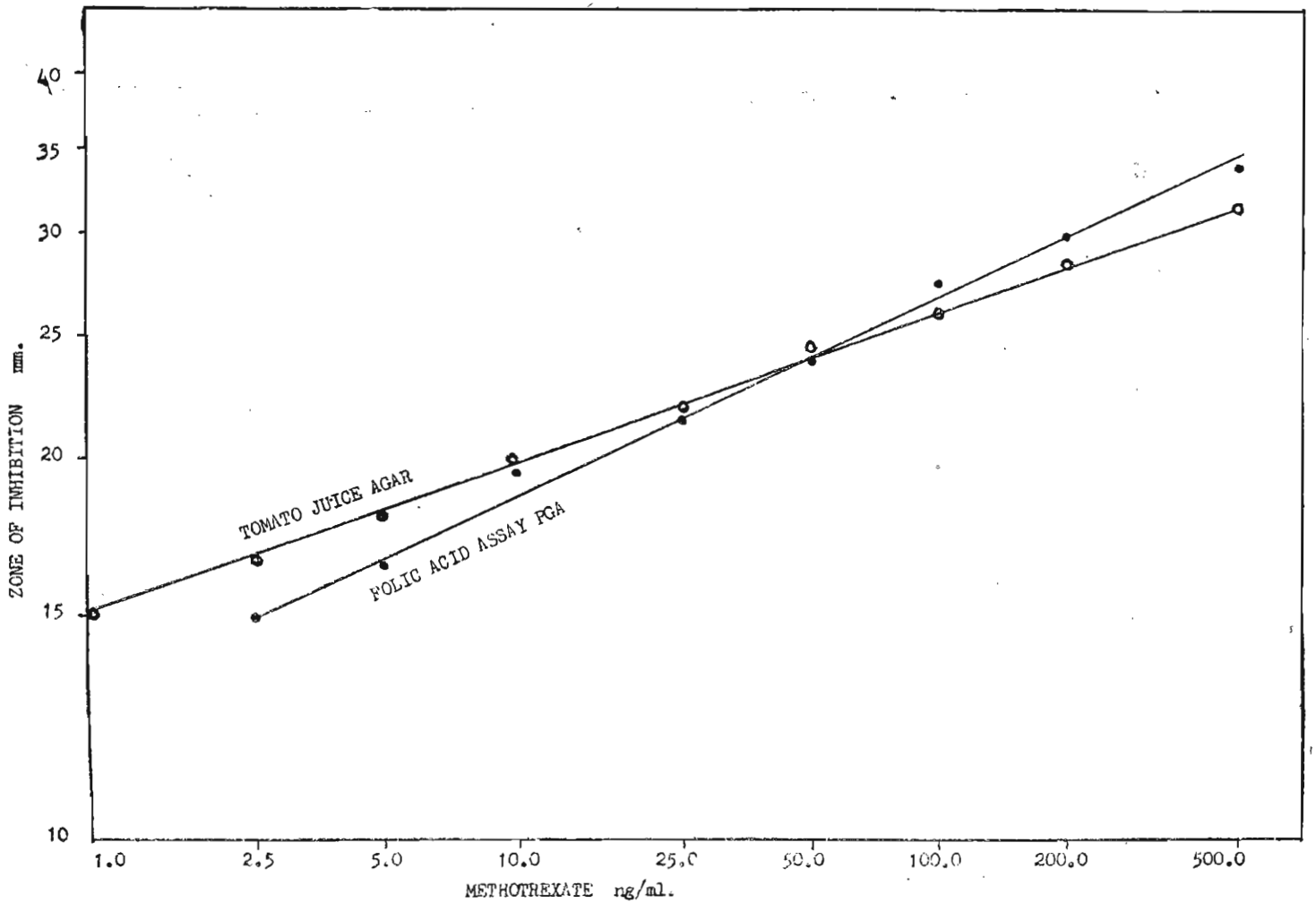
FIURE I.: CALIBRATION CURVES FROM DIFFERENT TYPES OF MEDIA.



