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RADIOIMMUNOASSAY FOR THE DETERMINATION OF DIGOXIN AND RELATED COMPOUNDS IN *DIGITALIS LANATA**

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Key Word Index—*Digitalis lanata*; Scrophulariaceae; radioimmunoassay; cardenolides; digoxin; distribution of digoxin; screening techniques.

Abstract—A radioimmunoassay technique has been developed for the measurement of digoxigenin glycosides in crude extracts from both fresh and dried leaf material of *Digitalis lanata*. The antibody, obtained by immunizing rabbits against a conjugate of digoxin with human serum albumin, had a high affinity ($K_a = 0.8 \times 10^{10}$ l/mol) for digoxin and permitted detection of as little as 60 fmol digoxin (45 pg) per 0.1 ml of sample. The antiserum was highly specific for digoxigenin and its glycosides, with only diginatin showing a substantial cross reactivity (~30%). The use of [^{3}H]-labelled and [^{125}I]-labelled digoxin as tracer and of dextran-coated charcoal or ammonium sulfate for separation did not change the specificity of the assay nor the properties of the standard curve. This method has been found to correlate with the usual fluorimetric determination of digoxin, but is more sensitive by a factor of 10^4. A correlation analysis of 8 and 30 different *D. lanata* plants (leaf discs and drugs analysed with both methods) gave correlation coefficients of $r = 0.989$ and $r = 0.907$ respectively. The analysis of a single leaf disc, 3 mm in diameter (obtained from a fresh leaf), gave an exact measure of the digoxin content found in the dried leaf drug ($r = 0.973$). With a semi-automated technique, about 2000 quantitative analyses per week can be performed by one person, thus providing the potential to screen plants for use in breeding or tissue culture work. The distribution of digoxigenin equivalents in single seeds, seedlings and plants of different ages has also been investigated.

INTRODUCTION

In clinical biochemistry, radioimmunoassay (RIA) is presently the most powerful analytical tool for quantitative detection of molecules of diverse structure and function, in biological fluids of human or animal origin. RIA comprises a unique combination of sensitivity and specificity as well as precision and general applicability [1]. In spite of the fact that several RIA's have been developed and applied to the quantitative determination of drug constituents of plant origin such as colchicine [2], digoxin [3], morphine [4] and nicotine [5], this method has not yet been used in phytochemistry, plant physiology or pharmacognosy. Because of the unique features of the RIA method, this assay system should render itself exceedingly useful for the development of screening programs in plant breeding of chemical characters, the screening of plant cell cultures for secondary or primary metabolites, or investigations of biosynthesis, transport, deposition of plant constituents, and many other problems.

The principle of RIA is based on the highly specific reaction of antibodies with antigens against which the antibodies have been directed. Usually, compounds with a MW below 1000 are not immunogenic when introduced into the blood stream of an animal. However, if these low MW compounds are bound covalently, as haptenes, to protein carriers they become immunogenic and specific antibodies against these haptens are produced. The competition between labelled and unlabelled hapten, for a limited constant number of antibody binding sites in the assay tube, results in some of the hapten being bound while the rest remains free. Thus distribution of the known and constant amount of labelled hapten in the free and bound fractions will be a function of the amount of unlabelled hapten present in the assay tube. The immunologically bound antigen and free antigen are then separated and determination of the radioactivity in either fraction gives an exact measure of the unknown unlabelled antigen present as computed from a standard curve. This paper deals with the application of RIA systems using antibodies directed towards the cardiac glycoside, digoxin, which represents one of the most medicinally valuable and widely used plant constituents.

Digitalis lanata is the only plant which produces digoxin or the primary glycoside lanatoside C. Breeding for high yielding strains of *D. lanata* is therefore desirable and for this purpose large scale screening of individual plants is necessary. An efficient screening method should combine simplicity in handling (e.g. crude plant extracts without purification) with high specificity, precision and the possibility for automatization. These conditions are fulfilled in an ideal way by the radioimmunoassay. Also, in this paper, the use of tritium labelled vs ^{125}Iodine-labelled haptene in RIA as well as dextran-coated charcoal vs ammonium sulfate as antibody separating system are evaluated.

RESULTS

Properties of antiserum

Antiserum against digoxin was isolated from rabbits. The titre of the antiserum, defined as that dilution of

* Part 1 in the series "Use of Immunoassay in Plant Science".

