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GAS–LIQUID CHROMATOGRAPHY OF DIGITOXIGENIN AND DIGOXIGENIN AS ACETATES OF THEIR EPOXYGENINIC ACID METHYL ESTERS

J. W. MEILINK and J. B. LENSTRA
Pharmaceutical Department, Medical Faculty, Erasmus University, Rotterdam (The Netherlands)
and
A. BAERHEIM SVENDSEN
Department of Pharmacognosy, State University Leiden, Leiden (The Netherlands)
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SUMMARY

Gas chromatographic analysis of digitoxigenin and digoxigenin, the genins of the cardenolide glycosides digitoxin and digoxin, cannot be done without derivatization. However, during the derivatization, side-reactions often present serious problems. A procedure has been found for transforming digitoxigenin and digoxigenin into the corresponding acetates of their epoxygeninic acid methyl esters, which are stable compounds and suitable for gas chromatographic analysis.

INTRODUCTION

It is generally accepted that gas chromatographic procedures for the analysis of drugs, when applicable, surpass many other methods in sensitivity and selectivity. Reports of gas chromatographic methods for separation, identification and estimation of digitalis glycosides (cardenolides) are few, however, probably owing to the high molecular weights and low volatilities of such compounds. This is surprising, since Horning *et al.*[1] gas chromatographed other types of high molecular weight steroids using packed columns with siliconized supports, thinly coated with thermostable liquid phases.

So far, combinations of analytical techniques have been used in research on digitalis glycosides when qualitative as well as quantitative results were desired. Recently, the combination of thin-layer chromatography (TLC) with the "RbCl uptake method" was used in studies of digitoxin and its metabolites in man (Storstein[2,3]).

Gas–liquid chromatography (GLC) of digitalis glycosides is difficult for two reasons. First, cardenolides and cardenolide genins cannot be gas chromatographed as such. Second, the sensitivity for cardenolides and cardenolide genins that can be obtained with common GLC detectors is too low for many investigations. Therefore a solution has to be found in chemical processing to improve the volatility as well as the

detectability. It would be particularly elegant to use the same chemical reaction to achieve both aims.

The chemical instability of cardenolides in the presence of various reagents is often a complicating factor during such reactions. The close resemblance of cardenolides to their degradation products hampers the detection of decomposition. Of all the possible degradation routes, the cleavage of the glycosidic bond by acidic hydrolysis or by acetolysis, disconnecting the sugar moiety from the steroidal genin, is the most conspicious one. However, the genin released turns out to be a better substrate for GLC than the parent glycoside; hence cleavage of the glycosidic bond is likely to be a part of any useful method. In this way the glycosides are analysed in the form of the corresponding genins. The GLC procedures described by Wilson[4] and Watson and Kalman[5] are based on this principle.

As emphasized by Sondheimer[6], the genin itself can undergo further chemical changes. The most important ones are: (1) acid-catalysed conversion into α- and β-anhydrogenins; (2) base-catalysed conversion into epoxides; (3) inversion of the 17α side-chain to a 17β configuration. These reactions are inconvenient, but do not interfere with the analytical results as long as the yield is quantitative and they lead to just one conversion product.

It is a striking fact that all conversion reactions involving the steroidal genin give relatively stable products. This is a good reason for using them in the analysis of cardenolides. The only GLC procedure in which this principle has been applied was described by Tan[7]. His derivatization method could easily give rise to a mixture of derivatives of the genin and the corresponding α- and β-anhydrogenins. However, after converting the genin quantitatively and selectively into the β-anhydrogenin, Tan does not encounter any limitations concerning the choice of a derivatization procedure.

It should be possible to make similar use of the stability of the epoxides. This stability, for example, enabled Shah *et al.*[8] to split off the sugar moiety of the glycoside in a very strongly acidic medium. However, the great stability of epoxides has so far not been used in gas chromatography.

In order to investigate the usefulness of epoxides in GLC procedures, we carried out the experiments described below.

