SPECTROFLUOROMETRIC ANALYSIS OF CARDIOTONIC STEROIDS

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The sensitive and specific fluorimetric methods introduced by Jensen (1952; 1953) for digitalis estimation were re-evaluated for the assay of cardiotonic steroids and other steroids of biological interest. Characteristics of the excitation and fluorescence spectra were determined. Using a modification of Jensen's original digitoxin method and a spectrofluorometer, a linear relation was demonstrated between fluorescence intensity and concentrations from 0·01 to 6 μg./ml. for both digitoxin and digoxin. Application of these methods to the assay of gitoxin and cholesterol would seem to be equally practicable. In addition, scillaren A and B and strophanthin K can be made to fluoresce weakly. On the other hand, ouabain, cholestanol, testosterone, and hydrocortisone showed no fluorescence. Possible mechanisms of the reactions are discussed.

The dehydration of the cardiotonic steroids by treatment with strong acids or oxidising agents has long been recognised (Jacobs and Collins, 1924; Smith, 1935; Smith, 1936). Petit (1950) showed that the unsaturated anhydrogitoxigenin resulting from phosphoric acid treatment of gitoxin emitted fluorescence under ultra-violet irradiation which could be utilised for the quantitative estimation of the glycoside. Sciarini and Salter (1951) described a fluorometric method for digitalis estimation using hot sulphuric acid. These methods were superseded by the hydrochloric acid methods for gitoxigenin (Jensen, 1952) and digitoxigenin (Jensen, 1953).

The present investigation was made to extend the methods of Jensen to other cardiotonic steroids, to increase the sensitivity to levels usually found in biological fluids and tissues, and to define the excitation and fluorescence spectra of the compounds investigated.

Experimental

Methods and Results

Reagents and standards. The reagents were of standard reagent grade. Only glycerol, which had a very high blank fluorescence, required additional purification. This was achieved by passage through a column of activated charcoal. The steroids were obtained from commercial sources and were checked for homogeneity using the following paper chromatography systems: I. methyl isobutyl ketone and isopropyl ether (4: 1 v/v) with formamide as the stationary phase (Gisvold and Wright, 1957); II. dichloromethane with formamide as the stationary phase. Both systems were used with the descending technique for 2 to 4 hr. on Whatman No. 1 paper. System II was the only method of the many

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tested which moved gitoxin without tailing. Digitoxin and strophanthin K, which were found to contain appreciable amounts of impurities, were purified by chromatography of mg. quantities using the same solvent systems on large sheets.

Measurement and spectral analysis of fluorescence were made in the Aminco-Bowman Spectrofluorometer or the Aminco-Keirs Spectrophosphorimeter with fluorescence attachment. A few correlative measurements were made with the Farrand Fluorometer Model A. Slit arrangements were chosen for maximum sensitivity or maximum specificity.

Composition of the reaction mixture. Attention was focused on the digitoxigenin method of Jensen which depends on the action of hydrochloric acid in the presence of hydrogen peroxide and ascorbic acid in

TABLE I

EFFECT OF OMISSION OF COMPONENTS OF REAGENT ON SPECTRA AND ON FLUORESCENCE INTENSITY OF DIGITOXIN

Composition of reagent	Relative fluorescence (per cent)	Activation peak (mμ)	Fluorescence peak (mμ)
HCl, ascorbic acid, H_2O_2, MeOH	100	395	570
HCl, H_2O_2, ascorbic acid	42	395	580
HCl	10	400	580
HCl, H_2O_2, MeOH	11	395 380	570 535
HCl, H_2O_2	7	370	520
HCl, ascorbic acid	19	450	500

methanol. Table I summarises the effects of omitting one or more components of this mixture on the fluorescence of digitoxin. Acid alone is sufficient for the development of fluorescence. When acid was omitted no fluorescence could be detected. However, for maximum intensity, the other components are obviously important. The peak wavelengths fall into three general groups. In the experiment in which only ascorbic acid was omitted (Table I, row 4), there was a gradual shift of the activation and emission peaks to the lower wavelengths after about 10 min.

