A FLUORIMETRIC ASSAY FOR MINUTE AMOUNTS OF SOME THIOHYDANTOINS

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A fluorimetric assay sensitive to μg. quantities of certain thiohydantoins has been devised using dichloroquinone chloroimide as a fluorescence reagent. The nature of the buffer used is critical. Satisfactory results have been obtained for recoveries from urine, spinal fluid and plasma. 16 thiohydantoins and some related compounds have been examined by the suggested technique.

SEVERAL years ago, during an investigation of the chemical aspects of the suppression of tuberculous infection by a series of substituted 2-thiohydantoins, a search for sensitive assay methods was made. 5-*n*-Heptyl-2-thiohydantoin, the most active member of the series being studied, was used as the index compound in the search. Of the more promising chromogenic reactions, we chose to investigate the fine orange colour resulting from condensation with 2:4-dinitrochlorobenzene, and the deep purple-blue colour formed by condensation with 2:6-dichloroquinone chloroimide. The latter reagent, in pH 8 borate buffer, has been reported by McAllister to yield a yellow colour with 4-methyl-2-thiouracil and 4-propyl-2-thiouracil[1,2], and yellow to red colours with 2-mercaptoimidazole and its derivatives[3,4]. However, neither of these colour reactions turned out to have much advantage over simple spectrophotometry.

EXPERIMENTAL AND RESULTS

The Fluorimetric Estimation of 5-n-*Heptyl-2-thiohydantoin*

During the course of exploratory tests with dichloroquinone chloroimide in which the optimum pH for reaction was sought, it was observed that maximum colour development occurred only within a narrow pH range. For example, 100 μg. portions of the drug reacted as follows. In phosphate buffer 7·2—faint purple; in borate buffer 8·0—moderately strong purple; in borate buffer 8·4—much stronger purple; in borate buffer 8·8—maximum colour—fine deep purple-blue; in ammonia-ammonium chloride, pH 9·2—brownish violet, and in ammonia-ammonium chloride, pH 10·2—brownish violet.

The dirty brownish-violet colour in the NH_4OH-NH_4Cl buffer exhibited the faint dichroic colour which is so often a sign of fluorescence. On inspecting the reaction mixture under filtered ultra-violet light, an intense green fluorescence appeared. None of the purple-blue coloured reaction compounds show fluorescence. It appears that the nature of the buffer used is critical. Thus at pH 8·7, in borate buffer, a purple-blue colour develops, but no fluorescence. At pH 8·7, in NH_4OH-NH_4Cl, a brownish-violet colour develops, with strong green fluorescence under ultra-violet light. Presumably the borate interferes with the formation of the

fluorescent derivative, and permits the formation of the purple coloured derivative. With 5-*n*-heptyl-2-thiohydantoin, the utilization of dichloroquinone chloroimide as a fluorescence reagent made possible a method which is sensitive to one microgram, or less.

Reagents. *iso*Propanol, reagent quality. *iso*Butanol, redistilled. pH 10·2 buffer. Dissolve 23 g. ammonium chloride in 100 ml. water and add 27 per cent ammonium hydroxide to adjust pH to 10·2. pH 6·0 buffer. Prepare two solutions A and B. (A) Dissolve 675 g. KH_2PO_4 in 3000 ml. water. (B) Dissolve 150 g. $Na_2HPO_4 \cdot 12H_2O$ in 500 ml. water. Mix about 11 parts of A with 14 parts of B, varying the ratio slightly, if necessary, to adjust to pH 6·0.

Dichloroquinone chloroimide reagent. (DCQ) (2:6-dichloroquinone chloroimide, Eastman). Purify as follows. Dissolve 1 g. in 50 ml. acetone. While stirring rapidly, add water slowly until a permanent precipitate is formed. Filter on a Buchner filter; dry briefly by suction, then in a vacuum chamber. Transfer to a glass-stoppered bottle and store in the refrigerator. Dissolve 50 mg. in 250 ml. of *iso*propanol. This solution, if kept cold, is stable for at least 2 weeks. *Standard solutions.* (*a*) Primary Standard. Dissolve 100 mg. of 5-*n*-heptyl-2-thiohydantoin in 200 ml. of 95 per cent ethanol. (Stable for at least 2 weeks in a cool dark place.) (*b*) Working Standard. Dilute 3 ml. of the primary standard to 250 ml. with water. Concentration is 6 μg./ml.; it must be prepared each day. Quinine reference standard. Prepare a solution containing 100 μg. of quinine sulphate per litre of 0·1 N sulphuric acid.