EXPERIMENTAL

Reagents

Acetic acid, acetic anhydride, potassium hydroxide, anhydrous sodium sulphate, dichloromethane, tetrahydrofuran and acetone were all Merck (Darmstadt, G.F.R.) p.a. quality. Pyridine p.a. (BDH, Poole, Great Britain) was distilled from solid NaOH and stored over solid NaOH. Methanol p.a. (Merck) was dried with Molecular Sieve 3A pellets (Union Carbide, New York, N.Y., U.S.A.). Diethyl ether p.a. (Merck) was distilled and dried over Molecular Sieve 4A pellets (Union Carbide). Diazomethane was prepared according to a method described by De Boer and Backer[9]: 900 mg of *p*-tolylsulphonylmethylnitrosamide (Merck) was dissolved in 14 ml of dry diethyl ether. The solution was cooled in ice and subsequently made alkaline with 4.5 ml of KOH (1 mole/l) in 96% ethanol. 10 min later a diazomethane–diethyl ether mixture was distilled off.

Apparatus

A Pye Unicam Series 104 gas chromatograph equipped with a single flame ionization detector was used. The column oven temperature was 250° and the detector oven temperature was 325°. The injection port temperature was kept about 40° above the column oven temperature. The attenuation setting was $5 \cdot 10^2$, corresponding to $5 \cdot 10^{-10}$ A for full-scale deflection of the recorder.

Two columns were used: (1) A 50 cm × 4 mm glass column fitted with a glass-to-metal joint for coupling to the detector, filled with 2% OV-1 on silanized Chromosorb W AW, 70–80 mesh; and (2) A 50 cm × 4 mm glass column fitted with a glass-to-metal joint for coupling to the detector, filled with 0.5% poly(ethylene glycol) 20M on silanized Chromosorb W AW, 70–80 mesh.

Nitrogen was used as a carrier gas with a flow-rate of 40 ml/min in all experiments, resulting in inlet pressures of 0.3 atm and 0.25 atm for the OV-1 and PEG 20M columns, respectively. Peak areas were measured with an Infotronics integrator CRS 204. Sonification was done in a Sonicor ultrasonic bath.

Methods

To 20 μg digitoxigenin (3β,14-dihydroxy-5β,14β-card-20(22)enolide) or digoxigenin (3β,12β,14-trihydroxy-5β,14β-card-20(22)enolide) was added 200 μl of 0.5 mole/l KOH in methanol, followed after 30 min by 400 μl of water. The reaction mixture was allowed to stand for another 30 min at room temperature. Then 30 μl of acetic acid was added, followed by 15 ml of dichloromethane. The mixture was kept in an ultrasonic bath. When a homogeneous opalescence indicated that the water phase was evenly emulsified in the dichloromethane, *ca.* 1 g of anhydrous sodium sulphate was added under continuous sonication. After centrifugation the dichloromethane was decanted off and evaporated at room temperature under a stream of air. To the residue were added 100 μl of tetrahydrofuran and 20 μl of methanol, followed by 100 μl of diazomethane in diethyl ether. If the yellow colour of the solution disappeared, more diazomethane was added. After 10 min the reaction mixture was evaporated to dryness under a stream of air at room temperature.

Acetylation was performed by dissolving the residue in 100 μl of pyridine and 100 μl of acetic anhydride and leaving the reaction mixture overnight at room temperature. The solution was then evaporated to dryness, 100 μl of acetone was added and 1 μl was injected into the gas chromatograph.

RESULTS AND DISCUSSION

The fact that epoxides of cardenolides can be formed by a base-catalysed reaction has been known[10] for a long time. After the molecule has been rearranged to the 14,21-epoxide, the double bond in the lactone ring, the characteristic feature of all cardioactive glycosides, is eliminated (Fig. 1). A good method for the preparation of the epoxide of digitoxigenin (3β-hydroxy-14,21ξ-epoxy-5β,14β,20ξ-cardanolide) has been given by Jacobs and Gustus[11]. They dissolved digitoxigenin in methanolic potassium hydroxide; after some time the epoxide crystallized out. Lindig and Repke[12] state that this rearrangement occurs instantaneously.