The effects of variation in the ascorbic acid and hydrogen peroxide concentration were also studied. As reported by Jensen (1953), variation in ascorbic acid concentration from 0·2 mg./ml. of final solution to over 1 mg./ml. had no effect on intensity or stability of fluorescence. However, in contrast to Jensen's optimum peroxide concentration of $3·75 \times 10^{-4}$ M in the final reagent mixture, we found the concentration range of $7·7 \times 10^{-5}$ to $1·5 \times 10^{-4}$ M to yield maximum intensity. Therefore, a concentration of $1·15 \times 10^{-4}$ M was used in all subsequent studies.

Increased temperature of incubation during the development of fluorescence (40°, 60° and 80°) was found to diminish the maximum fluorescence, in accordance with Jensen's results.

Deterioration from exposure to ordinary fluorescent and incandescent room light was ruled out by experiments in which samples prepared under a red darkroom safelight as the sole source of light were found to yield

the same intensity and spectral distribution of fluorescence as samples prepared under ordinary conditions. Nevertheless it was observed that constant exposure to the exciting radiation in the fluorometer did cause some deterioration as indicated by an initial fall off of 1 to 2 per cent in

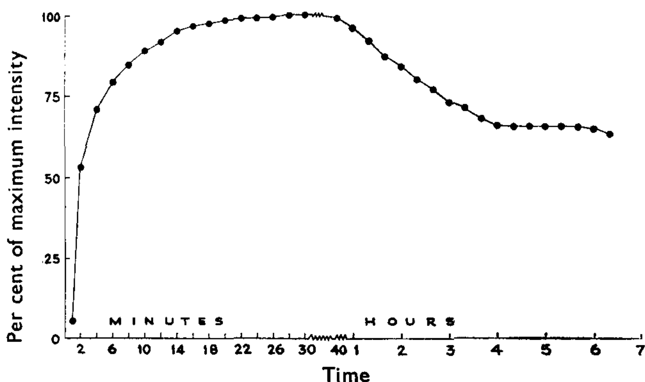

FIG. 1. Time course of fluorescence of digitoxin in the modified digitoxin reagent described in the text. Fluorescence intensity plotted as per cent of the maximum attained.

the galvanometer deflection during the first 2 min., followed by a slower decrease of 4 to 5 per cent over a period of 15 to 20 min. This deterioration was only partially reversible in the dark.

The development and decay of digitoxin fluorescence with time is shown in Fig. 1. Two stable periods were observed, one at the time of maximum fluorescence from 20 to 30 min. and a second after partial decay to approximately 70 per cent of maximum fluorescence in the 4 to 6 hr. period. The relatively earlier stable period compared to

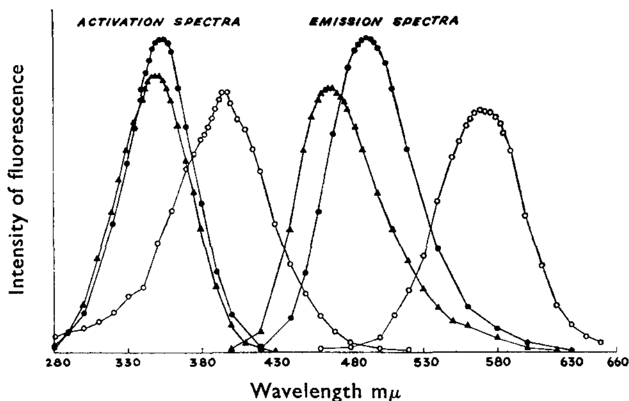

FIG. 2. Activation and fluorescence spectra of digitoxin, digoxin, and gitoxin using the modified digitoxin method. Activation spectra were measured with the fluorescence monochromator set at the previously determined fluorescence peak and vice versa. Fluorescence intensity in arbitrary units. Digitoxin, 10 μg./ml., ○—○; digoxin, 2·5 μg./ml., ●—●; gitoxin, 10 μg./ml., ▲—▲.