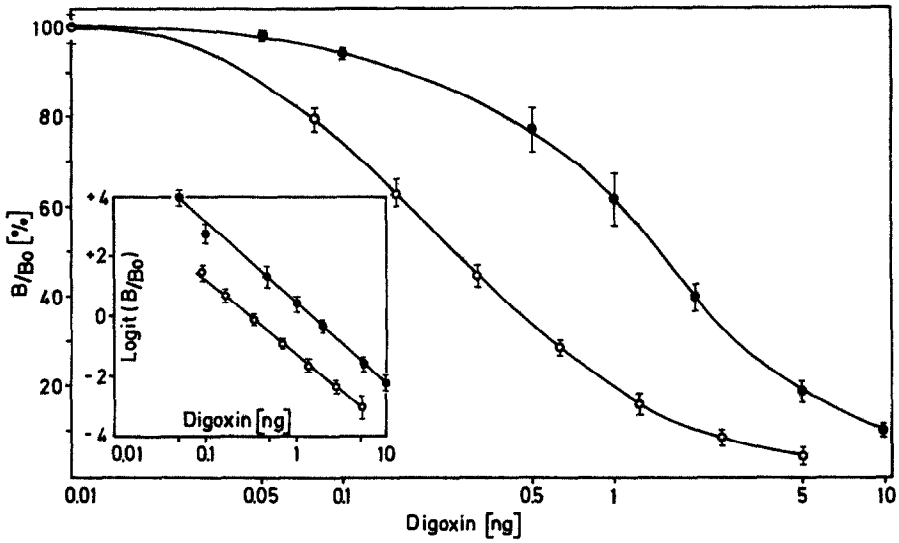

Fig. 1. Digoxin standard curve which is obtained by plotting *B/Bo* vs mass of unlabelled digoxin on semilogarithmic scale. The insert shows the linearised logit–log plot [8] using the same experimental data: ● digoxin-[G-^{3}H] as tracer in combination with charcoal separation ($n = 2$); ○ digoxin-[^{125}I]-tyrosine methylester as tracer in combination with $(NH_4)_2SO_4$-separation ($n = 25$); B = % binding in the presence of unlabelled digoxin; Bo = % binding in the absence of unlabelled digoxin; logit (B/Bo) = ln(B/Bo/100-B/Bo).

antiserum which binds 50% of a fixed mass of radioactively labelled haptene under constant assay conditions, was 1:1000 using 1 pmol digoxin-[G-^{3}H], and 1:4000 using 0.025 pmol digoxin-[^{125}I]tyrosine methyl ester as tracer. This corresponds to a final antiserum dilution of 1:9000 and 1:36000, respectively in the assay tube.

From a Scatchard plot [6] with digoxin-[^{125}I]-tyrosine methyl ester as tracer and a final antiserum dilution of 1:36000, a maximum affinity constant of $K_a = 0.8 \times 10^{10}$ l/mol and a value for the total number of binding sites $A_0 = 1.6 \times 10^{-10}$ mole/l were determined. The nonlinearity of the plot shows the occurrence of several antibody populations with different K_a values. Using the Michaelis–Menten plot [7] an average association constant of 0.33×10^{-9} mol/l. and a saturation concentration of digoxin of 1.9×10^{-10} mol/l. were calculated. The use of tracer, alone or in combination with unlabelled digoxin, yielded identical values for these constants. This shows, that in this system the reactivity of the tracer was indistinguishable from that of the unlabelled compound.

Sensitivity of the radioimmunoassay

The standard curves obtained with [^{3}H]-labelled digoxin and charcoal separation and with [^{125}I]-labelled digoxin and ammonium sulfate separation are shown in Fig. 1, expressed in two possible plots. Table 1 shows the most important parameters derived from the standard curves using different separation techniques and differently labelled antigens.

It is evident, that the mode of separation of bound from free antigen did not have any influence on the pattern and shape of the standard curve. Using the iodinated tracer, however, the sensitivity of the assay could be increased by a factor of 5. The limit of detection, which is defined as that value which can be safely distinguished from a zero sample corresponding to a B/Bo value of $B/Bo = 100\% - 3 \times S.D._{100\%}$ ($S.D._{100\%}$ = standard deviation of the B/Bo value), is 0.06 pmol/0.1 ml sample (45 pg digoxin/0.1 ml sample).

Evaluation of the radioimmunoassay

Effect of solvents. Using ethanol extracts no deviation in the assay was detected up to 20% ethanol. Since diluted plant extracts used for RIA contained a maximum of 8% ethanol, a correction was not necessary.

Specificity of antibodies. The ability of compounds other than digoxin to compete for the antibody is called cross reactivity. The percentage cross reactivity (CR %)

Table 1. Characteristic parameters of standard curves obtained with different tracers and separation techniques

Tracer	Digoxin-[G-^{3}H]	Digoxin-[^{125}I]-tyrosine methylester	
Separation technique:	Dextran-coated charcoal	Dextran-coated charcoal	$(NH_4)_2SO_4$
Usable range of standard curve	0.5–5 ng	0.1–1 ng	0.1–1 ng
Midrange (B/Bo = 50%)	1.5 ng	0.26 ng	0.26 ng
Slope of the logit–log plot	−2.730	−2.430	−2.400
Unspecific binding	4%	9%	14%
Blank values	0	0	0

Blank values were determined using water or solvent; unspecific binding was determined by replacing the antibody dilution with water

is defined [9] as:

$$CR\% = \frac{\text{pmol of digoxin yielding } B/Bo = 50\%}{\text{pmol of compound under investigation yielding } B/Bo = 50\%} \times 100$$

The cross reactivity is a measure of the disturbance of the immunoassay by compounds structurally similar to the haptene against which the antibodies have been produced. 75 compounds have been evaluated, among these the most important cardenolides, digitanols and saponins of *D. lanata*. Table 2 shows a summary of the cross reactivities of these components.

