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# AN IMPROVED METHOD FOR THE GAS CHROMATOGRAPHIC IDENTIFICATION OF *DIGITALIS* CARDENOLIDES

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SUMMARY

A simple and efficient method for the identification of steroid cardenolides as their "$\beta$"-anhydro derivatives has been developed, resulting in greatly reduced retention times and enhanced resolution. Retention data of eighteen cardenolides on three liquid phases are reported. Spectral evidence is presented showing that the tertiairy 14$\beta$-hydroxy group is neither affected by esterification nor etherification.

## INTRODUCTION

In connection with biogenetic studies of physiologically important natural products, the need arose for developing a reliable method for the detection of *Digitalis* cardenolides on a microgram scale, which may also be applied to radioactively labelled metabolites. Several colour reactions with 3,5-dinitrobenzoic acid[1–3], picric acid[4–8], *m*-dinitrobenzene[9] and 2,4,2',4'-tetranitrodiphenyl[10] are known for the characterisation of the free genins or their glycosides on thin-layer and paper chromatography[11]. However, none of these reactions can be regarded as specific. The criteria for a specific chemical reaction must be based upon the salient features of the compounds to be investigated.

The socalled cardioactive steroids are distinguished by the presence of a tertiairy hydroxy group at the C-14 ring junction. The pseudoaxial configuration of the alcohol group makes this class of naturally occurring compounds unique among the steroids. Together with the conjugated five membered lactone ring in the case of the cardenolides, or the doubly conjugated six membered pyrone ring in the case of the bufadienolides, this oxygenated substituent defines the strong biological activity of the cardiotonic steroids. We have thus focussed our attention to the 14$\beta$-hydroxy group. In particular its lability caused by 1,3-diaxial interaction with the 17$\beta$-butenolide moiety, and its tertiairy nature. Our approach consisted in utilising this very instability as a point of attack.

Because of its high sensitivity, reproducibility and resolution, as well as its

applicability for the measurement of labelled steroids, gas–liquid chromatography[12] appealed to us as the analytical method of choice.

## EXPERIMENTAL

### *Materials and methods*

Digitoxigenin, gitoxigenin and digoxigenin were obtained from K & K Laboratories, Plainview, N.Y. Their purity was verified by TLC with two solvent systems, ethyl acetate and chloroform–methanol (9:1). All solvents were analytical grade and redistilled before use. Thionyl chloride was freshly distilled shortly before use. Bis-trimethylsilylacetamide, hexamethyldisilazane and trimethylchlorosilane were purchased from Pierce Chemical Company, Rockford, Ill. All silylation reactions were conducted in glass vials under nitrogen atmosphere. Melting points were determined on a Kofler hot stage under microscopic magnification and were not corrected.

### *Thin-layer chromatography*

TLC was performed on 4 × 20 or 20 × 20 cm glass plates, coated with 0.2 mm of Silica Gel $HF_{254}$ (E. Merck, Darmstadt). The spots were visualized either by spraying with KEDDE reagent[13] at room temperature, or with 50% aqueous sulphuric acid and heating at 110° for 2 min.

### *Infrared spectroscopy*

IR spectra were recorded on a Perkin Elmer 457 double beam grating spectrometer equipped with a beam condensor. Micro disks of 1.5 mm diameter were used. The potassium bromide pellets were dried under an IR lamp before measurement. Cardenolide–potassium bromide weight ratios were maintained at 1:100.

### *Gas–liquid chromatography*

A Hewlett-Packard F & M, Model 402 high efficiency gas chromatograph, equipped with dual hydrogen flame ionisation detectors, was used. Column support consisted of 100–120 mesh silanized Gas-Chrom Q (Applied Science Laboratories, State College, Penn.). Three types of glass columns were used: column A, 90 × 0.4 cm, coated with 3% SE-30; columns B and C, 180 × 0.3 cm, coated respectively with 3% OV-1 and 3% QF-1. Operating conditions were: oven temperature 250°, injection port temperature 275°, detector temperature 285°, input attenuation 10, output attenuation 16. Helium carrier gas flow was held at 23 ml/min for column A and 33 ml/min for columns B and C at 40 p.s.i. inlet pressure. A Hamilton 10 $\mu$l syringe (Hamilton Company, Whittier, Calif.) was used for all injections.

