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A NEW THIN-LAYER DENSITOMETRIC TECHNIQUE FOR THE ASSAY OF CARDENOLIDES FROM *DIGITALIS PURPUREA*

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SUMMARY

A new thin-layer densitometric analysis is described for the estimation of A and B series glycosides of *Digitalis purpurea* as their respective aglycones. The method utilises the colour reaction with antimony trichloride after heating. One of the compounds to be estimated, gitoxigenin, was added as an internal standard for peak area ratio estimations by means of the Chromoscan densitometer. The method has a coefficient of variation of 4% and is compared to official colorimetric and biological assays.

INTRODUCTION

The convenience and sensitivity of estimation of solutes directly on chromatograms makes thin-layer densitometry an attractive method for quantitative microanalysis. Traditionally calibration curves are obtained for each of the compounds of interest. These may be direct plots of peak height or area, obtained from the densitometric trace, against concentration, producing a curved plot, or some function of area and concentration to obtain a linear regression[1]. This method may be subject to some variation between replicate analysis as has been fully discussed by Shellard[2]. We describe a more practical method which utilises the principle of internal standardisation and the measurement of peak area ratios to eliminate some sources of error incurred by the direct use of area–concentration regression lines.

The method has been applied to the analysis of cardenolides from *Digitalis purpurea*, at present assayed biologically[3]. Differential analyses of the glycosides have been published which involve paper chromatographic[4,5] and thin-layer chromatographic (TLC)[6] separation before densitometry. However, *D. purpurea* contains about 27 cardiac glycosides making it difficult to estimate individual compounds on chromatograms. In the European Pharmacopoeia[7] method the glycosides are hydrolysed to digitoxigenin and gitoxigenin before colorimetric analysis. We have followed this principle estimating the two steroids on TLC plates by densitometry.

EXPERIMENTAL

Determination of the relative weight response of digitoxigenin to gitoxigenin

Five solutions of cardenolides of varying weight ratios (Table I) were used. Thin layers (300 μm thick) of silica gel H were spread on glass plates (2.5 cm × 20 cm) and activated at 100° for 15 min. A 5-μl aliquot of redistilled tetrahydrofuran solution of cardenolides was applied by means of a scientific glass engineering microsyringe.

TABLE I

DETERMINATION OF RELATIVE WEIGHT RESPONSES OF DIGITOXIGENIN TO GITOXIGENIN

Mean relative weight response, 0.657; standard deviation, 0.0267; coefficient of variation, 4.06%.

Weight ratio	*Area ratio*	*Number of determinations*
0.477	0.3164	15
0.989	0.6489	14
1.525	1.046	16
2.101	1.360	15
3.055	1.924	15

The plates were developed 15 cm with dry ethyl acetate and visualised with 10% antimony trichloride in benzene by heating at 120° for 20 min. The peak areas were determined at 620 nm on the Chromoscan densitometer (Joyce, Loebl & Co.) by scanning in the direction of solvent flow through the centre of each spot using a 1-mm slit width. Areas were calculated as the product of height and width at half height.

Extraction of crude drug material

About 10 g of powdered crude drug accurately weighed was extracted with 70% ethanol in an ultrasonic disintegrator for 45 min. 10 ml of a strong solution of lead subacetate were added and the mixture was shaken. This was followed by addition of 40 ml of 6.3% sodium sulphate solution to remove excess lead. The mixture was shaken and filtered through a büchner using Celite 545 as a filter aid. The filtrate was reduced to 30 ml and the glycosides extracted with 20 ml of ethyl acetate. The ethyl acetate fraction was cooled on ice and shaken with 5-ml portions of 2 *N* sodium hydroxide at 2–4°, washed with ice-cold water until neutral and residual moisture removed over anhydrous sodium sulphate.

Hydrolysis of cardiac glycosides

Hydrolysis was effected by the method of Frey and Jacobson[8] using 0.002 *N* perchloric acid in redistilled tetrahydrofuran. The residues produced after removal of solvent were dissolved in 1.0 ml of tetrahydrofuran and the solution divided into two equal parts. Gitoxigenin (0.2 mg) was added to one portion as an internal standard. 5-μl aliquots of both standard and test solutions were applied to the plates as before.