These values are grouped in Fig. 2, which gives a clearer picture of the relationship between molecular structure and cross reactivity. The sterols tested, including the biosynthetic precursors of the digoxin molecule, progesterone and pregnenolone, as well as the saponins and digitanols show no or, in the case of progesterone only a very slight cross reactivity (group C in Fig. 2). Most of the binding specificity of the antibody is directed toward ring C and D of the aglycone, and is extremely specific for the β-OH group at C_{12}, the characteristic substituent of the digoxin series. Blocking this group by acetylation, as in 12-acetyldigoxin leads to a loss of reactivity. An epimeral α-OH group at C_{12}, as in 12-epidigoxin, is not recognized by the antibody. This substance shows a cross reactivity similar to digitoxin (12-desoxydigoxin). All the bufadienolides investigated show only slight cross reactivities (0.2–1.8%). This can be traced to specific binding of the five membered lactone ring of the cardenolides to the antibody, which is not the case with the six membered lactone ring of the bufadienolides. A β-OH group in 12 position, as in 12-β-hydroxyscillarenin, in the bufadienolide series results in a strongly increased cross reactivity of 17%. Generally, however, one can deduce that structural modification in ring A and B are not recognized by the antibody; thus, uzarigenin as well as canarigenin show a cross reactivity comparable to digitoxigenin. Furthermore, within the same series of aglycones, different sugar moieties are not distinguished by the antibody. This fact further supports the theory [10] that the antibody does not recognize the site at which the haptene is fixed to the carrier protein; since in our case, the digoxin molecule was attached to the human serum albumin via the third digitoxose molecule of digoxin, the antibody did not recognize different carbohydrate components linked to the 3-position of digoxigenin. Cross reactivities for the *Digitalis* aglycones and their glycosides were distributed as follows: digoxigenin: 100%; digitoxigenin: 4–10%; gitoxigenin: 0.2–5%; gitaloxigenin: 0.1–3%; diginatigenin: 30–40%. Of all the *Digitalis* glycosides tested, only diginatin shows substantial cross reactivity due to its β-C_{12} hydroxyl group. However, of the total cardenolides of *D. lanata*, diginatin (the tridigitoxoside of diginatigenin) comprises only about 0.05% [11], an amount which is too little to interfere with the digoxin determination. Therefore, the antibody used here reacts selectively with the total digoxigenin glycosides present in the crude extracts of *D. lanata*. Since digoxin is used as the standard in the study, the experimental values are expressed as digoxin equivalents.

Estimation of cross reacting material in crude plant extracts

The advantage of the RIA method is the high sensitivity and specificity by which a given molecule can be selectively and quantitatively determined in biological fluids or extracts. In our studies no purification or concentration of extracts of *Digitalis* was necessary for digoxin equivalent determination. In addition to determining the cross reactivity with pure compounds, we attempted to gain further information about the specificity of the antibody and investigated the possibility of nonspecific factors interacting with the assay system. We, therefore, tested plants of widely differing taxonomic ori-

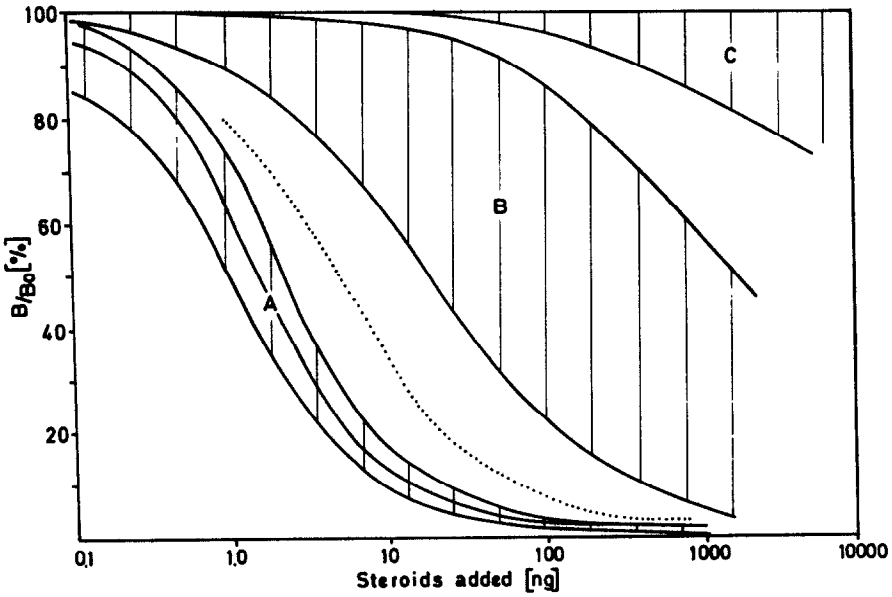

Fig. 2. Distribution of cross reactivities of the anti-digoxin antibody. Area A: Digoxigenin and digoxigenin glycosides. The digoxin standard curve (——) occupies a medial position; Area B: Cardenolides not β-hydroxylated at C-12 position and bufadienolides; Area C: Sterols, digitanols and saponins; (....) = Diginatin.