### *Preparation of "β"-anhydrodigitoxigenin (3β-hydroxy-5β-carda-14,20; 22-dienolide) IIa*

A solution of 20 mg of digitoxigenin (*Ia*) in 0.6 ml of anhydrous pyridine was cooled in an ice bath. Under nitrogen atmosphere was added 0.2 ml of trifluoroacetic anhydride and the esterification allowed to proceed at room temperature for 2 h. The yellow coloured reaction mixture was then cooled again to 0° and a solution of 0.04 ml of thionyl chloride in 0.2 ml of anhydrous chloroform added. After 16 h at 4°, 2 ml of methanol was added, the crude reaction product taken up in 150 ml of a mixture of chloroform–ether (2:1). The organic extract was washed successively with dilute

hydrochloric acid, aqueous sodium hydrogen carbonate and water, dried over sodium sulphate and evaporated in vacuum to give 16 mg of crude *IIa*. Recrystallisation from acetone–hexane gave 11 mg of colourless needles, m.p. 199–201°. $\nu_{max(KBr)}$: 3500 and 1030 $cm^{-1}$ (–OH); 1775, 1735 and 1620 $cm^{-1}$ (–butenolide ring). A mixed melting point with authentic material* gave no depression and their IR spectra were superimposable.

*Preparation of "β"-anhydrodigitoxigenintrimethylsilylether (IId)*

*(A) From "β"-anhydrodigitoxigenin (IIa)*

*Procedure 1.* 2 mg of *IIa* was dissolved in 0.2 ml of anhydrous pyridine. Under nitrogen atmosphere was added 0.2 ml of N,O-bis-(trimethylsilyl)-acetamide (BSA), followed by two drops of trimethylchlorosilane (TMCS). After 2 h at room temperature, the mixture was blown dry with a stream of nitrogen, the residue dissolved in 1 ml of anhydrous dichloromethane and filtered through a 2 ml syringe, equipped with a Swinny adapter and a membrane filter. 1 to 2 μl of the clear filtrate was then used for each gas chromatographic analysis. Evaporation of the solvent with a stream of dry nitrogen gas gave colourless crystals of *IId*. $\nu_{max(KBr)}$: 1780, 1745 $cm^{-1}$; no –OH absorption bands.

*Procedure 2.* The same as procedure 1, except that the BSA reagent was replaced by an equal volume of hexamethyldisilazane (HMDS).

*(B) Directly in situ from digitoxigenin (Ia)*

2 mg of *Ia* was silylated as described above. The crystalline material obtained was redissolved in 0.2 ml of anhydrous pyridine and cooled to 4°. To the cooled solution was then added 0.4 ml of a freshly prepared mixture of thionylchloride–benzene–pyridine (1:15:5). Dehydration occurred within 15 min and the reaction mixture gave after injection a peak with the same retention time as *IId*, prepared by silylation of *IIa*.

*Preparation of digitoxigeninacetate (Ig)*

2 mg of *Ia* was acetylated overnight with equal (0.2 ml) volumes of anhydrous pyridine and acetic anhydride at 35°. Excess of anhydride was then destroyed by addition of 0.4 ml of methanol. The organic solvents were evaporated over a stream of nitrogen to give slightly coloured crystalls, m.p. 218–224° (ref. 14, reported m.p. 222–225°); $\nu_{max(KBr)}$: 3400, 1025 (OH), 1780, 1620 (lactone), and 1730, 1260 (acetate) $cm^{-1}$.