Calculation of the proportions of cardenolides of the A and B series

For a chromatogram spotted with crude drug extract one can write

$$\frac{A_d}{A_g} = R \cdot \frac{W_d}{W_g} = A_R \qquad (1)$$

where A_d = area of digitoxigenin peak,
A_g = area of gitoxigenin peak,
W_d = weight of digitoxigenin in 0.5 ml of solution,
W_g = weight of gitoxigenin in 0.5 ml of solution,
R = relative weight response of digitoxigenin to gitoxigenin,

A_R = ratio of the peak areas, $\frac{A_d}{A_g}$.

For a chromatogram spotted with extract containing added gitoxigenin

$$A'_R = R \cdot \frac{W_d}{W_g} + Z_g \tag{2}$$

where A'_R = new peak area ratio, and Z_g = weight in mg of gitoxigenin added to 0.5 ml of extract. Substituting for R in eqn. 1 yields:

$$W_g = Z_g \cdot \frac{A'_R}{A_R - A'_R} \tag{3}$$

and further, when W_g has been calculated, W_d can be obtained from eqn. 1.

For extracts which contain only one component the equation becomes:

$$A'_R = R \cdot \frac{W_d}{Z_g} \tag{4}$$

Colorimetric analysis

Samples were analysed as described in the European Pharmacopoeia[7] using a Hilgar & Watts spectrometer. The total aglycone content was measured at 540 nm ($E^{1\%}_{1\,cm} = 107$) and gitoxigenin measured at 592 nm ($E^{1\%}_{1\,cm} = 720$). The total aglycone content calculated as digitoxigenin was also obtained by the method of Rowson[9–11].

RESULTS AND DISCUSSION

For the analysis of the A and B series glycosides as digitoxigenin and gitoxigenin they must be quantitatively hydrolysed to the true genins. When a 2-deoxy sugar such as digitoxose is attached to the steroid, mild hydrolysis conditions[12] may be used, but with a 2-hydroxy sugar such as digitalose more vigorous conditions are required producing C_{14} anhydro genins. We have therefore investigated traditional methods[13,14] by means of TLC and it proved impossible to determine a quantitative end point. Trace amounts of Δ^{14}-anhydrodigitoxigenin and Δ^{14}-anhydrogitoxigenin were produced before all the glycosides were hydrolysed. A recent method[8] was also investigated and demonstrated that primary and secondary cardiac glycosides could be converted to their true genins within 50 min at 50°. The sequence was temperature, concentration and acid-strength dependent. For the assay of plant samples, 0.002 *N* perchloric acid in fresh redistilled tetrahydrofuran at 50° was heated for 50 min with a total glycoside concentration of about 10 mg%.

When sprayed with antimony trichloride and heated, digitoxigenin produced

a blue colour with a maximum light reflectance at 620 nm and gitoxigenin a green colour with maximum reflectance at 403 nm. The colours were produced against a white substrate background and both were suitable for analysis. In the case of gitoxigenin the reflectance at 620 nm was strong enough to enable this wavelength to be used for both spots, thereby eliminating the problem of changing filters during the analysis. On heating the colour for both steroids developed evenly and reached a maximum at 120°. Heating beyond this point produced a charred effect due to decomposition of the coloured complex. The colour was stable in the dark for up to 30 min, and a standard cooling time of 20 min was allowed in the dark before readings were taken on the densitometer. Using these conditions the relative weight response of digitoxigenin to gitoxigenin was found to be 0.657 with a coefficient of variation of 4% for 75 determinations (Fig. 1). This analysis which employs an internal standard is

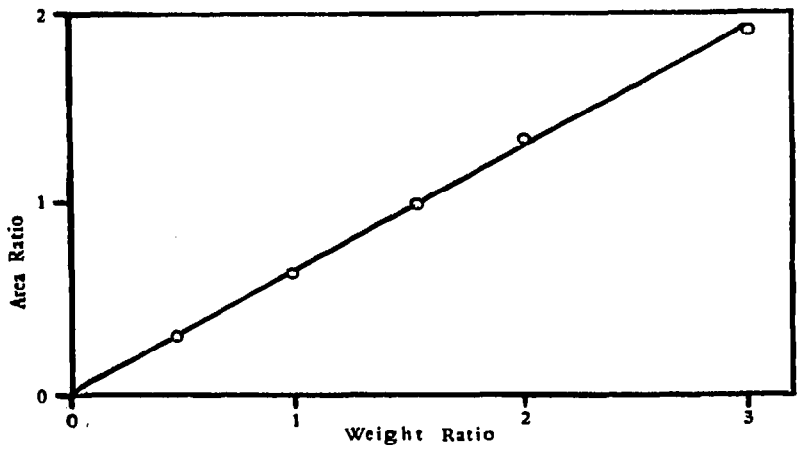

Fig. 1. Determination of the relative weight response of digitoxigenin to gitoxigenin.