Table 2. Cross reactivities determined using different tracers and separation techniques

Substance	Digoxin-[G-^{3}H] and dextran-coated charcoal		Digoxin-[^{125}I]-tyrosine methyl ester and $(NH_4)_2SO_4$	
	pmol (B/Bo = 50%)	CR (%)	pmol (B/Bo = 50%)	CR (%)
Digoxin	1.9	100	0.3	100
Digoxigenin	2.6	75	0.5	59
Digoxigenin-digitoxoside	2.7	71	0.3	103
Digoxigenin-bis-digitoxoside	1.7	114	0.3	128
Neo-digoxin	1.3	150	0.4	84
Digoxoside	1.4	134	0.2	188
α-Acetyldigoxin	1.8	105	0.2	152
β-Acetyldigoxin	2.7	72	0.5	69
Lanatoside C	1.7	113	0.2	133
Desacetyllanatoside C	2.0	96	0.2	152
12-Epi-digoxin	25.6	7.5	2.8	11.3
12-Dehydrodigoxin	160.5	1.2	10.0	3.2
12-Acetyldigoxin	315.9	0.6	43.4	0.7
Diginatin	5.9	32	0.8	41
Digitoxigenin	45.4	4.2	5.0	6.4
Evatromonosid	45.5	4.2	4.6	7.0
Digitoxigenin-bis-digitoxoside	31.5	6.1	7.7	4.1
Digitoxin	48.4	4.0	8.6	3.7
Acetyldigitoxin	29.7	6.5	9.6	3.3
Gluco-evatromonoside	19.5	9.8	9.6	3.3
Gluco-digitoxigenin-bis-digitoxoside	18.2	10.5	8.4	3.8
Desacetyllanatoside A	37.7	5.1	14.1	2.3
Lanatoside A	23.5	8.2	4.4	7.3
Odorobioside G	23.7	8.1	7.0	4.5
Neo-odorobioside G	94.8	2.0	35.9	0.9
Glucodigifucoside	30.8	6.2	19.2	1.7
Neo-glucodigifucoside	73.3	2.6	26.1	1.2
Gluco-digitoxigeninglucomethyloside	20.5	9.4	13.5	2.4
Odoroside H	48.7	3.9	14.6	2.2
*Digitoxigenin glucomethyloside	16.1	11.9	10.6	3.0
16-Anhydrodigitoxigenin	13400	~0	753	0.04
Gitoxigenin	1670	0.1	179	0.2
Gitoroside	384	0.5	184	0.2
Gitoxin	84	2.3	20.5	1.6
β-Acetylgitoxin	237	0.8	91	0.3
Gluco-gitoroside	337	0.6	198	0.2
Lanatoside B	28.4	6.8	3.1	10.3
Digitalinum verum	813	0.2	121	0.3
Neo-digitalinum verum	1010	0.1	266	0.1
Strospeside	1363	0.1	327	<0.1
16-Acetylgitoxin	1458	0.1	1130	0.03
Oleandrigenin	23100	~0	2315	~0
Gitaloxigenin	1914	<0.1	4545	~0
Gitaloxin	6700	<0.1	612	0.05
Verodoxin	969	0.2	190	0.2
Lanadoxin	492	0.4	397	0.08
Gluco-lanadoxin	76	2.5	9.7	3.3
Gluco-verodoxin	310	0.6	102	0.3
Strophantidin	606	0.3	69	0.5
Cymarin	493	0.4	160	0.2
Helveticoside	476	0.4	13.0	2.5
Ouabain	>1668	0.1	5128	~0
Uzarigenin	61.5	3.1	10.7	3.0
Uzarigenin canaroside	83.2	2.3	107	0.3
Uzarigenin digitoxoside	83.2	2.3	83.2	0.4
Canarigenin	62.7	3.1	21.0	1.5
Canarigenin canarobioside	72.1	2.7	22.6	1.4
3,5-Dianhydroperiplogenin	452	0.4	181	0.2
Hellebrin	1381	0.1	251	<0.1
Scillarenin	216	0.9	36.2	0.7
Scillarene A	303	0.6	310	<0.1
Proscillaridine A	311	0.6	543	≪0.1
12β-Hydroxiscillarenin	22.5	8.5	3.25	7.7
Digifolein	≫8500	~0	≫8500	~0
Lanafolein	≫7000	~0	≫7000	~0
Tigogenin	≫7700	~0	≫7700	~0
Digitonin	≫4700	~0	≫4700	~0

Table 2—*continued*

Substance	Digoxin-[G-^{3}H] and dextran-coated charcoal pmol (B/Bo = 50%)	CR (%)	Digoxin-[^{125}I]-tyrosine methyl ester and $(NH_4)_2SO_4$ pmol (B/Bo = 50%)	CR (%)
Gitogenin	⩾7900	~0	⩾7900	~0
Lanosterol	⩾8200	~0	⩾8200	~0
Cholesterol	⩾12000	~0	⩾12000	~0
Sitosterol	⩾9200	~0	⩾9200	~0
Stigmasterol acetate	⩾5100	~0	⩾5100	~0
Pregnenolone	>14200	~0	>14200	~0
Progesterone	>11000	~0	267	0.09

*Substance chromatographically impure.

gin in order to gain knowledge about the occurrence of nonspecific interfering material and/or factors.

Figure 3 shows the result of this study with the digoxin directed RIA using 250 species of 80 families of non-cardenolide plants (ranging from liverworts to orchids), 8 species of plants producing cardenolides but not the *Digitalis* glycosides, 46 samples of 25 species of *Digitalis* as well as several *D. lanata* plants of different origins. All plants which did not belong to the genus *Digitalis* showed values clearly lower than the average detection limit of the RIA. Inclusion of known amounts of digoxin to these extracts gave 100% recovery, proving that the presence of inhibitors for the RIA can be excluded. All *Digitalis* species (except for *D. lanata*) showed, on an average, 0.32 μg digoxin equivalents/per leaf unit. This low value is slightly above the detection limit of the assay and probably has to be attributed to cross reactivity of cardenolides which do not contain a β-OH group at the C_{12} position. In contrast, leaf extracts of 1-yr-old, fully developed *D. lanata* plants show average values of 2 μg digoxin equivalents/leaf unit. Industrially-used, inbred plants gave an average of 3–5 μg/leaf unit. From this latter population, individual plants were found which gave exceedingly low (1 μg/leaf unit) or high (12 μg/leaf unit) digoxin values.

