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COLORIMETRIC ASSAY OF DIGITOXIN AND GITOXIN IN MIXTURES AFTER THIN-LAYER CHROMATOGRAPHIC SEPARATION*

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SUMMARY

A colorimetric method is proposed for the determination of digitoxin and gitoxin in mixtures after TLC separation. After quantitative separation on silica-gel coated plates each glycoside is extracted and determined colorimetrically by the dixanthylurea reaction. The proposed method is rapid and more reliable than the standard methods used up to now and is thus very useful for routine control work. The limits of error are ± 1.44 for digitoxin and ± 1.46 for gitoxin.

INTRODUCTION

The control of pure digitalis glycoside solutions on the market which contain digitoxin and gitoxin in the ratio 3:2 is usually carried out by standard biological or chemical methods.

The biological method generally applied for testing peroral solutions utilizes an intravenous route of administration, whereas therapeutic use of these drugs requires absorption from the intestinal tract. The matter of absorption of these glycosides from the intestinal tract is thus important.

However, according to results of biological assays using oral application on animals and on men reported by various authors[1–4] (*i.e.*, including absorption from the intestinal tract) digitoxin is absorbed completely, while gitoxin is not absorbed at all or only to a very small extent. This means that intravenous biological testing introduces a bias for orally administered mixtures of cardiatonic drugs containing digitoxin and gitoxin, and digitalization carried out by means of such a drug does not fulfill the expectations of the physician. Moreover, standard chemical methods permit only the determination of both glycosides together and that again may give misleading results.

In our opinion, therefore, a more efficient control of such oral preparations should be carried out either by a chemical assay that permits determination of each glycoside separately (*i.e.* after separation of digitoxin and gitoxin) or by a biological assay which

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involves oral administration. In this way the labeled amount of the therapeutically active digitoxin can be controlled efficiently. The scope of our study, therefore, was to separate digitoxin from gitoxin and subsequently to determine each of the separated glycosides by a suitable micromethod.

Separation of digitoxin from gitoxin by paper chromatography was first achieved by SVENDSEN AND JENSEN[5]. The determination of the separate glycosides was carried out by these investigators as well as by others who proposed other possibilities of PC separation, *e.g.*, by the colorimetric Baljet reaction after extraction of each glycoside from the paper. However, all these procedures are time consuming and the limits of error are significant, making them unsuitable for routine work.

For all the well-known advantages of TLC over PC, separation on thin layers proved to be a very useful and elegant tool in solving the problems of separating the digitalis glycosides.

Partition TLC was first used by STAHL[6] for the separation of the main digitalis glycosides using methylene chloride–methanol–formamide (80:19:1) as the solvent system. Much work has been reported since on the improvement of TLC separation of digitalis glycosides[7,8]. For the quantitative determination of digitoxin in digitalis leaves, HEUSSER[9] proposed a method based on the TLC separation of digitoxin from the extract and colorimetric determination of the extracted glycoside by the xanthydrol method.

EXPERIMENTAL

Reagents

Reagent solution. In a 100 ml volumetric flask 10.0 mg of dixanthyl urea were dissolved in 50 ml glacial acetic acid and 1 ml hydrochloric acid with glacial acetic acid being added to fill the flask to the mark.

Spray reagent. 1 % solution of iodine in chloroform.

Solvent system. Chloroform–methanol (88:12).

Reference standards. Digitoxin and gitoxin (E. Merck A.G., Darmstadt).

Standard solution digitoxin. 15.0 mg of digitoxin were dissolved in the chloroform–methanol (1:1) mixture and the solvent was used to bring the volume to 100 ml.

Standard solution gitoxin. 10.0 mg gitoxin were dissolved in the chloroform–methanol mixture (1:1) and the solvent was used to bring the volume to 100 ml. All reagents used were of p.a. purity grade.

