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DETERMINATION OF HETRAZAN IN BIOLOGICAL FLUIDS

BY

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Hetrazan (1-diethylcarbamyl-4-methylpiperazine hydrochloride) was introduced by Hewitt and co-workers (1947a, b, c ; 1948) for the treatment of filariasis and applied to filariasis in man caused by *Wuchereria bancrofti* by Santiago-Stevenson and co-workers (1947). It is effective in small doses, and blood concentrations seldom exceed 0.5 mg. per 100 ml. The chemical structure of hetrazan makes difficult the finding of a specific, sensitive colour test, but it was found possible to estimate it by an application of the general method for the determination of organic bases described by Brodie and Udenfriend (1945). In this method, a coloured salt of the base is formed with a dye and is extracted into a solvent in which the dye itself is insoluble. The specificity of the method can be increased by a proper choice of solvent, dye, and conditions of extraction.

METHOD

Reagents

(1) 30 per cent (w/v) NaOH (A.R. grade).

(2) Ethylene dichloride. The technical grade usually supplied must be purified for use. Shake 1 litre of ethylene dichloride with two 200 ml. portions of N-HCl. Wash three times with 500 ml. portions of distilled water.

(3) 0.05 per cent (w/v) bromthymol blue, *p*H 7.0. Weigh 0.500 g. bromthymol blue powder into a litre standard flask. Add 6.810 g. KH_2PO_4, dissolved in distilled water, and swirl the flask to dissolve the dye. Then add 30.0 ml. N-NaOH, mix well, and make up to 1 litre with distilled water. For use, wash 100 ml. of the dye solution three times or more with 20 ml. portions of ethylene dichloride. There should be no extractable colour seen with the naked eye when 1 ml. bromthymol blue is shaken with 5 ml. of ethylene dichloride.

(4) Stock standard hetrazan. This contains 20 mg. of hetrazan base per 100 ml. Dissolve 0.2366 g. of the hydrochloride in distilled water to make 1 litre. The solution keeps well.

(5) Urine standard. This contains 2 mg. hetrazan base per 100 ml.

(6) Blood standard. This contains 1 mg. hetrazan base per 100 ml.

Both these dilute standards should be made fresh as required.

(7) Carbon disulphide. Use the fresh liquid. Discard it when deeply yellow.

(8) N-NaOH. This need be only approximately normal.

Apparatus

Extractions of blood can be conveniently carried out in a 1 ounce screw-capped bottle (" universal container "), the rubber liner in the cap being guarded by a disc of

cellophane. Extraction and centrifugation can thus be carried out in the same vessel. For urine extractions, a 4 ounce screw-capped bottle is satisfactory. Mechanical shaking can be carried out in a Kahn shaker.

In this work, optical densities were measured by means of a Spekker photoelectric absorptiometer, fitted with micro-cups. An adaptation of the method, enabling it to be used with the usual types of visual colorimeter, is described in this paper.

Procedure for blood

To 5 ml. of serum or plasma in a stoppered vessel, add 1 ml. of 30 per cent NaOH and 5 ml. of ethylene dichloride. At the same time, treat 5 ml. of distilled water and 5 ml. of blood standard in the same way. Shake mechanically, or by hand, for 5 minutes and then centrifuge for 10 minutes as fast as possible. Three layers are formed, a clear ethylene dichloride layer (at the bottom), a turbid upper aqueous layer, and a thin solid middle layer. Remove the aqueous layer with a teated pipette, pierce the solid layer, and filter the ethylene dichloride layer through a small Whatman No. 41 filter paper into a small tube. About 3.5 ml. of extract should be obtained. Occasionally, an emulsion is formed in the bottom layer. This can be broken by brisk stirring with a glass rod, followed by fast centrifugation.

Measure 3 ml. of the ethylene dichloride extract into a small centrifuge tube, add 1 ml. of bromthymol blue, stopper (using a rubber bung wrapped in cellophane), and shake by hand for two or three minutes. Centrifuge for 5 minutes at a moderate speed. Two layers are obtained. Remove and discard as much of the upper layer as possible and filter the lower, yellow, ethylene dichloride layer through a small paper. About 2.5 ml. of extract should be obtained.

To 2 ml. of this extract in a small centrifuge tube, add 1 ml. of N-NaOH, stopper, and shake for a few minutes by hand. Centrifuge for 5 minutes and collect the clear supernatant blue layer.

Read the optical density of the blue solution in the Spekker, using a red filter. Set the blank obtained from water carried through the procedure at zero. The concentration of hetrazan can be read from a previously prepared calibration curve or calculated from the formula:

$$\frac{\text{reading of test}}{\text{reading of standard}} \times 10 = \mu\text{g. hetrazan base/ml. serum or plasma}$$

The calibration curve over the range 0–20 μg. hetrazan base/ml. is a straight line.