It should be noted that serial dilutions of leaf extracts of *D. lanata* covering the entire range of the standard curve yielded values exactly paralleling those of the standard curve. This demonstrates that a linear relation exists between the extract volume used for RIA and the digoxin value measured; thus from each diluted sample the same concentration of reactive material within the plant could be calculated. This again shows the high specificity of the antibody used.

Correlation of RIA and chromatographic method

In order to prove that the radioimmuno-reactive material was actually due to digoxigenin glycosides, a quantitative comparison was made between the RIA and the conventional chromatographic method for determination of *Digitalis* aglycones [11]. Plant leaves were harvested and dried. Aliquots of the drug were extracted for RIA determination or hydrolysed. The hydrolysates were separated by TLC and the quantity of each aglycone was determined fluorimetrically. The results of these experiments using 8 or 30 individual plants are shown in Table 3.

It is clearly and convincingly shown, that a close correlation exists between digoxin or digoxigenin content of the extract assayed by either method. The correlation coefficients [12] comparing RIA and fluorimetry were determined to be $r = 0.989$ (experiment 1) and $r = 0.902$ (experiment 2). It should be stressed that those individual plants with the highest digoxigenin glycoside concentration used for experiment 1 were selected from a random population using the RIA method. Fluorimetry has, in each case, confirmed this selection based on RIA determination. Conformity of values determined by the RIA method, with the actual concentration of digoxin equivalents within the plant, is therefore proven. It can now be concluded that the RIA method can be used successfully for the selection of plants with a desired high or low content of a given plant product, and should, therefore, prove very valuable for plant breeding and tissue culture work.

Distribution of digoxigenin-glycosides in D. lanata

Due to the extreme sensitivity of the assay it was also possible to investigate the distribution of digoxigenin glycosides within single seeds which are only 1 mm in length. Seedcoat, endosperm and embryo were analysed separately. Analysis of 10 seeds of 2 different origins showed (Fig. 5) that more than 80% of the digoxin equivalents of the whole seed are located in the testa, 5%

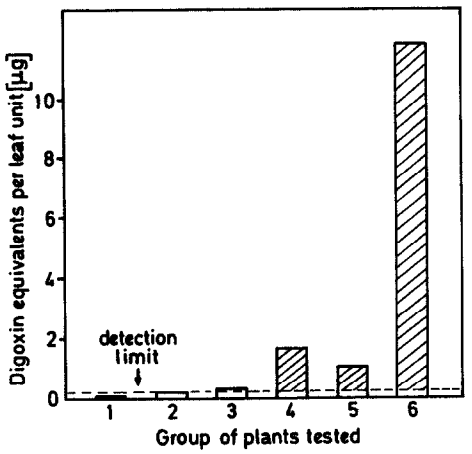

Fig. 3. Radioimmunologically determined digoxin values in plant material of different origins: 1 = plants of different systematic categories known to contain no cardenolides (250 species); 2 = cardenolide producing species other than *Digitalis* (9 species); 3 = *Digitalis* species other than *D. lanata* (25 species, 46 origins); 4 = *Digitalis lanata*, wild population (Turkey); 5 = *Digitalis lanata*, selected plants with low digoxin values: 6 = *Digitalis lanata*, selected plants with high digoxin values. Of each species, two (group 1 + 2), four (group 3 + 4) and five (group 5 + 6) individual plants were tested.

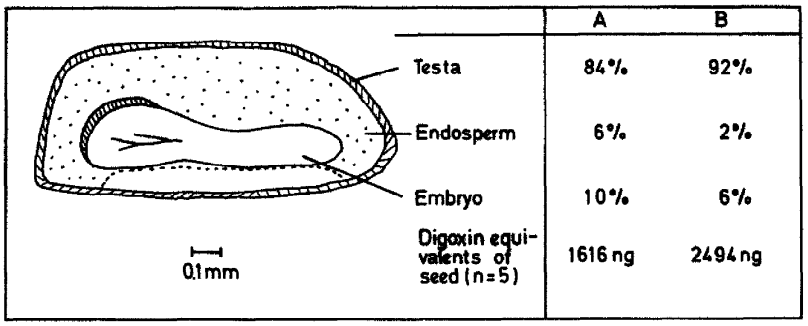

Fig. 4. Distribution of digoxin equivalents in seeds of two origins. Note that, despite quite different total amounts, the distribution of digoxin equivalents within the seed is rather similar.

Table 3. Correlation data for fluorimetric and radioimmunoassay analyses

	Experiment 1 $n = 8$		Experiment 2 $n = 30$
	RIA drug	RIA leaf discs	RIA drug
Fluorometry Digoxigenin	$r = 0.989$	$r = 0.998$	$r = 0.907$
Fluorometry Digoxin	$r = 0.978$	$r = 0.973$	$r = 0.864$
RIA drug	—	$r = 0.987$	—

Collected from two different experiments with (1) $n = 8$ RIA-selected plants. Leaf discs and leaf material for drug preparation was harvested at the same time. (2) $n = 30$ drug specimens collected at random. The fluorometric determination of digoxigenin was performed as described in the Experimental and the digoxin measurements were conducted using published methods [11]. r = correlation coefficient.

in the endosperm and 8% in the embryo (2–3 pmol digoxin equivalents per total seed).