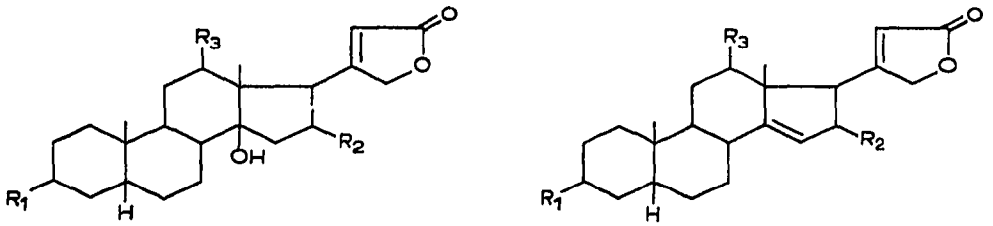
In analogous manner were prepared gitoxigeninacetate (*Ih*) and digoxigeninacetate (*Ii*).

*General procedure for the preparation of "β"-anhydro derivatives (IIa–i)*

For gas chromatographic analysis, compounds *IId–i* were prepared *in situ* directly from the corresponding 14β-hydroxylated parent cardenolides *Id–i*, by addition of the thionylchloride–benzene–pyridine reagent[15] at 4°, as described previously under procedure (B).

The cardadienolides *IIa*, *IIb* and *IIc* were prepared respectively from *Ia*, *Ib* and *Ic* via their TMS-derivatives, followed by dehydration with the thionylchloride–

* Kindly furnished by Prof. K. MEYER, University Basle, Switzerland.



(I) (II)

Fig. 1. Structural formula of the cardenolides studied.

| *No.* | *Name of parent cardenolide (I)* | *Substituents* |
|---|---|---|
| *a* | Digitoxigenin | $R_1$ = β-OH; $R_2$ = $R_3$ = H |
| *b* | Gitoxigenin | $R_1$ = $R_2$ = β-OH; $R_3$ = H |
| *c* | Digoxigenin | $R_1$ = $R_3$ = β-OH; $R_2$ = H |
| *d* | Digitoxigenin-TMS | $R_1$ = β-OTMS; $R_2$ = $R_3$ = H |
| *e* | Gitoxigenin-TMS | $R_1$ = $R_2$ = β-OTMS; $R_3$ = H |
| *f* | Digoxigenin-TMS | $R_1$ = $R_3$ = β-OTMS; $R_2$ = H |
| *g* | Digitoxigeninacetate | $R_1$ = β-OAc; $R_2$ = $R_3$ = H |
| *h* | Gitoxigeninacetate | $R_1$ = $R_2$ = β-OAc; $R_3$ = H |
| *i* | Digoxigeninacetate | $R_2$ = $R_3$ = β-OAc; $R_2$ = H |

benzene–pyridine reagent in the cold and subsequent hydrolysis of the TMS-group by treatment with 90% methanol at room temperature.

## RESULTS AND DISCUSSION

The gas chromatographic analysis of cardenolides and bufadienolides on a non-selective phase has been reported previously with more or less success[16–18]. Thus, on a 360 × 0.4 cm SE-30 column, JELLIFFE AND BLANKENHORN[16] found the trimethylsilyl (TMS) ethers of digitoxigenin (*Id*) and of digoxigenin (*If*) to exhibit retention times of 37.5 and 47.5 min respectively. Silylation of the 14β-hydroxy group in both compounds was thereby assumed. The validity of this critical postulate was not