Procedure for urine

To 10 ml. of urine in a stoppered vessel, add 2 ml. of 30 per cent NaOH and 10 ml. ethylene dichloride. Shake mechanically, or by hand, for 10 minutes and allow to stand until partial separation into two layers has occurred. Remove and discard about 10 ml. of the upper layer and transfer the remainder to a 15 ml. centrifuge tube. Spin for 5 minutes to get two layers. Remove as much of the supernatant as possible and filter the ethylene dichloride layer through a small Whatman No. 41 paper into a centrifuge tube. Carry out the same procedure with 10 ml. of distilled water (water blank) and 10 ml. of urine standard. Emulsion formation is uncommon and easily dealt with as described for blood.

To the ethylene dichloride extract (there is no need to measure its volume) add 1 drop (about 0.1 ml.) of CS_2, mix by tapping, stopper, and allow to stand for 15 minutes. Add 4.0 ml. N-NaOH, shake well for two or three minutes, and centrifuge to obtain two layers. Remove the upper layer and filter the lower ethylene dichloride

layer through a small filter paper. About 8 ml. should be obtained. Transfer 5 ml. of this to a centrifuge tube, add 3 ml. bromthymol blue, shake by hand for a few minutes, and then centrifuge. If the supernatant is very pale or colourless, remove most of it, add a further 3 ml. of bromthymol blue, and shake once more. Repeat the process until a deeply coloured supernatant is left. Remove the upper layer as completely as possible and filter the yellow ethylene dichloride layer as before.

Using a violet filter, read the optical density of the yellow extract in the Spekker, setting the water blank at zero. Dilute deeply coloured extracts with ethylene dichloride, if necessary. Read the concentration from a calibration curve or calculate from the formula:

$$\frac{\text{reading of test}}{\text{reading of standard}} \times 20 \times \text{dilution factor} = \mu\text{g. hetrazan base/ml. of urine}$$

For other fluids, e.g., hydrocoele fluid, the procedure is the same as for urine.

Discussion of Method

In the method described, normal whole blood has an appreciable apparent hetrazan content sometimes reaching 3 μg./ml. With plasma or serum, however, this blank value is usually zero and seldom exceeds 1 μg./ml. Slight haemolysis is of no importance. Recovery experiments show 95–100 per cent extraction. Deproteinization of whole blood, serum, or plasma by the usual methods gives rise to an enhanced blank value and a diminution in the recovery of added hetrazan, less than 70 per cent being recovered.

The apparent hetrazan content of normal urines is higher, being usually 2–3 μg./ml. but sometimes exceeding 10 μg./ml. If the step involving addition of CS_2 is omitted, much higher blank values are obtained. It is probable that much of the apparent hetrazan in urine consists of primary and secondary amines, which can be made to combine with CS_2 and extracted by alkali as thiocarbamates. No hetrazan is removed by this procedure.

Adaptation of method for use with visual colorimeter

Urine.—No special modification is required, as adequate volumes of extract are obtained. The water blank, which is extremely pale, can be disregarded. Compare test and standard in the usual way, using a blue or violet filter.

Blood.—To 2 ml. of the final ethylene dichloride extract add 1 ml. of absolute ethyl alcohol and a small drop (about 0.02 ml.) of 30 per cent NaOH. A clear blue solution results, the intensity of which can be read against a neutral grey screen (density 0.25), using a red filter. Readings should be carried out within 10 minutes as a slight turbidity sometimes develops after this period. If the readings of blank, test, and standard are respectively B, T, and S, then

$$\frac{B-T}{B-S} \times \frac{S}{T} \times 10 = \mu\text{g. hetrazan base/ml. serum or plasma}$$

When the blank is very pale, i.e., B is large compared with T and S, the formula reduces to

$$\frac{S}{T} \times 10 = \mu\text{g. hetrazan base/ml. serum or plasma}$$

Hetrazan administration in the human

Blood and urine hetrazan levels were determined in normal male volunteers after the oral administration of hetrazan hydrochloride. During the experiments all the subjects carried on with their normal activities and diets, but took no medicines.