Jensen's period of 50 to 100 min. may be the consequence of the lower peroxide concentration which we used. Digoxin fluorescence, on the other hand, was found to remain stable for at least 24 hr.

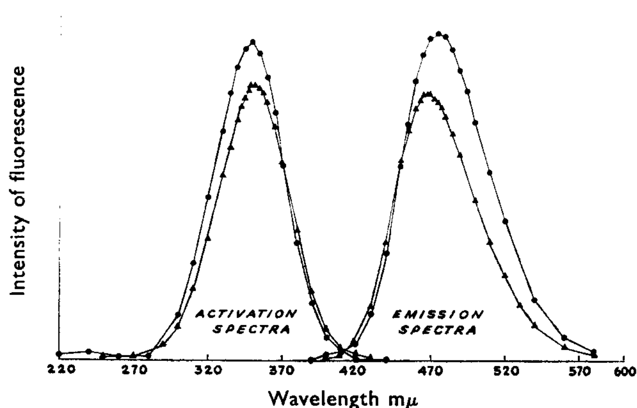

FIG. 3. Activation and fluorescence spectra of digoxin and gitoxin using Jensen's gitoxin method. Procedure as described in Fig. 2. Digoxin, 20μg./ml., ●—● ; gitoxin, 10 μg./ml., ▲—▲.

From the preceding results the following optimal procedure was chosen. To the sample, dry or dissolved in a few μl. of methanol, are added 2 ml. methanol containing 2 mg. ascorbic acid, 3 ml. concentrated hydrochloric acid, and 0·2 ml. 0·003 M aqueous hydrogen peroxide. The tubes are immediately agitated to mix the contents and are then

TABLE II

FLUORESCENCE OF VARIOUS STEROIDS USING THE MODIFIED JENSEN TECHNIQUES

	Digitoxin method			Gitoxin method		
Steroid tested	Relative intensity (per cent)	Activation peak (mμ)	Fluorescence peak (mμ)	Relative intensity (per cent)	Activation peak (mμ)	Fluorescence peak (mμ)
Digitoxin	100	395	570	0	—	—
Digoxin	400	350	490	4	350	495
Gitoxin	125	350	470	100	350	470
Scillaren A ..	5	465	535	0	—	—
Scillaren B	0	—	—	4	465	535
Ouabain	0	—	—	0	—	—
Strophanthin K ..	0	—	—	1	350	470
Acetyl-strophanthidin ..	0	—	—	0·2	405	475
Cholesterol ..	21	470	530	0	—	—
Cholestanol ..	0	—	—	0	—	—
Testosterone ..	0	—	—	0	—	—
Hydrocortisone ..	0	—	—	0	—	—

allowed to stand at room temperature. After 20 to 40 min. the samples and a reagent blank are read in the fluorometer.

With the above procedure, activation and emission (fluorescence) spectra were determined for several cardiotonic steroids and for a few other steroids of biological interest. Spectra were similarly plotted for those compounds which were found to fluoresce with the gitoxin method of Jensen (1952) (equal volumes of glycerol and concentrated hydrochloric acid incubated with the sample at room temperature for 30 to 60 min.). Representative spectra are shown in Figs. 2 and 3. Table II summarises the results for all the steroids tested.

Application of the method to the assay of digitoxin and digoxin. Using the digitoxin method described above with activation and emission at the appropriate peak wavelengths, the concentration: fluorescence relation was found to be linear from 0·01 to over 6 μg. digitoxin per ml. of reaction mixture. A similar range of linearity was found for digoxin.