The Standard Curve

To each of a series of six fluorimeter tubes transfer, 0, 0·1, 0·2, 0·3, 0·4 and 0·5 ml. of the working standard. Add to each tube sufficient water to dilute to exactly 2·0 ml. To each add 4·0 ml. of a mixture of 3 volumes *iso*propanol and 1 volume of *iso*butanol. Then add 0·5 ml. of the pH 10·2 buffer and 0·5 ml. of the DCQ reagent. Mix and let stand for 2 hours. Read in the fluorimeter, using B-1 as the primary filter and P-C-1 as the secondary filter (Coleman photofluorimeter and Coleman filters) and setting the instrument at a convenient point with the quinine reference standard. Plot the net instrument readings against μg. of 5-*n*-heptyl-2-thiohydantoin. A linear plot results.

Extraction of Micro Amounts of 5-n-*Heptyl-2-thiohydantoin from Water*

To each of a series of five small separators, which must be scrupulously clean and stopcocks lubricated only with water, transfer 8·0 ml. of chloroform. Add, 0, 0·2, 0·4, 0·6 and 0·8 ml. of the working standard. Add to each separator 2 ml. of the pH 6·0 buffer and sufficient water to dilute the aqueous phase to 5 ml. Shake each funnel for two full minutes, let stand until the layers separate and draw off the chloroform layer into a clean centrifuge tube. Centrifuge only if necessary to clarify. Transfer 5 ml. by pipette to a fluorimeter tube and cautiously evaporate all of the chloroform on a steam bath. Cool and add 4·0 ml. of a mixture of 3 volumes of *iso*propanol and 1 volume of *iso*butanol. Add 2·0 ml. of water, then

0·5 ml. of the pH 10·2 buffer and 0·5 ml. of the DCQ reagent. Mix, let stand for 2 hours and read in the fluorimeter. The plot of net instrument readings against samples taken corresponds closely to that of the standard series, and demonstrates quantitative extractability of the compound from water.

Extraction from Urine, Spinal Fluid and Plasma

Precisely the same procedure given above may be used for extraction from urine, spinal fluid or plasma. Recoveries from human urine, 2 ml. samples with 0·6 to 1·2 μg. added drug per ml., are 100 to 104 per cent. One test using 1 ml. of monkey spinal fluid with 2 μg. added drug gave a recovery of 97 per cent. This technique has been found to yield 97 to 113 per cent recovery of amounts of drug of 6 to 30 μg. added to 2 ml. of human plasma*.

The *iso*propanol and *iso*butanol mixture is used to keep fats in solution. If the sample is known to be fat-free, it is possible to use a simpler solvent system. Thus, with water, urine or spinal fluid, extract with chloroform, evaporate, then take up the residue in 1 ml. 95 per cent ethanol and 5 ml. of water. Add 0·5 ml. of the pH 10·2 buffer and 0·5 ml. of the DCQ reagent. In this solvent fluorescence develops faster, so that only 1 hour of standing is required. The total intensity of developed fluorescence is also slightly greater in the ethanol medium than in the higher mixed alcohols.

Selectivity of the Fluorescenc Test

A start has been made on experiments which may eventually yield dependable information on the selectivity of the test, and the mechanism by which the fluorescent derivative is formed. Of the large number of compounds prepared in these laboratories by S. Archer and associates, 16 were selected for test. A few important related compounds were also tested. The figures in the last column of Table I refer to net fluorimeter readings equivalent to 2 μg. of the respective compound, by the technique already described. As noted, in the case of some compounds, much larger samples were taken (marked with star).