Analyses of cotyledon, hypocotyl (with plumule) and primary root of 14-day-old seedlings of two origins showed that about 80% of digoxin equivalents are located in the cotyledon, 15% in the hypocotyl and 7% in the radicle. An absolute amount of 0.35–0.50 pmol has been found in the seedlings as compared to about 0.20 pmol in the embryo. This means that digoxin glycoside synthesis has occurred in the seedling and the content of the embryo has doubled 14 days after germination. It is noteworthy that the empty seed coat still contained nearly the amount of digoxin equivalents which had been present originally in the ungerminated seed. Translocation from the testa into the seedlings has, therefore, not taken place.

A complete analysis of a 2-month-old plant is shown in Fig. 6. The average content of digoxigenin glycosides increases with the age of the leaf; however, a small but consistent drop is found in very old leaves. The apical region shows a slightly higher content of β-C_{12}-OH compounds than the youngest leaves. Within a given leaf, the relative distribution of digoxin equivalents, independent of its age, is the same. The glycosides are concentrated in the green parenchymatous parts of the lamina and are found in highest concentration in the distal third and the marginal parts of the leaf. The glycoside content drops near the central vein and also toward the base of the leaf. No detectable amounts are found within the stem and the whole root system.

Lanatoside C and its secondary glycoside, digoxin, are extracted from mature leaves harvested in the fall of the first year of growth. A single mature leaf was cut into 200 pieces of about equal size; each piece was extracted and the digoxin equivalents determined by RIA. The resulting pattern is shown in Fig. 7. The relative distribution is identical to the leaves of a 2-month-old plant; however, the absolute content is much higher. For selection work, leaf samples can be removed from the distal third of the leaf between the leaf margin and the peripheral vein. The digoxin value thus obtained is a direct measure of the content of the leaf drug obtained after harvest.

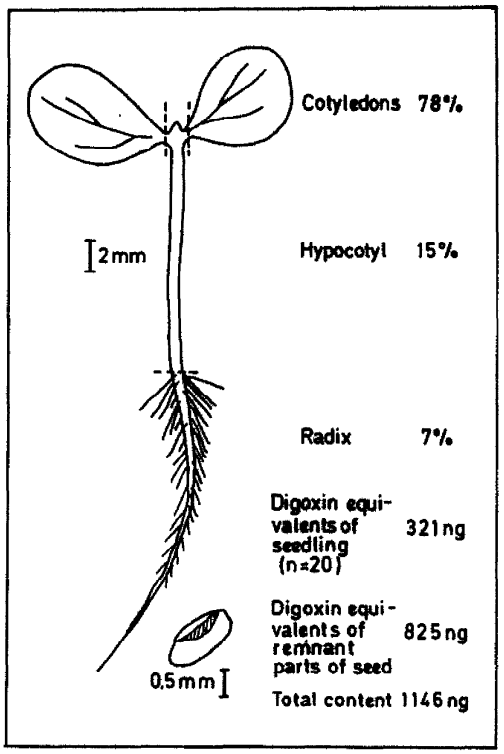

Fig. 5. Distribution of digoxin equivalents in 14 day old seedlings of *Digitalis lanata* and remaining parts of seed after germination. The % values are given on the basis of total digoxin equivalents of the seedling.

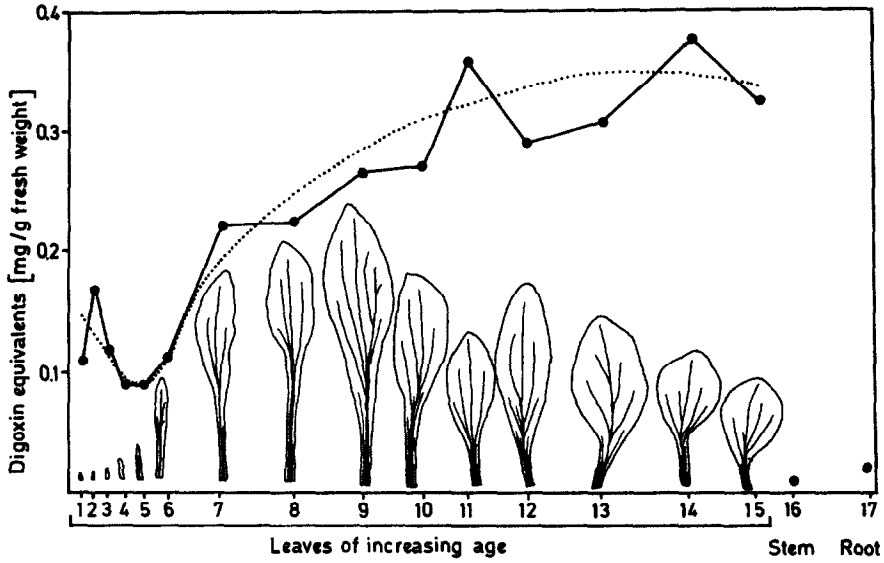

Fig. 6. Distribution of digoxin equivalents within a 2-month-old plant of *D. lanata*.