TABLE I

IR ABSORPTION DATA OF SEVERAL CARDENOLIDES

| *Cardenolide* | $\nu_{max(KBr)}$ *in* $cm^{-1}$ | |
|---|---|---|
| | *OH-group* | *Butenolide and acetate groups* |
| *Ia* | 3400–3510, 1025 | 1780, 1725, 1620 |
| *Ib* | 3320–3480, 1030 | 1780, 1750, 1730, 1620 |
| *Ic* | 3400, 1030 | 1775, 1725, 1620 |
| *Id* | 3350, 1070, 1030 | 1780, 1750, 1650, 1620 |
| *Ie* | 3400, 1030 | 1780, 1720, 1655, 1620 |
| *If* | 3350, 1025 | 1730, 1660, 1620 |
| *Ig* | 3400, 1025 | 1780, 1730, 1620, 1260 |
| *Ih* | 3450, 1030 | 1775, 1730, 1620, 1260 |
| *Ii* | 3480, 1025 | 1780, 1725, 1620, 1245 |
| *IIa* | 3500, 1030 | 1775, 1735, 1620 |
| *IId* | no absorption | 1780, 1745 |
| *IIg* | no absorption | 1780, 1750, 1725, 1250 |

further clarified by the paper of WILSON, *et al.*[17]. Our spectral analysis (Table I) indicate beyond doubt that neither the bulky trimethylsilyl ether group, nor the smaller acetyl cation are able to attack the tertiairy C-14 alcohol substituent. All acetylated and silylated 14$\beta$-hydroxy cardenolides studied (*Id–i*), showed hydroxyl absorbtion. However, no hydroxyl absorbtion bands were observed with the spectra of those compounds, in which the 14$\beta$-OH group has been removed, *i.e. IId* and *IIg*. Although under more drastic conditions in the presence of a strong acidic catalyst, angular hydroxy groups at the steroid nucleus are known to be acetylated, for instance at position 17$\beta$ in 17$\alpha$-methyltestosterone[19] and 17$\alpha$-ethinyl-19-nortestosterone[20], and at the epimeric $\alpha$-position in 17$\alpha$-hydroxyprogesterone[21,22], in the case of the steroid cardenolides, approach to C-14 from the $\beta$-side of the molecule is severely hindered by the bulky 17$\beta$-butenolide moiety. Thus, in the presence of the strong reagent thionyl chloride, nucleophilic attack at the 15$\alpha$-hydrogen atom from the rear is favoured and the cardenolides undergo an elimination reaction with release of steric strain to yield derivatives containing a planar trigonal C-14 atom.

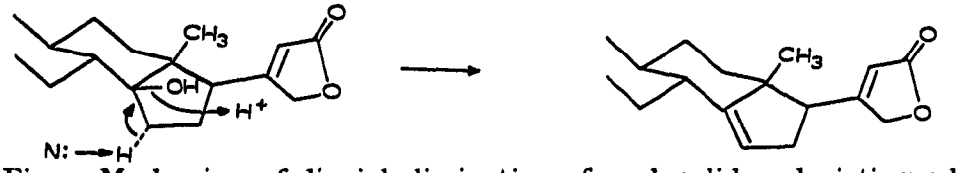



Fig. 2. Mechanism of diaxial elimination of cardenolides, depicting release of steric compression. N = nucleophilic species.

In our approach for a sensitive and specific identification method of *Digitalis*