Apparatus

Thin-layer chromatographic apparatus with thickness regulating spreader (DeSaga, Heidelberg). Agla micrometer syringe (Burroughs Wellcome & Co., London).

Preparation of plates

Thickness of layer: 0.50 mm. Activation: 1 h at 105°. Adsorbent: Silica Gel G (E. Merck A.G., Darmstadt).

Procedure

By means of a micrometer syringe, 200 μl of the solution (about 30 μg of digitoxin and about 20 μg of gitoxin) and 200 μl of each standard solution were applied

along the starting line as 4-cm horizontal bands. The chromatogram was run by the ascending technique until the solvent moved within 2 cm of the upper edge of the plate (about 50 min). The plate was removed from the chamber and the separated glycosides were located by spraying with the reagent. Each yellow zone containing the glycosides from the sample and from the standard solutions was immediately marked with an ample margin and quantitatively scraped off the plate into a centrifuging tube. After addition of 10.0 ml of the chloroform–methanol mixture (1:1), the tubes were shaken thoroughly for about 15 min and centrifuged for 15 min (5000 r.p.m.). 7.0 ml of each supernatant clear solution was pipetted into a 50-ml flask and the solvent evaporated to dryness by heating on a boiling water bath. The residues were dried for 10 min at 105°. After cooling to room temperature 5.0 ml of the reagent solution was added, the flask stoppered and immersed in a boiling water bath for exactly 3 min. Care had to be taken that the surface of the solution in the flasks was under that of the water in the water bath and that the flasks were tightly stoppered. After cooling for a few minutes in cool water the flasks were left at room temperature for 10 min and the absorbencies of the colour obtained were measured at 535 mμ in 2-cm glass cells against water as a blank. The percentage of each glycoside in the sample was calculated relative to the concentration of the glycoside in the corresponding standard solution.

DISCUSSION AND RESULTS

Preparations of purified digitalis glycosides in solution on the market contain 0.15 mg of digitoxin and 0.10 mg of gitoxin in 1 ml. To the solution about 10% of glycerol is added for the purpose of adjusting the surface of the solution, since these preparations are administered dropwise.

Taking into account the solubility of both glycosides in relation to their structural characteristics, various solvent systems were tested which were thought to be appropriate for the quantitative separation of both glycosides as well as glycerol.

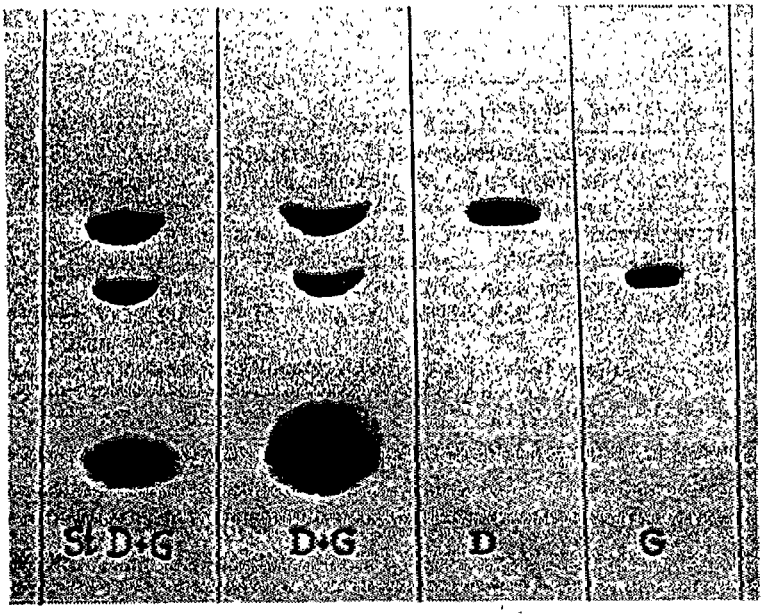

Fig. 1. Separation of digitoxin from gitoxin.