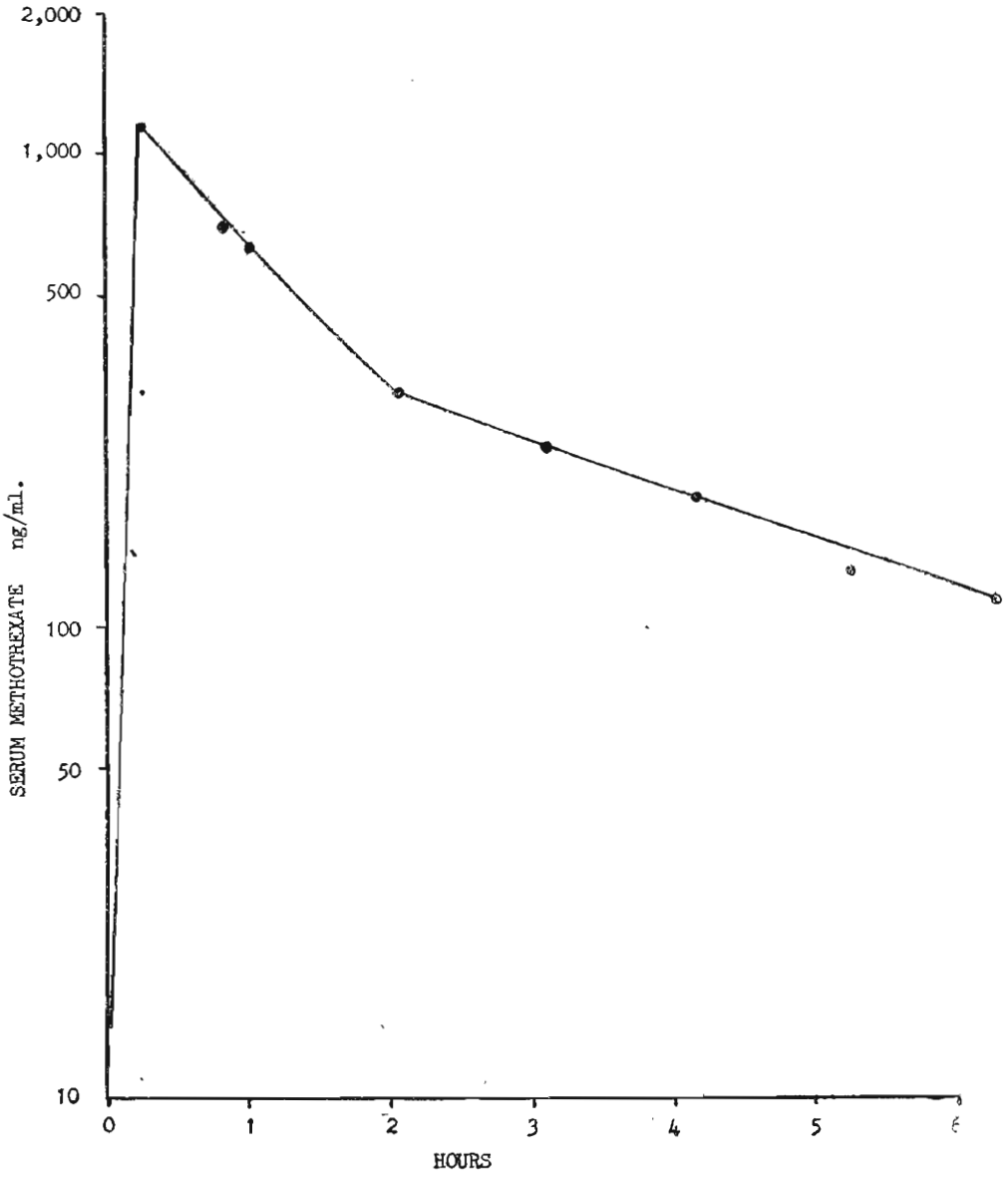
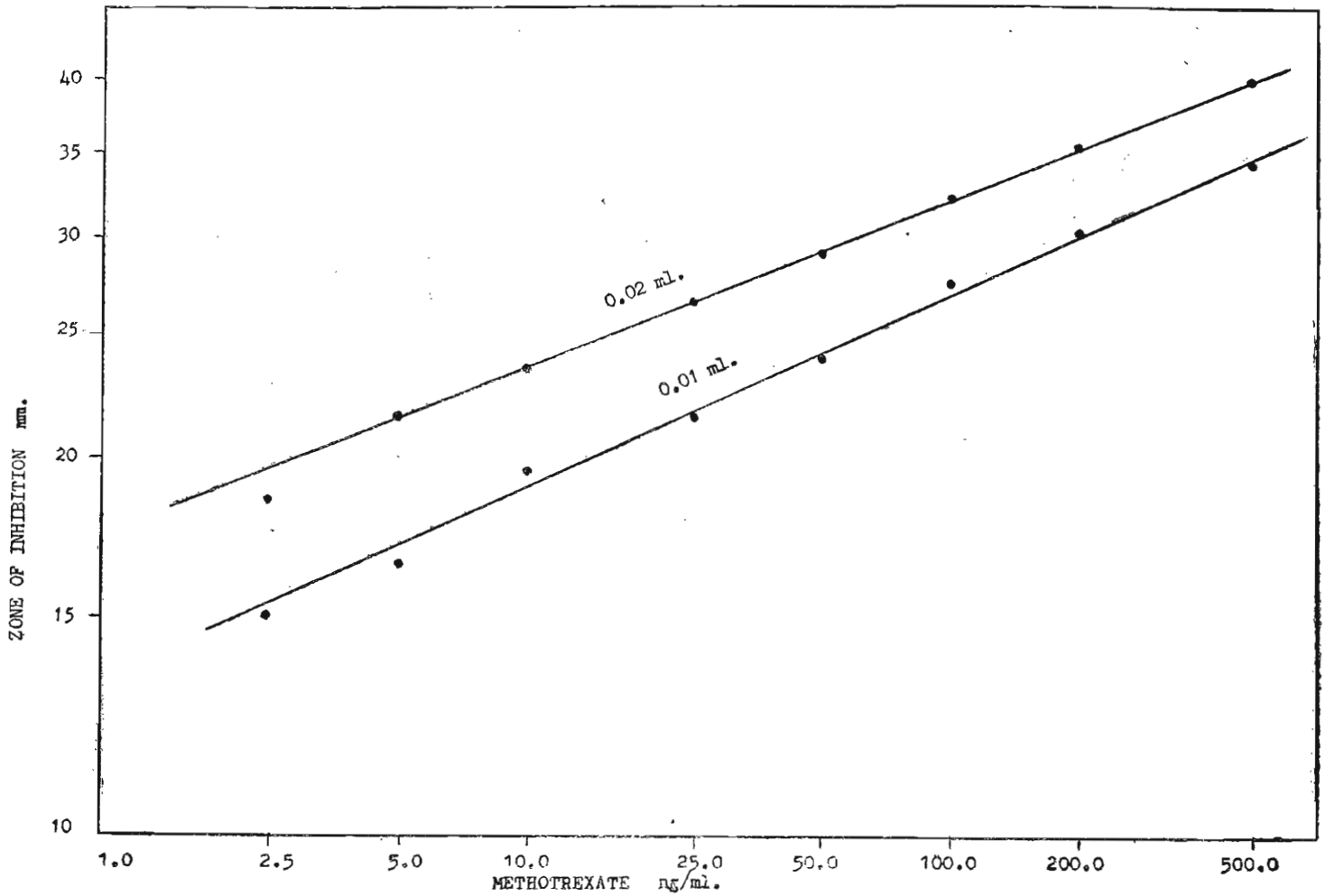