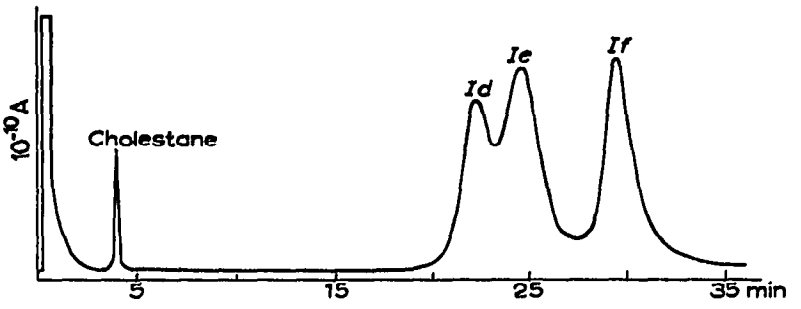
TABLE II

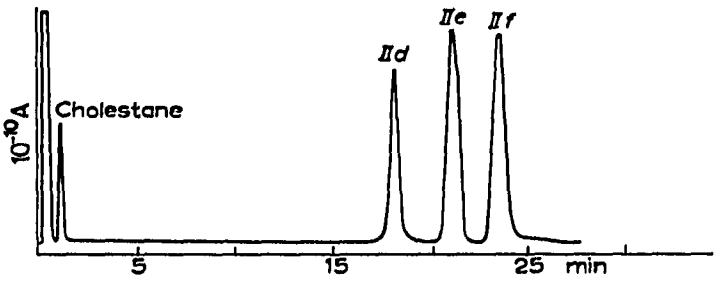
RETENTION TIMES, RELATIVE TO CHOLESTANE, CO-INJECTED AS AN INTERNAL STANDARD

| *Compound* | *Relative retention* | | |
|---|---|---|---|
| | *Column A (SE-30)* | *Column B (OV-1)* | *Column C (QF-1)* |
| Cholestane | 1.00 (2.56 min)[a] | 1.00 (4.35 min)[a] | 1.00 (1.08 min)[a] |
| *Ia* | 5.04 | 5.22 | 49.43 |
| *Ib* | 4.56 | 4.98 | 61.59 |
| *Ic* | 8.78 | 6.83 | 47.96 |
| *Id* | 5.02 | 5.19 | 39.85 |
| *Ie* | 5.84 | 5.69 | 46.52 |
| *If* | 6.12 | 6.69 | 42.69 |
| *Ig* | 6.39 | 6.61 | 102.00 |
| *Ih* | 6.03 | 6.26 | 85.71 |
| *Ii* | 9.24 | 9.00 | 138.76 |
| *IIa* | 3.13 | 3.18 | 18.64 |
| *IIb* | 1.92 | 2.02 | 12.07 |
| *IIc* | 2.51 | 2.66 | 9.66 |
| *IId* | 3.14 | 3.29 | 16.60 |
| *IIe* | 3.66 | 3.75 | 20.00 |
| *IIf* | 4.33 | 4.45 | 21.34 |
| *IIg* | 4.03 | 4.11 | 41.66 |
| *IIh* | 4.89 | 5.05 | 50.38 |
| *IIi* | 6.19 | 6.46 | 39.69 |

[a] Absolute retention time.

cardenolides, we have made use of the inherent instability, offered by the unique combination of a tertiairy C-14 hydroxy group and a C-17 lactone ring, present in a 1,3-pseudodiaxial steric relationship. The possibility of selectively acetylating or silylating only the secondary hydroxy groups at positions 3$\beta$, 12$\beta$ or 16$\beta$, while leaving the 14$\beta$-OH group intact, provided us with a facile method for preparing *in situ*, the desired $\Delta^{14(15)}$-cardadienolides *IIa–i*, suitable for gas chromatographic analysis.



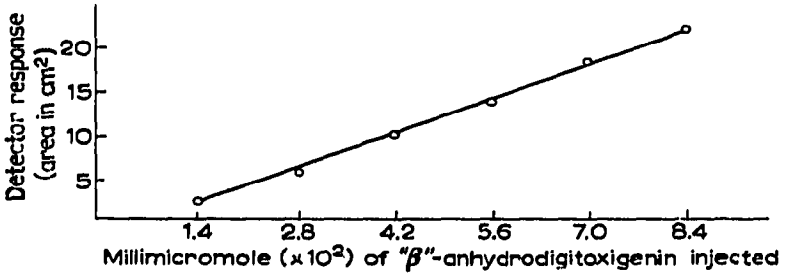




Fig. 3. Incomplete separation of the TMS-ethers of digitoxigenin, gitoxigenin and digoxigenin on OV-1 at 250°. He: 33 ml/min. Glass column, 180 × 0.3 cm.

Fig. 4. Complete separation of the "$\beta$"-anhydro TMS-ethers of digitoxigenin, gitoxigenin and digoxigenin on QF-1 at 250°. He: 33 ml/min. Glass column, 180 × 0.3 cm.