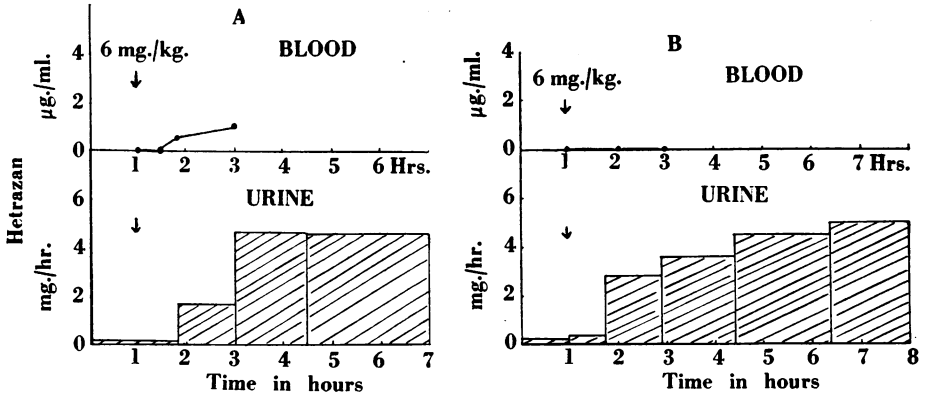

FIG. 1.—Response to dose of 3 mg. hetrazan per kg. body weight. Abscissae: Time in hours. Ordinates: mg./hr. (lower part); µg./ml. (upper part).

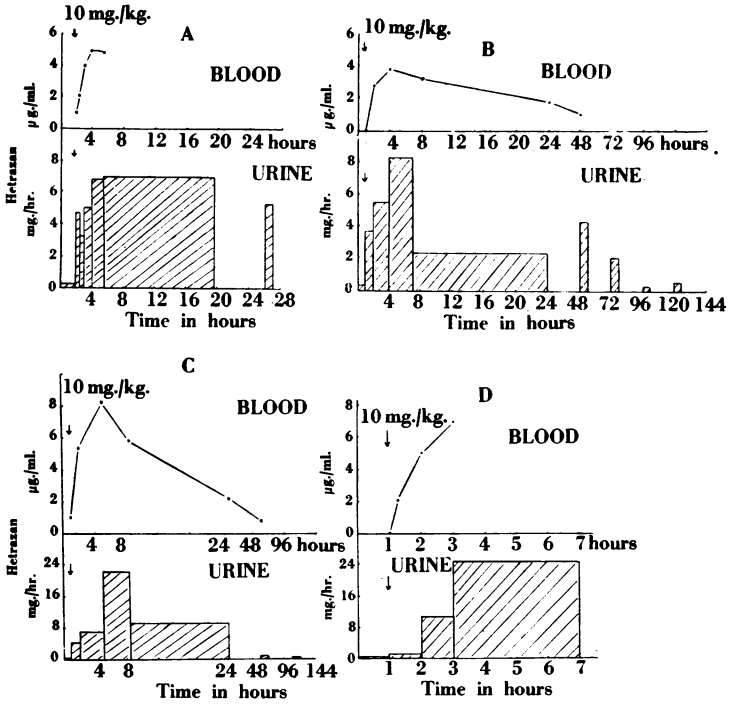

FIG. 2.—Response to dose of 10 mg. hetrazan per kg. body weight. Abscissae and ordinates as in Fig. 1. Scale of abscissae is different in C. Scale of ordinates is different in C and D.

Effect of a single dose

In two subjects a dose equivalent to 6 mg. base/kg. body weight was given. Less than 1 μg./ml. base could be detected in the blood. Urinary excretion started almost at once and reached a steady rate of 4–5 mg./hr. after a few hours (Fig. 1A, B). There were no toxic effects.

In four subjects, a dose of 10 mg./kg. body weight was given. In all of them, the blood level rose appreciably over several hours, reaching maximum values of 4–8 μg./ml. (Fig. 2A, B, C, D). Urinary excretion started quickly and, after a few hours, a fairly steady rate of excretion of 4–8 mg./hr. was established, although one case (Fig. 2D) showed a rate of 24 mg./hr. The drug could be detected in the urine for 2–3 days after administration and in the blood for at least one day after (Fig. 2B, C). About 20 per cent of the ingested dose could be accounted for in the urine. The only untoward effects produced were slight nausea and lassitude.

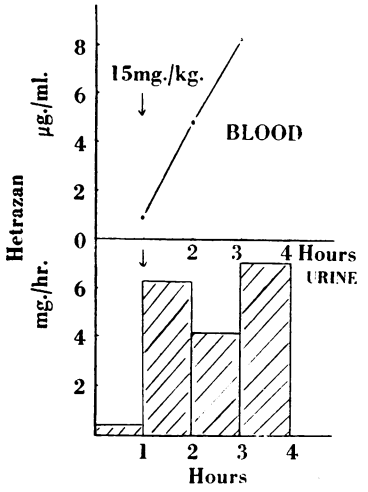

FIG. 3.—Response to dose of 15 mg. per kg. body weight.

