A Simple Method for Determination of Levels of Amethopterin in Blood and Urine.* (19154)

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The marked variation in the amounts of amethopterin or other folic acid antagonists which different patients can tolerate suggests that there may be considerable differences in their ability to absorb and metabolize these compounds. Swendseid *et al.*(1) have used turbidometric methods to study the excretion of aminopterin in leukemic patients. In order to facilitate our studies of the absorption and excretion of amethopterin, a simpler assay method, similar to those used to determine levels of penicillin and streptomycin, was devised. Such a technic is herein described.

Method. Seventy-five g of Difco Folic Acid Assay medium(6), 30 g agar, and 20 μg of crystalline pteroylglutamic acid§ are added to 2,000 ml of distilled water. This is heated to dissolve the agar, autoclaved 15 minutes at 15 lb pressure, cooled to 45°C in a water bath, and inoculated with 2 ml of a 24-hour-old broth culture of *Streptococcus faecalis* (ATCC No. 8043). After shaking thoroughly to mix the organisms throughout the media, 20 ml aliquots are pipetted into 100 specially pressed, flat bottom Petri dishes (pyrex dishes 100 mm diameter, Corning No. 3162) arranged on a level surface, and allowed to solidify. These plates are then stored in the refrigerator at 4°C and used when desired 1 to 96 hours later. Using a specially purified sample of 4-amino-N^{10}-methyl pteroylglutamic acid (Amethopterin)(7)¶, 30 standard tubes are prepared containing 5 cc each of a solution of the compound at a concentration of 1,000 mγ/ml. These stock tubes are then frozen at —40°C, and a tube removed every 3 days when new series of standards are required. By diluting this stock solution with distilled water, standards containing 10, 20, 30, 40, 60, 80, 120, and 200 mγ/ml are prepared. These standard solutions are stored in the cold room at 4°C when not in actual use. A standard plate is prepared by placing 4 special filter paper discs, 12.7 mm diameter (Schleicher and Schuell penicillin assay discs), on the agar, and delivering 0.09 ml of one of the various standards to each disc with an automatic penicillin pipette. Since the disc absorbs moisture rapidly from the agar, it is essential that the solution be delivered to the disc immediately after it touches the agar. Several plates are made to cover the various concentration ranges of the standard. The plates are then incubated for 18-24 hours, and the diameter of the zone of inhibition read with a Bermuda Colony Counter (American Optical Co.), containing an etched glass scale graduated in millimeters for measuring zone size. The diameter of the zone is plotted against the logarithm of the concentration of the drug to give a standard curve. Since the concentrations of amethopterin in the urine

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1. Swendseid, M. E., Swanson, A. L., Miller, S., Bethell, F. H., *Abst. Fed. Proc.*, 1950, v9, 372.

6. Capps, G. F., Hobbs, N. L., Fox, S. H., *J. Bact.*, 1948, v55, 869.

§ We wish to acknowledge the kindness of the Lederle Laboratories Division of the American Cyanamid Co., in supplying us with this compound.

7. Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., Hultquist, M. E., *J. Am. Chem. Soc.*, 1949, v71, 1753.

¶ This sample was supplied to us through the kindness of Dr. H. P. Broquist of Lederle Laboratories Division of the American Cyanamid Co.

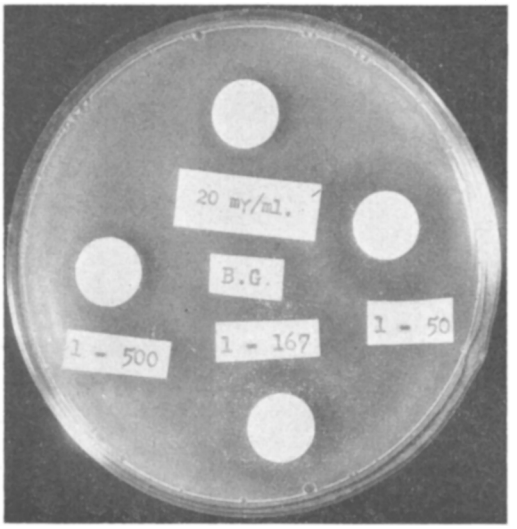

FIG. 1. Urine amethopterin determination with amethopterin standard, 20 mγ/ml, and urine dilutions.