DISCUSSION

A special requirement for the successful use of a radioimmunoassay in complex plant extracts is the availability of a highly specific antibody. It has been shown here that an antibody directed against digoxin is specific for the recognition of a β-OH group at carbon atom 12 of the cardenolide moiety. For example epimerisation of the β-OH group into the α-position drastically decreases the cross reactivity to 1/13 in 12-epidigoxin. The site of highest antibody specificity is diametrically opposed to the site at which the haptene is bound to the protein carrier. This observation is in agreement with results obtained with other antigens [10, 13]. The nearer the site of coupling, the less alternation in the molecular structure of the haptene is recognized by the antibody. Thus a change of the five membered lactone ring of the cardenolides to the six membered ring of the bufadienolides leads to a lowering of the cross reactivity to 1/50 that of digoxin. Changes at ring A or B of the basic skeleton of the steroid are not recognized, even if these changes cause a drastic stereo-chemical modification. Glycosides of the same aglycone cannot be distinguished, and the aglycones generally show slightly higher reactivity on a mass basis. It can, therefore, be concluded that the RIA described here allows a highly specific and extremely sensitive determination of digoxigenin and its glycosides in crude plant extracts. The cross reactivity of cardenolides not hydroxylated at C_{12} position is not a serious handicap and yields only slightly increased background values.

The radioimmunoassay offers a series of advantages over present, conventional analytical methods [11, 14–18] for the quantitative determination of *Digitalis* glycosides. Especially for screening problems, the RIA seems extraordinarily useful, since it permits the quantitative determination of large numbers of samples. With automation of the assay, one person can analyse 2000 samples within 5 days. Using this method it is also possible to select interesting types of digoxigenin glycoside-containing plants from large plant populations. Due to

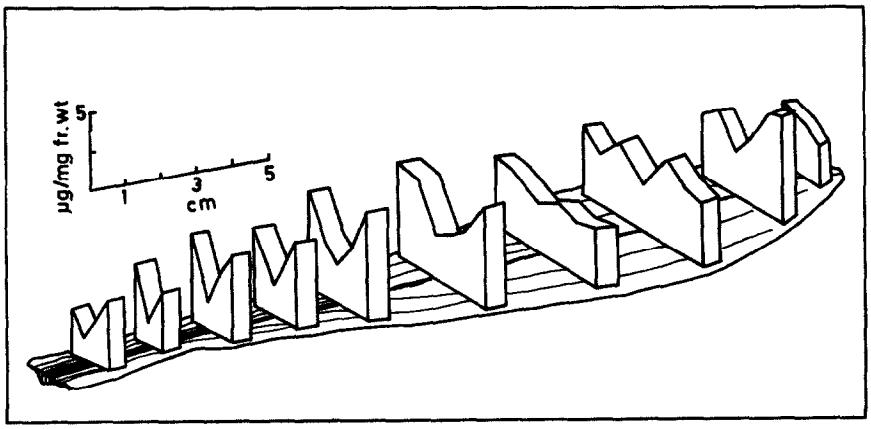

Fig. 7. Distribution of digoxin equivalents within a fully matured leaf of a 1-yr-old *D. lanata* plant ready for harvest. For better visualization, the graph depicts only characteristic zones of the distribution. The entire leaf was measured by cutting the leaf into 200 pieces for individual analysis.

the high sensitivity of the assay, analysis of a small sample (3 mm leaf disc) is sufficient. In theory, the content of one single cell, containing about 0.15% of digoxigenin glycosides, is sufficient to be measured accurately by this method.

Careful comparison of the values determined with the RIA system and by chromatographic and fluorimetric analysis of leaf drugs showed a close correlation between both methods. It should be stressed, however, that the RIA is specific for the whole digoxigenin glycoside population. For the selection of industrially used lanatoside C- or desacetyl-lanatoside C-containing plants, the plants selected by the above RIA method must be evaluated chromatographically to determine whether undesirable compounds e.g. digoxigenin-bis-digitoxoside, are present.

Three variations of the RIA method have been compared. The best method for plant screening proved to be the use of ^{125}I-labelled digoxin as tracer and ammonium sulfate for the separation of bound and free antigen. Ammonium sulfate precipitation is not time dependent, thereby allowing the simultaneous work-up of large numbers of samples. The use of γ-labelled antigen has the advantage of higher sensitivity, shorter counting time and avoidance of costly scintillators, thus compensating for the short half-life of the tracer.

The high sensitivity of the assay (45 pg digoxin per test corresponding to 6×10^{-14} mol) which can be achieved only by radioimmunoassay, enables the use of the method in areas of plant sciences which have not yet been entered due to the lack of adequate analytical methods.

EXPERIMENTAL

Materials. Human and bovine serum albumin were obtained from Serva, calf serum from Behringwerke, Marburg. Freund's complete adjuvant was supplied by Difco. Norit A charcoal, radioimmunoassay grade, was from Schwarz/Mann. Digoxin-[G-^{3}H] was obtained from NEN (NET-222 sp. act. 9 Ci/mol), digoxin-[^{125}I]-tyrosine methyl ester was purchased from Wellcome Reagents Ltd. (sp. act. 500 Ci/mol). The scintillator used was Unisolve 1 from Koch-Light. (All other reagents used were of highest purity commercially available). The steroids used were kind gifts of Dr. F. Kaiser, Mannheim, Prof. K. Meyer, Basel, and Prof. R. Tschesche, Bonn. Digoxin was purchased from Merck and the stock soln contained 10 μg/ml dissolved in 35% EtOH.