TABLE III

PRECISION OF THE MODIFIED DIGITOXIN METHOD

Steroid	Conc. (μg./ml.)	Mean fluorescence	Standard error n = 4
Digitoxin	0·02	5·1	0·23 (5 per cent)
	0·2	54·8	0·16 (0·3 per cent)
	2·0	514·0	2·12 (0·4 per cent)
Digoxin	0·02	24·4	0·48 (2 per cent)
	0·2	262·5	1·56 (0·6 per cent)
	2·0	2513·0	25·00 (1 per cent)

The reproducibility of the method was estimated from replicate determinations at three different glycoside concentrations and the results are shown in Table III.

The maximum sensitivity was arbitrarily defined as the least concentration of glycoside yielding a fluorescence intensity twice that of the reagent blank. Using the procedure outlined previously, with no special precautions, the least concentration meeting the above conditions was between 0·005 and 0·010 μg./ml. of reagent mixture for both digitoxin and digoxin.

Comparison of the fluorescence of digitoxigenin and digoxigenin with that of the parent glycosides demonstrated identical spectra and equivalent fluorescence intensity when calculated on a molar basis.

DISCUSSION

Of the non-isotopic digitalis assay methods in the literature, the fluorometric methods would seem to offer the maximum of both sensitivity and specificity. The modified Jensen method described in this paper offers a further increase in specificity if a spectrofluorometer is available. Thus, digitoxin and either digoxin or gitoxin can be measured separately in the same sample by selecting the appropriate activation and fluorescence

wavelengths. Jensen (1954) has described a simple and effective technique for the assay of glycosides on paper chromatograms. Application of the method to *direct* analysis of extracts of biological fluids and tissues is in progress but has been hampered by the presence of interfering fluorescent compounds.

Mechanism of the reaction. Bellet (1950) and Sasakawa (1959) have shown that the bluish-green fluorescent product of acid treatment of gitoxin is the Δ^{14}, Δ^{16}-dianhydro derivative of gitoxigenin. On the other hand, treatment of digitoxin and digoxin with concentrated hydrochloric acid yields the $\Delta^{8,(14)}$ anhydro compounds (Smith, 1935; Smith, 1936) which also fluoresce, according to our results. Thus it would appear that the orientation of the double bond at position 14 is not critical to the development of fluorescence. However, it may well be that the intensity and possibly the spectral characteristics of the fluorescence are related to the double bond position. From the three sets of peak wavelengths found for digitoxin by omitting various components of the reaction mixture (Table I), it would appear that there are at least three different fluorescent derivatives of digitoxin.

In the strongly hydrolytic conditions prevailing in the reaction mixture conversion of the glycosidic compounds to their genins is likely. That the sugars do not in any way influence the nature of the final product or its fluorescence is indicated by the identical spectra and intensities, mole for mole, which we obtained for two pairs of genins and glycosides. In the digitoxin method the fate of the hydroxyl group at position 3 after hydrolysis of the glycosidic linkage has not been established. Dehydration at this position with the formation of a Δ^{2} or Δ^{3} double bond may occur although we found no precedent in the literature for dehydration at this position under these conditions. This interpretation would seem to be supported by the observation that cholesterol which has only the single hydroxyl group at carbon 3 is converted in this reaction mixture from a non-fluorescent to a fluorescent compound whereas no fluorescence is produced in the gitoxin reagent which, as we have noted, dehydrates gitoxin at the 14 and 16 positions but not at position 3. However, other possibilities such as formation of an unusual complex at the 3 position remain to be ruled out. Isolation of the fluorescent products for subsequent structural analysis is in progress.

From the absence of fluorescence when cholestanol is treated with the digitoxin reagent mixture it would appear that more than one double bond is required in the final product. Every compound in this series which fluoresced had at least two hydroxyl groups or one hydroxyl group and a double bond in the steroid nucleus. However, some steroids with several hydroxyl groups or double bonds showed no fluorescence in either reagent mixture. In addition, since both squill and digitalis derivatives were capable of fluorescence, it is apparent that the number of double bonds in the lactone ring is not critical and may not contribute to the fluorescence of the final product at all.

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