FIGURE II.: SERUM METHOTREXATE LEVELS AFTER INTRAVENOUS INJECTION

FIGURE II.: EFFECT OF DIFFERENT SIZE OF SAMPLES.



REFERENCES

1. Li, M.C., Hertz, R., and Spencer, D.B.: Effect of Methotrexate Therapy Upon Choricarcinoma and Chorioadenoma. Proc. Soc. Exp. Biol. Med. 93:361, 1956.
2. Hertz, R., Lewis, J., and Lipsett. M.B.: Five years Experience with Chemotherapy of Metastatic Choriocarcinoma and Related Trophoblastic Tumors in Women. Amer. J. Obst. Gynec. 82, 631, 1961.
3. Evans, A.E., D. Angio, G.J., and Mitus, A.: Central Nervous System complications of Children with Acute Leukemia. An Evalution of Treatment Methods. J. Pediat. 64 . 94, 1964.
4. Newton, W.A., Jr., and Sayers, M.D.: Intrathecal Methotrexate Therapy of Brain Tumors of Childhood. Proc. Am. Ass. Cancer Res. 6 : 48, 1965.
5. Sullivan, R.D. et al: Clinical Effects of Continuous Intravenous and Intra-Arterial Infusion of Cancer Chemotherapeutic Compounds. Cancer Chemother. Rep. 16 : 499, 1962.
6. Burchenal, J.H., Waring, G.B., Ellison, R. R., and Reilly, H. C.: A Simple Method for Determination of Lavels of Amethopterin in Blood and Urine. Proc. Soc, Exper. Biol. & Med. 78 : 603, 1951.
7. Freeman, M.V.: A Fluorometric Method for the Measurement of 4-amino-10-methyl pteroylglutamic acid (Amethopterin) in plasma. J. Pharm. Exp. Ther. 120 : 1, 1957.
8. Rothenburg, S. P.,: A Radioenzymatic Assay for Folic Acid Antagonists in Biological Fluids. J. Lab. Clin. Med. 66 : 294, 1965.
9. Werkeiser, W.C.: Biochemical, Cellular and Pharmacological Action and Affects of Folic Acid Antagonists. Cancer Res. 23 : 1277, 1963.
10. Liguori, V.R., et al: Effects of Different Dose Schedule of Amethopterin on serum and Tissue Concentrations and Urinary Excretion Patterns. Clin. Pharmacol. Ther. 3 : 34, 1962.