In one case, a dose of 15 mg./kg. body weight was given. Blood and urine were collected hourly, but the experiment was discontinued after three hours because of the intense nausea, headache, and lassitude experienced by the subject. After a further two hours, he vomited and was not fully recovered until about 10 hours after taking the drug. At the end of two hours the blood level of hetrazan had reached 8.2 μg./ml. and it is very probable that it rose to much higher levels later. Urinary excretion of the drug was between 4 and 7 mg./hr. (Fig. 3).

Effect of repeated doses

In four subjects, 250 mg. hetrazan hydrochloride, equivalent to about 3 mg./kg. body weight, were given by mouth at 12-hour intervals for five days. During the experiment, a blood sample was taken 2½ to 3 hours after the morning dose and 24 hour urine samples collected as far as possible. For three or four days after the last dose of hetrazan, blood samples were taken at the same time as before and samples of urine collected over a measured time. The subjects carried on with their normal routine, but avoided medicines, during the period of observation (Fig. 4A, B, C, D).

In general, there was little change in the blood level on the first day, after which it rose to a maximum of 3–5 μg./ml. on the third or fourth day. Hetrazan could be detected in the blood for about two days after the drug was stopped. In the urine, a steady rate of excretion of 4–6 mg./hr. was set up during the period of administration and for the first day after this. Excretion continued at a diminished rate for a further 2–3 days. The total amount of hetrazan excreted was about 30 per cent of the ingested dose. No toxic effects were noted during the experiments, apart from slight lassitude.

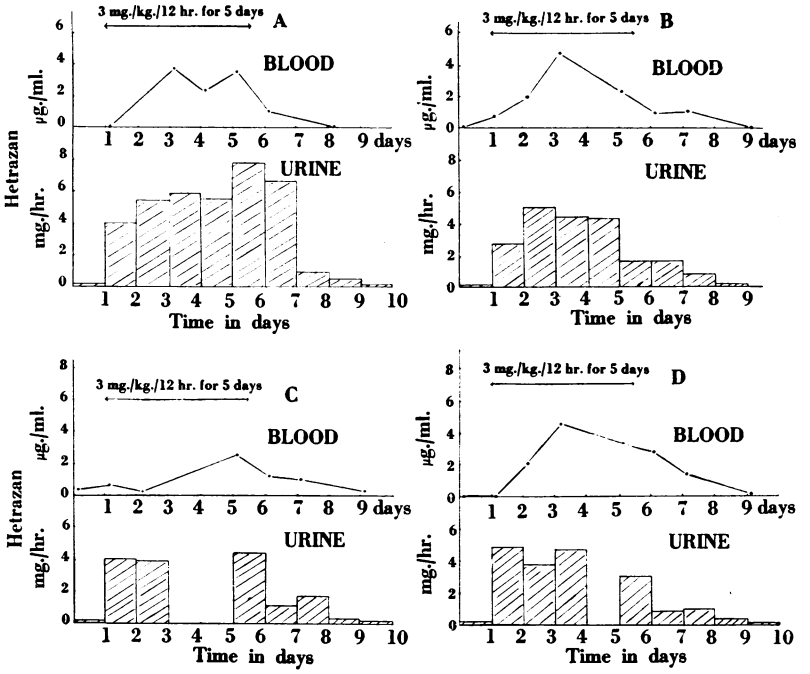

FIG. 4.—Response to repeated doses of 3 mg. per kg. body weight. Abscissae: Time in days. Ordinates: as before.

Conclusions

These results suggest that a single dose of hetrazan produces a maximum blood level in 2–3 hours and that a dose of about 10 mg./kg. body weight is required to give a blood level of 3–5 μg./ml. at this time. In one subject a level of probably over 9 μg./ml. was associated with nausea and malaise. Africans undergoing treatment with hetrazan for filariasis seem able to tolerate higher blood levels without marked discomfort (Hawking, private communication).

Repeated doses lead to an accumulation of the drug in the blood. This may be related to the fairly steady rate of excretion in the urine, which seems to be independent of the dose within the range tried. The highest blood level determined by the author was 15 μg./ml. in an African who had had 500 mg. of the hydrochloride twice daily for two days preceding the taking of the blood sample. The blood was taken after the morning dose on the third day (blood sample sent by air mail).

SUMMARY

1. A method is described for determining hetrazan levels in blood and urine.

2. The levels of hetrazan in blood and urine are described in seven subjects who had a single dose and in four subjects who had repeated doses.

3. Administration of a single dose of 10 mg./kg. body weight results in a maximum blood level of 3–5 μg./ml.

4. Administration of 3 mg./kg. body weight twice daily for five days results in a maximum blood level of 3–5 μg./ml.

5. About 30 per cent of the drug is excreted in the urine.

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