Plant extracts. Freshly harvested leaves or leaf disc (0.3 cm ϕ, called "leaf-units") were extracted with 5 ml 80% EtOH for a period of 4 hr at 74° in a closed water-bath. These extracts were then diluted with H_2O between 10- and 1000-fold for the RIA. *D. lanata* drug was prepared by drying fresh leaves at 60° for 48 hr. The leaves were subsequently powdered in a mortar.

Immunization procedure. Digoxin was coupled to human serum albumin using the method of Smith *et al.* [3]. 3.6 mol of digoxin were coupled to 1 mol of serum albumin as determined by spectroscopy. 4 mg of the lyophilyzed conjugate was dissolved in 1 ml H_2O and emulsified with 1.5 ml complete Freund's adjuvant. Rabbits were immunized by one injection of 0.4 ml freshly prepared antigen emulsion into the foot pads followed by weekly intradermal injections. Antisera were obtained, usually 8 weeks after the first injection, by serial bleedings of the animals. The best sera were pooled and stored at −18°. The sera showed no change in activity or specificity within a period of 3 y.

Radioimmunoassay procedure. The following reagents were pipetted into a test-tube in an icebath: 0.5 ml buffer (0.01 M KPi, 0.15 M NaCl, pH 7.4) 0.1 ml 1% bovine serum albumin, 0.1 ml tracer (11 nCi digoxin-[G-^{3}H] corresponding to 1.2 pmol, or 13000 cpm digoxin-[^{125}I]-tyrosine methyl ester corresponding to 0.025 pmol, and 0.1 ml digoxin standard or plant extract in an appropriate dilution. Blanks for determination of non-specific binding contained 0.1 ml dil solvent. Components were mixed and 0.1 ml antibody soln added. After mixing, the reaction was started by raising the temperature to 24°. After 30 min equilibrium was achieved. Samples were cooled in ice and 0.5 ml of a cold dextran-coated charcoal suspension (prepared by freshly diluting a stirred suspension of 62.5 mg dextran and 2.5 g charcoal in 25 ml buffer pH 7.4 twofold with that buffer) was added, the contact time being 10 min at 0°. Within 60 min at 0° no dissociation of the antigen–antibody complex could be observed; at 22°, however, after being in continous contact with charcoal, a 36% dissociation of the complex was noted. Therefore, sufficient cooling of the incubation mixture was necessary. Separation of free and antibody-bound haptene was achieved by centrifugation for 10 min at 2700 rpm in a labofuge II, and the supernatant, containing the bound digoxin, was decanted; in the case of the ^{3}H-haptene, into a scintillation vial containing 10 ml scintillator (Unisolve 1) and counted in a scintillation counter. For iodinated haptene, incubation time was increased to 2 hr. After removal of charcoal, the supernatant was decanted into a polystyrene vial and counted in a γ-spectrometer, equipped with a 2″ × 2″ NaI crystal. Data were collected with a programmable on-line calculator type alphatronic (Diehl). For the semi-automated assay an Analmatic preparation unit (Searle Co.) was used for pipetting. Since difficulties were encountered pipetting the charcoal suspension with motor driven glass-pipettes, 1 ml freshly prepared $(NH_4)_2SO_4$ soln (10 parts saturated soln $(NH_4)_2SO_4$ and 1 part H_2O) was added to give a final dilution of 48% $(NH_4)_2SO_4$. Instead of 0.1 ml 1% bovine serum albumin, 0.1 ml of a 1:10 diluted calf serum preparation was added to facilitate precipitation of the antigen–antibody complex, the free digoxin staying in the supernatant. Centrifugation was done at 4000 rpm. 1.5 ml supernatant was removed and added to together with 2 ml H_2O into the counting vial. An important facilitation was the fact that using the ammonium sulfate precipitation, all steps of the RIA could be conducted at room temp, and the timing of the separation step was not critical, since, within 1 hr. an increase of the *Bo* value (% binding of tracer in absence of unlabelled haptene) of only less than 3% was observed; thereafter the value remained constant for at least 20 hr.

Chromatographic procedure. For the correlation experiments to compare RIA with the conventional chromatographic estimation of *Digitalis* cardenolides, 0.5 g of dry leaf drug and 5 mg standard digoxin were used. Preparation (a): The samples were extracted with 25 ml MeOH for 30 min under reflux. For the radioimmunoassay, aliquots of these extracts were diluted 1:10000 with H_2O. Preparation (b): To the sample was added 25 ml MeOH and 25 ml 0.1 N H_2SO_4 and the mixture heated under reflux for 30 min [19]. After cooling and filtration the extract was brought to pH 7.0 by addition of 1 N KOH and saturated $NaHCO_3$ then extracted with $CHCl_3$(40 and 20 ml). $CHCl_3$ extract was dried and solvent removed under vacuum. Residue was taken up in 25 ml MeOH. For RIA, an aliquot, which was diluted 1:10000, was used. Digoxigenin was determined by TLC using Si gel. 20 μl of the extract was streaked (1 cm) on the plate and internally standardized. The plate was developed in $CHCl_3$–MeOH (9:1) [20], dried and sprayed with chloramine-T-reagent. After activation of the plate for 15 min at 110° the fluorescence was determined with a Camag-T-Scanner. Under these conditions, the standard curve was linear in a range from 1 to 7 μg digoxigenin.

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