19. Toennies, G., Gallant, D. L., *J. Biol. Chem.*, 1948, v174, 451.

20. MacLeod, C. M., *J. Exp. Med.*, 1940, v72, 217.

21. Roepke, R. R., Libby, R. L., Small, M. H., *J. Bact.*, 1944, v48, 401.

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Comparison of New Chemical Method of Determining Isonicotinoyl Hydrazide in Serum with Microbiological Assay. (24048)

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No satisfactory chemical method of determining isoniazid (INH) in blood of patients is as yet available, due to the insensitivity of present colorimetric methods. Extraction with organic solvents as proposed by Kelly and Poet(1) is difficult because of an unfavorable distribution coefficient between water and solvent. 1-chloro-2, 4-dinitrobenzene has been used by Ballard and Scott(2) for pharmaceutical assays of INH, and 1-fluoro-2, 4-dinitrobenzene by Yamaguchi(3) for determination of INH in rabbit urine for quantities far beyond the doses used clinically. No method for determination of INH in blood was mentioned. The present paper describes a spectrophotometric method for determination of INH in blood using 1-fluoro-2, 4-dinitrobenzene. Preliminary tests in aqueous medium showed that the color intensity was influenced by pH; therefore, serum deproteinization methods using either alkali or acid were not applicable.

Methods. Aliquots of 2 cc INH free serum or plasma were mixed with 1 cc absolute alcohol containing graded quantities of INH, 7 cc alcohol was added, the mixture shaken for 5 minutes in a mechanical shaker, centrifuged and 5 cc of clear supernatant (representing 1 cc serum) was used for colorimetry. After addition of 0.5 cc 0.4% alcoholic solution of reagent and approximately 0.1 g borax, the mixture was shaken, heated in screw cap-sealed tube 5 minutes at 80° and decanted from the excess borax for colorimetry. Concentrations of 0.0, 2.5, 5 and 10 μg INH in these samples (= μg/cc serum) were read at wave lengths from 450 to 520 mμ using the 0.0 μg/cc concentration as the blank. Fig. 1 shows the optical densities at various wave lengths on common coordinate paper. Higher degrees of absorption were observed at lower wave lengths, but readings, conforming to the Beer-Lambert Law were found at 500 mμ, which was used for subsequent work.

Results. Line 1, Fig. 2 shows the relation between concentration/cc serum and optical transmission by this method for quantities up to 10 μg per ml of INH. The points were averages of 10 determinations which showed very small deviations. At low INH content of the serum, the readings became less accurate. Experiments were carried out to use a double quantity of serum (4 cc) to be deproteinized with alcohol. In this case the ratio had to be 4 : 6, giving 60% alcohol dilution. Reading in this case had to be done at a temperature >60°. Line 2, Fig. 2 shows the readings for double quantities of serum and INH but is plotted for μg/cc serum. The curves obtained in 60% and 80% alcohol give

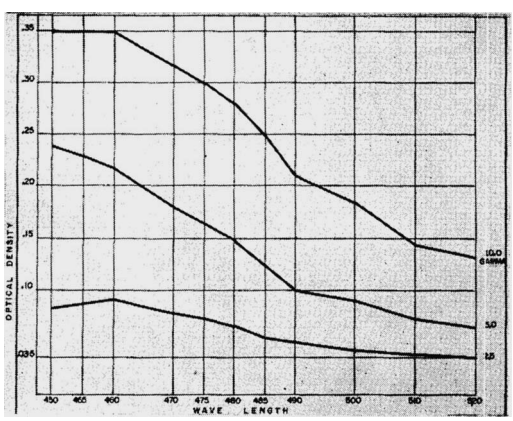

FIG. 1. Absorption spectra for 3 concentrations (μg/ml).

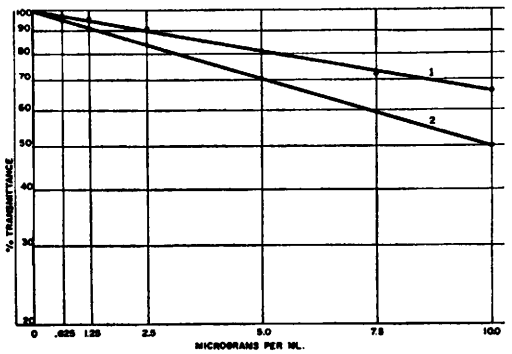

FIG. 2. Transmittance vs concentration ratios on semi-logarithmic paper. Serum to alcohol ratio: Line (1) 2:8; (2) 4:6.

straight lines, but have different coefficients. Recovery tests were carried out by precipitating the serum with 4 volumes of alcohol and adding to the deproteinized liquid measured amounts of INH in 80% alcohol, making allowance for the increased volume. The values of INH found when it was added after deproteinization were insignificantly higher than those obtained when the addition was made before deproteinization (Table I). INH free serum or plasma that is used in preparation of blank and standards gives a slightly yellow coloring with the fluoro reagent, so it is recommended that a pre-medication serum be used as the blank in assaying sera of elevated icterus indices. In present clinical practice, the microbiological assay of INH in serum is the accepted method for evaluation of efficacy of treatment(4). While this method measures the actual bacteriostatic efficacy of the serum, it admits a large margin of error, because of the fact that only concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 are tested (Bell and Riemensnider 5). This should be considered in the appreciation of Table II. It was not possible to obtain enough serum in every case to run both tests, and in many cases samples for chemical and biological assays were taken on different days. If both assays were performed on the same sample, the case is marked with an asterisk. The microbiological assays were carried out at Colorado Fn. for Research in Tuberculosis, Denver. Table II shows that there is sufficient agreement between the micro-biological and the chemical methods so that the latter might be used in clinical work.