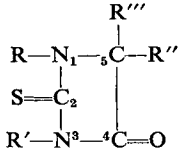
Some tentative generalizations may be drawn from the data in the Table. Hydantoins (i.e., oxygen instead of sulphur in the 2-position) develop no measurable fluorescence. (Tests 15, 16, 17). Neither do thiouracil or 6-propyl thiouracil. (Tests 21, 22). In the thiohydantoin series, it seems possible (Tests 1, 23) that, to achieve fluorescence, one of the 5-position hydrogens must be substituted. Apparently any monoalkyl substitution in the 5-position results in fluorescence. (Tests 1, 2, 3, 4.) But if both 5-position hydrogens are substituted, fluorescence is not measurable. (Tests 12, 13.) With the only dithiohydantoin available to us (Test 20), the "monoalkyl-5" rule fails, and no measurable fluorescence results. Even the presence of one aromatic substitution in the 5-position seems to quench fluorescence. (Test 8.) When the benzene ring is separated from the nucleus by a methylene group (Tests 14, 9)

* Human plasma test by E. W. McChesney and associates of these laboratories.

fluorescence re-appears. However, a saturated cyclic substituent at the 5-position acts like an alkyl group (Test 5). In fact, note that 5-*cyclo*hexyl-2-thiohydantoin shows twice the fluorescence of the 5-alkyl compounds. It is equally interesting that when the *cyclo*hexyl group is insulated from the hydantoin nucleus by a single methylene group (Test 6) the fluorescence is drastically quenched; when the insulation is lengthened

TABLE I

COMPOUNDS TESTED SHOWING NET FLUORIMETER READINGS EQUIVALENT TO 2 μ μG. OF COMPOUND



Test No.	Compound	R	R′	R″	R‴	Instrument reading
1	5-*n*-Heptyl-2-thiohydantoin	H	H	H	*n*-heptyl	30
2	5-*n*-Butyl-2-thiohydantoin	H	H	H	*n*-butyl	33
3	5-*n*-Pentyl-2-thiohydantoin	H	H	H	*n*-pentyl	30
4	5-*n*-Hexyl-2-thiohydantoin	H	H	H	*n*-hexyl	31
5	5-*cyclo*Hexyl-2-thiohydantoin	H	H	H	*cyclo*hexyl	66
6	5-*cyclo*Hexylmethyl-2-thiohydantoin	H	H	H	*cyclo*hexylmethyl	6
7	5-(2-*cyclo*Hexylethyl)-2-thiohydantoin	H	H	H	2-*cyclo*hexylethyl	38
8	5-Phenyl-2-thiohydantoin	H	H	H	phenyl	0
9	5-(4-Methoxybenzyl)-2-thiohydantoin	H	H	H	4-methoxybenzyl	3
10	5-Propylmercaptomethyl-2-thiohydantoin	H	H	H	propylthiomethyl	0
11	5-Butylmercaptomethyl-2-thiohydantoin	H	H	H	butylthiomethyl	0
12	5:5-Dimethyl-2-thiohydantoin	H	H	methyl	methyl	0
13	1-Acetyl-5:5-dimethyl-2-thiohydantoin	acetyl	H	methyl	methyl	0
14	1-Acetyl-5-benzyl-2-thiohydantoin	acetyl	H	H	benzyl	22
15*	Hydantoin					0
16*	5-Heptyl hydantoin					0
17*	5:5-Diphenyl hydantoin					0
18*	Thiourea					0
19*	Tibione (4-acetamidobenzaldehyde thiosemicarbazone)					10
20*	5-Hexyl-2:4-dithiohydantoin					0
21*	Thiouracil					0
22*	6-Propylthiouracil					0
23*	Thiohydantoin					0

* Used samples of 100 μg.

NOTE: Most of the compounds indicated as showing zero fluorescence, do indeed fluoresce at much higher levels. However, in the latter cases, it remains to be established whether the compound itself is fluorescing, or some impurity.

to ethylene (Test 7) the fluorescence intensity returns to the range shown by the simple 5-alkyl derivatives in the Table. If the 5-alkyl substituent is interrupted by sulphur (Tests 10, 11) so that only one carbon insulates S from the nucleus, fluorescence is lost. It is possible that if two or more carbons separated S from the nucleus, the fluorescence would return, but this has not been checked.

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