The method of preparation described by Jacobs and Gustus[11] was chosen as the starting point for our experiments. It also proved to be suitable for the prepa-

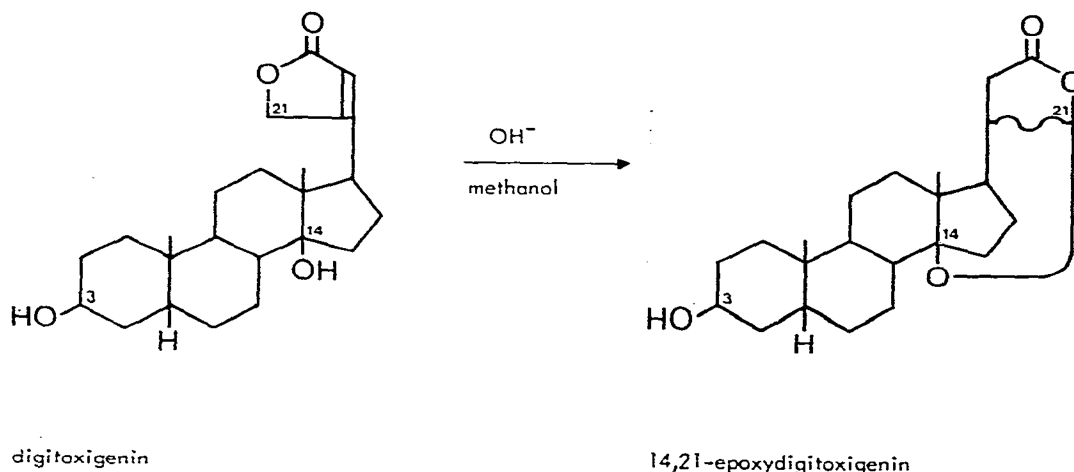

Fig. 1. Conversion of digitoxigenin into 14,21-epoxydigitoxigenin.

ration of epoxydigoxigenin (3β,12β-dihydroxy-14,21ξ-epoxy-5β,14β,20ξ-cardanolide). In alkaline media, the lactone ring of the epoxide readily opens on the addition of water. When the solution is subsequently made acidic by adding acetic acid, the lactone ring closes, but not at once if care is taken that the pH does not become too low. On the other hand, low pH values are needed for good extraction, keeping the acids undissociated. If the pH is kept at about 4, it is possible to extract the epoxide in the acid form with an organic solvent, such as dichloromethane. The formation of potassium acetate in the solution helps to control the acidification step.

When the extraction procedure is performed with small volumes as described here, it is advisable to dry out the aqueous phase. Emulsification of the aqueous phase prior to the addition of anhydrous sodium sulphate prevents incomplete extraction. Evaporation of the dichloromethane at room temperature leaves the epoxygeninic acid. According to Schindler and Reichstein[13], this substance can be stored at room temperature without any relactonization. In the procedure proposed, relactonization is also prevented by esterification of the carboxyl group. According to Schlenk and Gellerman[14] the reaction proceeds instantaneously when the diazomethane is dissolved in a mixture of diethyl ether and methanol (9:1): moreover, no side-products are formed.

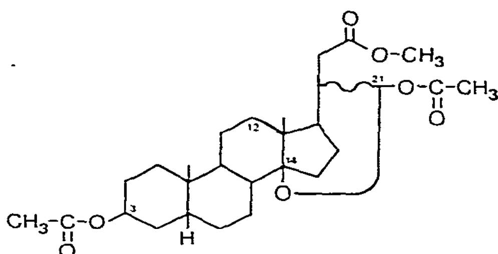

Fig. 2. Structure of 3,21-diacetoxy-14,21-epoxydigitoxigeninic acid methyl ester.

Just like the parent genin, the epoxygeninic acid methyl esters cannot be gas chromatographed as such. They have to be further processed to give better GLC properties. Masking of the hydroxyl groups by acetylation was considered to be the most effective means to this end. As far as the methyl ester of epoxydigitoxigeninic acid is concerned, it is the hydroxyl groups at C-3 and C-21 that have to be masked; for the digoxigenin derivative the hydroxyl group at C-12 has to be dealt with too (Fig. 2).