TABLE I. Recovery of INH following Deproteinization.

INH added before deproteinization (μg)	Optical density	INH added after deproteinization (μg)	Optical density
5	.067	5	.071
10	.136	10	.136
10	.136	10	.138
10	.137	10	.142
20	.274	20	.283

TABLE II. Comparison of Chemical and Microbiological INH Assay.

Bioassay, μg/ml serum		Chemical assay, μg/ml	
After 2 hr	After 6 hr	After 2 hr	After 6 hr
3.2	.8	3.5	1.3
3.2	.8	3.0	1.3
.4	<.2	1.0	.17
.8	<.2	.4	.0
.8	<.2	3.6	.6
3.2	.8	3.7	1.7
1.6	.2	1.8	.18
1.6	.8	3.8	2.5
3.2	1.6	3.3	1.5
1.6	.8	3.0	.6
1.6	.8	3.7	1.6
*3.2	.8	2.0	1.3
<.2	<.2	.6	.0
1.6	1.6	1.5	1.2
1.6	.2	2.6	.9
*1.6		1.3	
3.2	1.6	2.8	.7
*3.2	.4	3.7	.4
*3.2	.8	3.0	.6
*3.2	1.6	2.9	1.7
1.6	.2	1.1	.0
1.4	.8	1.5	.9
*3.2	.8	4.0	1.6
3.2	.8	2.1	.9
*3.2	1.6	2.1	1.9
*1.6	.2	2.0	.0
* .8	<.2	1.7	.2
	.2	2.0	.7
*3.2		3.0	2.0
*3.2	1.6	3.4	1.9

Discussion. The question may be raised whether both methods determine the same chemical compounds. The microbiological method obviously determines INH and such possible metabolites as are still active as tuberculostatics. In the conditions as used in our tests, the fluoro reagent does not give reactions with isonicotinic acid and acetylisonicotinoyl hydrazide, the most prominent metabolites of INH, which both are therapeutically inert. A reactivity with the pyri-

dine N, therefore, is not probable. Since the reagent gives a color reaction with phenylhydrazine, we are inclined to believe that the free NH_2 group of the hydrazine is essential. If there be tuberculostatic metabolites of INH present they either do not react with the reagent, or their amount is too insignificant to influence the readings noticeably. Of importance is, however, that the fluoro compound does not react with p-aminosalicylic acid, which agent is so frequently given together with INH.

The method can easily be adapted to determination of INH in urine. Since the INH concentration in urines is rather high, the urine must be diluted which usually goes to a point, when the yellow color does not interfere. At lower concentrations, the patient's urine may be matched in color with a normal urine at 500 mμ by proper dilution, then the estimation may be carried out in alcoholic or aqueous medium. In the latter case 0.5 cc 0.1N NaOH should be used as alkali. The advantages of the chemical method consist mainly in saving of time. About 8 determinations can be carried out in one hour, so that the results are available on the day when the serum has been taken, whereas the microbiological test requires several weeks.

Summary. A chemical determination of INH in patient's blood has been devised which can be carried out within one hour. The serum is deproteinized with 4 volumes of alcohol, centrifuged, and quantities corresponding to one cc serum are heated with 0.5 cc 0.4% 1-fluoro-2.4-dinitrobenzene solution in alcohol and with 0.1 g borax for 5 minutes at 80°. The developed color is read at a wave length of 500 mμ. The method gives good agreement with results obtained by the microbiological methods within the errors allowed for clinical purposes.

1. Kelly, J. M., Poet, R. B., *Am. Rev. Tuberc.*, 1952, v65, 484.

2. Ballard, C. W., Scott, P. G. W., *Chem. and Indust.*, 1952, v29, 715.

3. Katsu Sestti Yamagushi. *Fol. Pharmacol. Japan*, 1955, v51, 631; Breviaria 37.

4. Monograph: How much INH reaches the tubercle bacilli in your patient? Nat. Jewish Hosp., Denver.

5. Bell, J. Carroll, Riemensnider, D. K., *Am. Rev. Tuberc.*, 1957, v75, 922.

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Plasminogen Activator in Animal Meninges.* (24049)

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Presence of an activator of plasminogen in animal tissues was demonstrated by Astrup and Permin(1), and a method for its quantitative estimation in various organs has been published(2). With this method animal and human organs could be divided into groups of high, medium and low concentrations of plasminogen activator, although large individual variations were encountered(3,4). Lewis and Ferguson(5) found moderate fibrinolytic activity in dog meninges caused by an activator of plasminogen. Their method was not quantitative. Using the quantitative method, Roberts and Astrup(6) found large concentrations in meninges of monkeys. We have recently observed surprisingly high fibrinolytic activities of membranes of the brain(7). This observation has prompted a more thorough examination of the meninges.

Methods. Brains from freshly slaughtered, healthy animals were immediately examined or stored at −20° for later use. Dog brains were obtained from dogs used for experiments on vascular surgery (courtesy of Dr. H. H. Wandall). The Rhesus monkey brains were kindly supplied by Dr. Preben von Magnus

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