This was attained when the mixture chloroform–methanol in the ratio 44:6 was used, the adsorbent being silica gel. Due to the solubility rate in the solvent used, digitoxin migrated faster than gitoxin, while glycerol was left at the start (Fig. 1).

The most commonly used method for the determination of minute quantities of digitoxin and gitoxin are based on the color reaction of Baljet and of Kedde in an alkaline solution or with xanthydrol in an acid solution. Although the xanthydrol reaction is the most sensitive one and is specific for digitoxose, it has received much criticism because of the poor stability of the colored reaction product formed. Since the results of our experiments concerning these drawbacks confirmed the findings of

TABLE I

ANALYSIS OF STANDARD GLYCOSIDE SOLUTIONS

Analysis No.	*Digitoxin (21 μg added)*		*Gitoxin (14 μg added)*	
	Found (μg)	*Difference (%)*	*Found (μg)*	*Difference (%)*
1	21.59	+2.8	14.63	+4.5
2	20.73	−1.3	13.94	−0.4
3	21.00	ø	14.47	+3.3
4	20.53	−2.2	14.14	+1.0
5	20.43	−2.7	14.32	+2.3
6	20.43	−2.7	14.32	+2.3
7	20.47	−2.5	14.53	+3.8
8	20.81	−0.9	13.89	−0.9
9	20.89	−0.5	14.43	+3.1
Mean		20.76	Mean	14.29
Standard error		0.37	Standard error	0.26
Standard deviation ($P = 0.05$)		0.30	Standard deviation ($P = 0.05$)	0.21
Limits of error		±1.44%	Limits of error	±1.46%

TABLE II

ANALYSIS OF COMMERCIAL GLYCOSIDE SOLUTIONS

Digitoxin (0.15 mg labeled)		*Gitoxin (0.10 mg labeled)*	
Found (mg)	*Difference (%)*	*Found (mg)*	*Difference (%)*
Sample A			
0.1574	+ 4.9	0.0951	− 4.9
0.1554	+ 3.6	0.0947	− 5.3
0.1545	+ 3.0	0.0950	− 5.0
Sample B			
0.1476	− 1.6	0.1314	+31.4
0.1527	+ 1.8	0.1230	+23.0
0.1515	+ 1.0	0.1250	+25.0
Sample C			
0.1846	+23.1	0.0888	−11.2
0.1863	+24.2	0.0886	−11.4
0.1830	+22.0	0.0880	−12.0

TABLE III

COMPARISON OF RESULTS OBTAINED WITH BIOLOGICAL AND CHEMICAL ASSAYS[a]

Sample (0.25 mg labeled)	*Biological assay (i.v. titration on pigeon)*		*Chemical assay*
	Found (mg)	*Fiducial limits of error (%)*	*Found (mg)*
1	0.242	88.7–112.8	0.252
2	0.254	88.4–113.2	0.270
3	0.254	89.4–111.9	0.265
4	0.228	88.7–112.8	0.256

[a] For results of biological assay we are grateful to Mrs. LUDMILA HOLIK, Klarić.

earlier authors, our method of choice was the colorimetric method proposed by PÖTTER[10]. This method is based on the color reaction of digitoxin and gitoxin with dixanthylurea. The reaction product was found to be stable under the given conditions for a period of 90 min while Lambert–Beer's law was obeyed in the concentration range from 1 to 25 μg in 5 ml of the solution to be measured.

To confirm the accuracy and reproducibility of the procedure the determination of the glycosides was carried out with a standard solution mixture containing digitoxin and gitoxin, as well as glycerol in the same concentrations as those of preparations commonly used in therapy.

Results were statistically treated, and as can be seen in Table I, satisfactory limits of error were obtained.

In Table II results of determination with commercial samples are given.

In Table III results of biological assay using parenteral application and chemical assay including colorimetric determination of both glycosides together are given. As can be seen results obtained with both methods are in good agreement within their limits of error.

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