Fig. 5. Relative detector response to "$\beta$"-anhydrodigitoxigenin in the 1.4–8.4 m$\mu$mole range on OV-1 at 250°. Glass column, 180 × 0.3 cm.

Of the free genins, digitoxigenin (*Ia*) has two hydroxy groups, whereas gitoxigenin (*Ib*) and digoxigenin (*Ic*) have each three. Since *Ia* has the smaller molecular weight, it was to be expected that on the non-polar phases SE-30 and OV-1, *Ia* would be eluted before *Ib* and *Ic*. On the other hand, based on polarity considerations, *Ib* and *Ic* should have longer retention times on the selective QF-1 phase. Table II shows that this prediction is indeed true for *Ia* and *Ic*, as well as for the derivatives *Id*, *If*, *Ig* and *Ii*. However, *Ib* and *Ih* exhibited an anomalous pattern on both columns A

and B; they were eluted before *Ia* and *Ig* respectively. The overall behaviour of the cardenolides on the QF-1 phase was even more unpredictable. *Ia* and *Id* were eluted before *Ib* and *Ie* respectively, and *Ic* before *Ia*, but *Ig* was retained longer than *Ih* by the fluorosilicone polymer. It should further be noted that *Ia* and its TMS-ether *Id* displayed nearly identical retention times, both on the SE-30* and OV-1 columns. This was also the case for the "$\beta$"-anhydro compounds *IIa* and its TMS-ether *IId*. The slight difference in molecular weight was apparently not sufficient to cause their separation on a non-selective phase.

All the "$\beta$"-anhydro cardadienolides obtained showed greatly reduced retention times, and were well resolved, when compared with the 14$\beta$-hydroxylated parent cardenolides. The peaks obtained on all three columns were symmetrical; a prerequisite for quantitative measurements.

TABLE III

CALCULATED RETENTION TIME RATIOS CARDENOLIDE/CARDADIENOLIDE

| *Compounds* | *Retention time ratio* | | |
|---|---|---|---|
| | *Column A (SE-30)* | *Column B (OV-1)* | *Column C (QF-1)* |
| *Ia/IIa* | 1.61 | 1.64 | 2.65 |
| *Ib/IIb* | 2.37 | 2.46 | 5.10 |
| *Ic/IIc* | 3.30 | 2.05 | 4.96 |
| *Id/IId* | 1.59 | 1.57 | 2.40 |
| *Ie/IIe* | 1.59 | 1.51 | 2.32 |
| *If/IIf* | 1.41 | 1.50 | 2.00 |
| *Ig/IIg* | 1.58 | 1.60 | 2.44 |
| *Ih/IIh* | 1.23 | 1.24 | 1.70 |
| *Ii/IIi* | 1.49 | 1.39 | 3.49 |

Table III demonstrates the remarkable similar behaviour of the steroids investigated on the SE-30 and OV-1 columns, the only exception being digoxigenin (*Ic*). The greatest reduction in retention times were obtained with column C, which is the better column for the separation of the free genins *Ia*, *Ib* and *Ic*.

The response of a flame ionisation detector to the jet stream of a gaseous organic substance is, when operated under the same parameters of temperature, carrier gas flow and concentration of sample, essentially dependent only on the number of carbon atoms present in the molecule. Since by the dehydration reaction no carbon atoms are lost, our method is suitable for activity measurements of labelled cardenolides as well; with the exception of 15$\alpha$-tritiated compounds. To our knowledge, however, tritiated cardenolides, specifically labelled at position 15$\alpha$, have not been described so far.

We conclude that while no satisfactory separation could be achieved between the cardenolides digitoxigenin and gitoxigenin on a SE-30 or OV-1 column, and between gitoxigenin and digoxigenin on a QF-1 column, after dehydration, in the form of the "$\beta$"-anhydro derivatives, the cardadienolides thus obtained were easily separated on a selective, as well as a non-selective phase.

* We confirm in this respect the findings of WILSON *et al.*[17] with the same two compounds on a SE-30 column.

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