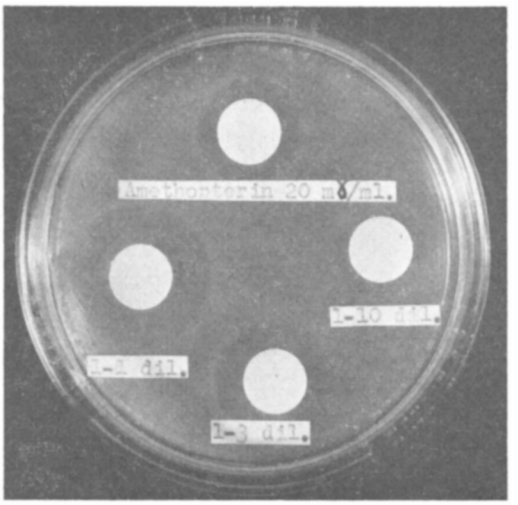

FIG. 2. Serum amethopterin determination.

specimens obtained during a 24-hour period after the oral administration of 5 mg of amethopterin usually range from 1 to 8 γ/ml, considerable dilution is necessary to reach the range (20-40 mγ/ml), at which determinations are most accurate. The usual procedure is to use 4 discs per plate to which are added 0.09 ml portions from a standard amethopterin solution containing 20 mγ/ml, and from 1-500, 1-167, and 1-50 dilutions of urine, respectively (Fig. 1). One to 3 ml samples of blood are collected without anticoagulant at various times after the administration of amethopterin to determine the levels in the serum. The bloods are allowed to clot, rimmed with a wooden applicator stick, centrifuged, and the serum removed. Full strength serum and 1-3 and 1-10 dilutions of this in distilled water are used for most studies. By such dilutions, serum levels of from 10 to 400 mγ/ml can be determined with relative accuracy. As with the urine determination, 4 discs are used per plate, onto which are pipetted 0.09 ml quantities of an amethopterin standard at 20 mγ/ml and of 1-1, 1-3, and 1-10 dilutions of serum, respectively (Fig. 2).

Results. In an attempt to study the recovery of amethopterin, 5 mg were added to 1,000 ml of normal pooled human urine to give a concentration of 5 γ/ml. This was then diluted 1-50, 1-167, and 1-500 and 10 separate plates were set up each containing a standard and all 3 dilutions of urine. A standard plate with concentrations of amethopterin in distilled H_2O was set up at the same time. Recovery values of from 4.8 to 5.6 γ/ml were obtained. On the basis of this experiment, the method would appear to have an accuracy of ±12%. A similar experiment was done in which 1 γ of amethopterin was added to 15 ml of normal serum, giving a concentration of 66.6 mγ/ml, and 1 γ of amethopterin was added to another 10 ml to give a concentration of 100 mγ/ml in the full strength sera. The results on 10 plates of the 1-3 and 1-10 dilutions of the serum containing 100 mγ/ml varied from 81 to 130 mγ/ml and the average of each plate varied from 94 to 111 mγ/ml, giving an accuracy of ±11%. Only the 1-3 dilutions of the serum containing 66.6 mγ/ml were within the satisfactory range for these determinations. With these dilutions on 10 plates, recovery values of 60 to 69.670 mγ/ml were obtained indicating an accuracy of ±10%.

Metabolic studies. Fig. 3 shows the levels of amethopterin in the serum, and the excretion of the drug in the urine after the oral administration of 5 mg of amethopterin. Two normal adult male volunteers in fasting condition (no food for 12-14 hours), and one normal adult female and 3 males, 3, 3, 2 and

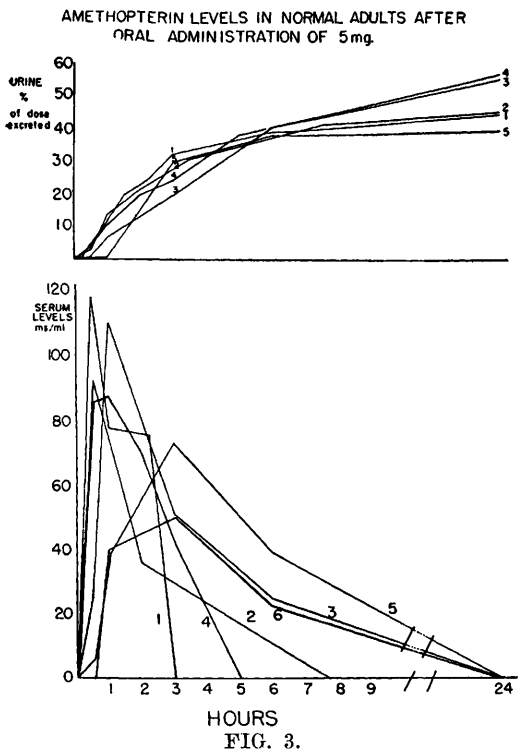

FIG. 3.

2 hours post cibum, were used in these studies. In the fasting adult, serum levels of approximately 100 mγ/ml were attained in the first 30 to 60 minutes after ingestion of the drug, and there was a rapid fall to below detectable levels in from 3 to 7½ hours. In 2 of the individuals (No. 5 and No. 6), who had eaten heavy breakfasts of bread, cheese, and milk 2 hours before the test, there appeared to be delayed absorption and the serum levels rose more slowly, and to a lesser degree. These in turn fell more slowly than in the fasting individuals (No. 1 and No. 2), or in those who had eaten less heartily, or in whom a longer period had elapsed since the last meal (No. 3 and No. 4). In individuals No. 5 and No. 6 a relatively high level was found 6 hours after the administration of the drug, but in none of the 6 volunteers was any detectable amount of the drug present 24 hours after ingestion. Similarly, the urinary excretion of the drug was delayed in No. 5 and No. 6, but the total 24-hour excretion in all individuals was approximately the same, varying from 40 to 57% of the ingested dose.