Acetic anhydride in pyridine, commonly used for the acetylation of a great number of compounds, proved to be effective. The acetylation of the 3-hydroxyl group proceeds much faster than that of the 21-hydroxyl group, as was demonstrated by Krasso *et al.*[15] who found that the 3-monoacetate was formed after "gentle acetylation", followed by the 3,21-diacetate after "vigorous acetylation". Indeed, with short acetylation times we found two peaks in the gas chromatogram of the digitoxigenin derivative, indicating that the reaction was incomplete. When the areas of the two peaks are considered as a function of acetylation time, the 3-monoacetate and the 3,21-diacetate can easily be identified, as the area of the monoacetate peak decreases with time, while that of the diacetate increases (Fig. 3). It may thus be concluded that the 3-monoacetate has a shorter retention time than the less polar 3,21-diacetate when OV-1 is used as stationary phase. Using a polar stationary phase such as poly(ethylene glycol), we find that the retention time of the monoacetate is greater than that of the diacetate.

Complete acetylation was achieved at room temperature by keeping the reaction mixture overnight. This was deduced from the absence of monoacetate and

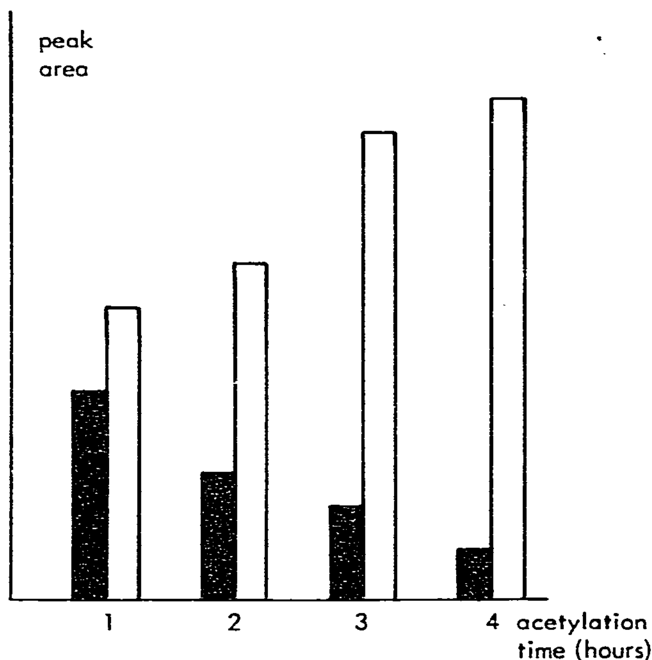

Fig. 3. Influence of the acetylation time on the degree of acetylation of epoxydigitoxigeninic acid methyl ester. The bars represent the areas of the peaks of the 3-monoacetate (black bar) and of the 3,21-diacetate (white bar) obtained after gas chromatography of the reaction mixture.

from the fact that the area of the diacetate peak did not increase on further acetylation. Raising the temperature during acetylation to 50° speeds up the reaction, giving good results after a reaction time of 4 h. The use of temperatures above 50° is not advisable, because it increases the risk of dehydrating the diacetate[15]. The dehydration product, if present, shows up in the gas chromatogram: on OV-1 it has a longer retention time than the diacetate itself.

Under the conditions described above, a peak giving full-scale deflection on the recorder is equivalent to 0.3 μg digitoxigenin or 0.5 μg digoxigenin. Amounts of 0.02–0.30 μg of digitoxigenin and 0.04–0.50 μg of digoxigenin gave a linear relationship between amount and peak area. The peaks were all well separated on both stationary phases used (Fig. 4).