independent of the volume of solution applied (eqn. 3) and because area ratios are estimated on the same plate, replicate analyses are also less sensitive to plate-to-plate variation due to differences in chromatographic conditions. The internal standard used was gitoxigenin which was one of the two compounds to be estimated, thereby eliminating the difficulty of finding a standard which reacts with the colour reagent and migrates in the solvent system used. It also has the advantages that only one set of determinations for the relative weight response (R) needs to be made and that the linear weight–area regression is similar for standard and test.

Recovery experiments on two gitoxigenin samples gave values of 97.44 and 98.26% respectively, each for 25 determinations (Table II). When applied to the analysis of *D. purpurea* of English and Dutch origin, the reproducibility was within 7% (Table III). Errors were due mainly to spot diffusion during the chromatographic elution, resulting from slight variations in layer thickness. This could be reduced still further by measuring the colour intensity of the whole solute spot with a more modern densitometer rather than by taking a 1-mm strip through the spot centre. Two pharmaceutical preparations of Digitalis were examined by the TLC method and compared to existing colorimetric methods (Table IV). All three methods examined gave results

TABLE II

ESTIMATION OF STANDARD GITOXIGENIN SOLUTIONS

Gitoxigenin weighed (mg)	*Gitoxigenin estimated (mg)*	*Percentage error**
0.860	0.838	2.56
1.376	1.352	1.74

* Mean of 25 determinations.

TABLE III

DENSITOMETRIC ANALYSIS OF *D. PURPUREA*

	Total aglycones (μg/g dry wt.)*	*Total aglycones (μg/g fresh wt.)*	*Digitoxigenin (μg/g)*	*Gitoxigenin (μg/g)*
English leaf	318		185	133
	354		201	153
English seed	302		205	97
	291		182	109
	300		193	107
	270		171	99
Dutch plants				
1st-year leaf		116	75	41
2nd-year leaf		55	25	30
Flowers		93	31	62
Roots		20	13.4	6.6
Seeds		480	282	198

* Mean of 5 estimations.

TABLE IV

COMPARISON OF ASSAY METHODS

E.P. = European Pharmacopoeia; B.P. = British Pharmacopoeia.

Total aglycones (μg/g dry wt.)*		*Digitoxigenin (μg/g)*		*Gitoxigenin (μg/g)*	
E.P.	*Rowson*	*E.P.*	*Densitometer*	*E.P.*	*Densitometer*
Digitalis leaf B.P. 8.11 units/g by biological analysis					
198.4	234	103.9	194	95	Nil
196.3	231	102.3	193	94	Nil
172.8	202	72.6	171	100.2	Nil
Digitalis tablets B.P. 10.00 units/g by biological analysis					
202.4	213	79.8	209	122.6	Nil
236.0	223	107.6	216	128.4	Nil
246.7	245	109.4	234	137.3	Nil
238.3	230	102.3	220	136.0	Nil

* Mean of 3 estimations.

which were well below the biological analysis, an observation reported by Rowson[9]. For both the European Pharmacopoeia[7] method and the TLC method, however, the ratio leaf:tablets for total aglycones expressed as digitoxigenin was 0.820 and 0.840, respectively, which compares well with the ratio of 0.811 obtained by biological analysis. By means of TLC these samples were found to be deficient in B series glycosides, and gitoxigenin was absent in the chromatograms of the hydrolysed products. This was confirmed by eluting from TLC plates and obtaining mass spectra. The colorimetric analysis of the same extracts indicated that approximately 50% of the glycosides were of the B series, thereby demonstrating the necessity for the TLC screening of crude drug extracts as a control in their evaluation.

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