Serum levels and urinary excretion after 5 mg of amethopterin administered orally on fasting stomach were compared with the values on the same patient obtained after intramuscular administration of the same amount of amethopterin one week later. No significant difference was apparent between the two.

Fig. 4 shows the serum levels after ingestion of 5 mg amethopterin in a fasting male patient with impaired renal function (B.U.N. 58) contrasted with 2 fasting normal males of about the same age. In this patient a serum level of 100 mγ/ml and above persisted for the first 6 hours, and detectable amounts were present in the serum even 48 hours after the ingestion of the drug.

Discussion. A thorough discussion of the advantages and disadvantages of the disc method for antibiotic assay has been given by Loo *et al.*(5). The advantages of this particular technic over the turbidometric tube method for the assay of amethopterin are: (1) that absolute sterility of blood and urine samples is not required, (2) that colored or cloudy solutions or suspensions do not interfere with the accuracy of the test, and (3) that very small blood and urine samples can

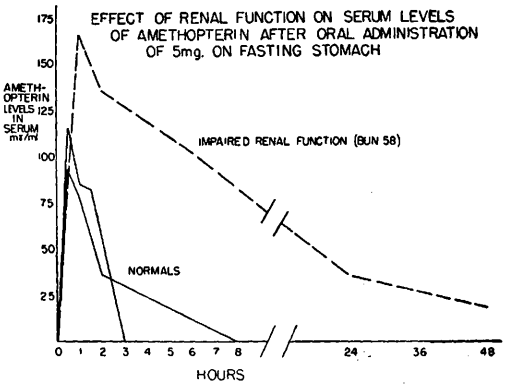

FIG. 4.

2. Vincent, J. G., Vincent, H. W., PROC. SOC. EXP. BIOL. AND MED., 1944, v55, 162.

3. Sherwood, M. B., Falco, E. A., de Beer, E. J., *Science*, 1944, v99, 247.

4. de Beer, E. J., Sherwood, M. B., *J. Bact.*, 1945, v50, 459.

5. Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., Sylvester, J. C., *J. Bact.*, 1945, v50, 701.

be used if desired, 0.5 ml serum being adequate for duplicate determinations. It is probably true that the accuracy achieved by this technic is somewhat less than that attained by turbidometric assay methods when clear solutions are being tested, but this plate technic compares favorably with similar penicillin assay methods using either discs or cups. Replicate determinations will considerably increase the accuracy of this technic.

In the serum of a patient who has been given amethopterin, all that can be said with certainty at present is that this technic detects a substance with growth inhibitory properties for *Streptococcus faecalis* similar to those of amethopterin. Probably this is the unchanged amethopterin, but it is possible that it may be a metabolite of that drug, rather than amethopterin itself. Chromatographic studies to determine the actual identity of this inhibitory substance are in progress at the present time.

The maintenance of a high level of amethopterin in the serum for the first 6 hours, and the presence of the drug in the serum 24 and 48 hours after ingestion in a patient with impaired renal function, gives a physiologic basis to the clinical finding that relatively small repeated doses of this compound are likely to cause severe toxic manifestations in such patients(8).

Preliminary results here reported suggest that the condition of the stomach (fasting, post cibum) may have considerable effect on the rate of the absorption of ingested amethopterin, but has little effect on eventual absorption as revealed by total 24-hour excretion of the drug.

Although in the one patient studied there was very little difference between the oral (fasting), and the intramuscular routes, it seems probable that for the most accurate metabolic studies an intravenous injection of the drug on a weight basis would be preferable. Metabolic studies by such a technic on patients with acute leukemia are in progress at the present time in the hopes of elucidating the mechanisms of the natural and acquired resistance of this disease to amethopterin.

Summary. (1) A simple disc technic has been devised for determining concentrations of amethopterin in blood and urine. (2) This test has an accuracy which compares favorably with similar assay methods for antibiotics. (3) Amethopterin appeared in the blood and urine in detectable amounts 15 to 30 minutes after the ingestion of a 5 mg dose on a fasting stomach by 2 normal individuals, and 30 to 60 minutes after ingestion at the same dose by 4 normal non-fasting individuals. In some of the 6 it was still detectable at 6 hours, but in none after 24 hours. (4) Six normal adults excreted 40 to 57% of the ingested dose in the urine within 24 hours. (5) In one patient little difference in serum levels or total excretion could be demonstrated whether the drug was given by the oral or intramuscular route. (6) In a patient with impaired renal function, higher initial serum levels were obtained and detectable amounts were present in the blood serum 48 hours after ingestion of amethopterin.

Addendum. Since this paper was submitted, further studies have shown that by decreasing the final concentration of crystalline pteroylglutamic acid in the medium from 10 mγ/ml to 1 mγ/ml a considerable increase in the sensitivity of the technic may be attained.

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