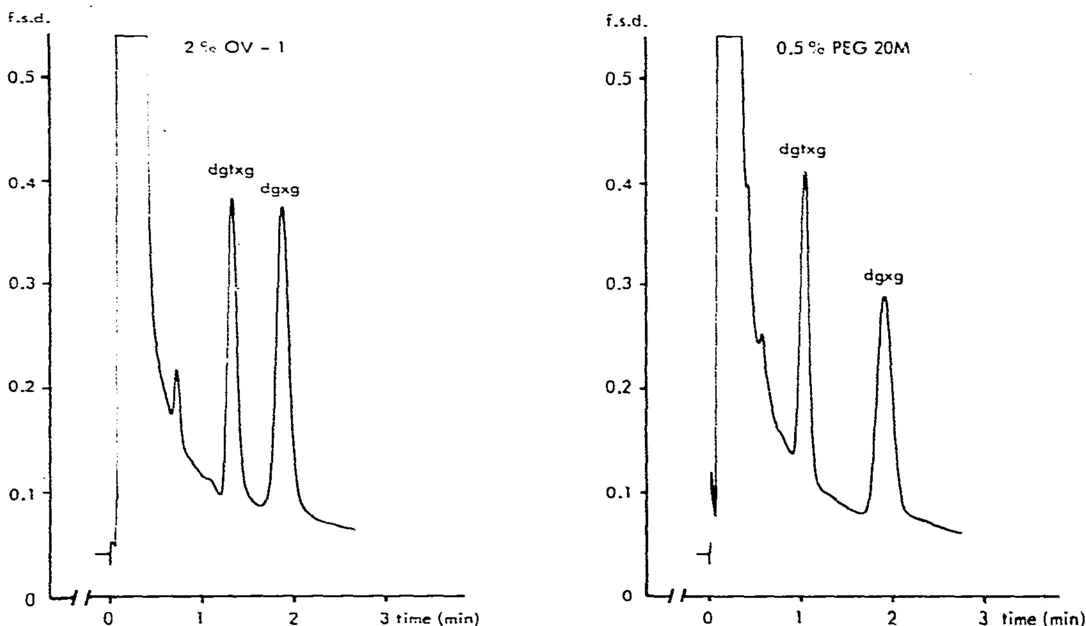

Fig. 4. Gas chromatograms of 0.1 μg digitoxigenin (dgtxg) and 0.16 μg digoxigenin (dgxg) after conversion into the acetates of the corresponding epoxygeninic acid methyl esters. The integrator counts are 2000 for the digitoxigenin derivative and 2500 for the digoxigenin derivative. Columns: 50 cm × 4 mm glass columns filled with 2% OV-1 and 0.5% PEG 20M, respectively, on silanized Chromosorb W AW, 70–80 mesh. Oven temperature: 250°. Detection: flame ionization.

The usefulness of this method arises from the fact that, though four stereoisomers of the epoxygenin could theoretically be formed, apparently only one is obtained. Other papers[13,15,16] give detailed information about this phenomenon. Krasso *et al.*[15] state that the diacetate of epoxydigitoxigeninic acid methyl ester will also be formed in just one steric configuration, if the right method of preparation is used.

The low melting points of the compounds formed certainly improve their GLC behaviour. The melting points of digitoxigenin and its derivatives are listed in Table I. No data are given in literature on the corresponding digoxigenin derivatives,

TABLE I

MELTING POINTS OF SOME DIGITOXIGENIN DERIVATIVES AS GIVEN IN THE LITERATURE

Compound	*Melting point (°C)*	*Reference*
Digitoxigenin	243	
Epoxydigitoxigenin	275	13
Epoxydigitoxigeninic acid	250	13
Epoxydigitoxigeninic acid methyl ester	128	11
Epoxydigitoxigeninic acid methyl ester diacetate	174	13

possibly because of the difficulty of isolating epoxydigoxigenin from the reaction mixtures.

For the determination of smaller amounts of digitoxigenin and digoxigenin, the sensitivity of detection would have to be improved. This could be done by applying a derivatization procedure other than the acetylation, in order to obtain products with greater sensitivity. Alterations to the procedure described can readily give other end-products, such as other steric configurations or dehydrated compounds, thus diminishing the reliability of the method.

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