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# AN IMPROVED METHOD FOR THE ISOLATION OF HEART GLYCOSIDES FROM TISSUES BY USING SEPHADEX G 200*

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**Abstract**—A method is described, by means of which the glycosides digitoxin, digitoxigenin-*bis*-digitoxoside, digitoxigenin-mono-digitoxoside, digoxin, digoxigenin-*bis*-digitoxoside, digoxigenin-mono-digitoxoside as well as the genins digoxigenin and digitoxigenin are isolated from animal tissue (the blood and heart of rats) after extraction by chloroform and cleaning through Sephadex G 200 on the basis of thin-film chromatography; these compounds can be prepared separately.

Compared with conventional methods, this procedure is about one decimal power more sensitive and permits the isolation as well as the test reaction of each 25 ng of the mentioned compounds in the heart or the blood of an animal.

INVESTIGATIONS by Repke[7] have shown that by i.v. application of digitoxin to rats less than 1 per cent of the injected dose is bound in the heart. Förster, Grade and Schulzeck[1] have found out that after i.p. injection even only 0·3–0·4 per cent are bound in the hearts of rats and a maximum of only 0·15 per cent of the injected dose in the heart of guinea-pigs. Due to these low amounts of glycoside bound in the heart it has been impossible so far to differentiate between digitoxin and its possible metabolites, i.e. digitoxigenin-*bis*-digitoxoside, digitoxigenin-mono-digitoxoside, digoxin, digoxigenin-*bis*-digitoxoside, digoxigenin-mono-digitoxoside and the corresponding genins.

The methods which have been adopted so far for the reconditioning of tissue and the detection of the mentioned compounds have to be regarded as insufficient for two reasons: (1) The method of paper chromatography is not sensitive enough, since, the lower limit for detecting the mentioned compounds is within about 10 $\mu$g per stain, when using visual fluorescence analysis and when spraying the chromatograms with chloramine.[4] (2) The ballast materials carried along in the extraction process interfere with the reconditioning of the tissues by affecting the shape of stains and showing a self-induced fluorescence. For the purpose of eliminating these ballast materials the various authors[2, 6, 8] have suggested the use of protracted and elaborate extraction processes which are even followed by a cleaning of the extracts through an absorbent (e.g. aluminium oxide).

Despite this it is known from experience that all the methods recommended so far have not achieved a complete elimination of the ballast materials. Therefore, it was

* Part of the results was already read out at the 7th meeting of Industrial and College Pharmacologists held in September 1965, in Weimar.

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our aim to devise a chemical method which was sensitive enough to serve as a test reaction on glycosides for all digitoxin metabolites traceable in the heart muscle and in the blood in the nanogramme range.

To solve this problem the method of thin-film chromatography was used instead of paper chromatography. The higher detection sensitivity secured by this approach required, however, an improved technology to remove the ballast materials. Since apart from aluminium oxide, infusorial silica, anionic and cationic exchangers, activated carbon, cellulose or silica gel have proved to be insufficient as a cleaning agent for the extracts, it was found that Sephadex G 200 alone was suitable to remove quantitatively the tissue ballast materials. The use of Sephadex made it possible to reduce the great number of extracting and cleaning operations to only four, so that the new method, despite its increased sensitivity, can more easily be applied than the hitherto described processes. The test limit lies within or below 1 $\mu$g of glycoside per test preparation.

## METHODS

In recovery tests and investigations *in vivo* 20 hearts of rats with a body weight of about 100–150 g each were reconditioned. The processes described in literature[2, 6, 8] were modified as follows.

The hearts were first divided by using a pair of scissors, cleaned of adhering residual blood by immersing them into physiological saline solution and frozen on solid $CO_2$. The frozen muscle parts were homogenized in 40–50 ml of physiological saline solution by means of a glass homogenizer, the homogenate was agitated with the fourfold amount of petroleum ether and carbon tetrachloride in a 500 ml round-bottomed flask on the electronic mechanical shaker of VEB Labortechnik Ilmenau at maximum frequency for 30 min, once for the elimination of fats and once for that of fat-similar compounds and, afterwards, centrifuged at 6000 *g* for 15 min in an enclosed laboratory centrifuge to separate the layers. The separated solvents were rejected. Afterwards, the homogenate was shaken twice each time for 30 min using double the amount of chloroform, centrifuged each time for 15 min and the chloroform phase carefully separated by means of an injection syringe which was connected with a pipette through a silicone rubber hose. The homogenate was rejected. Residual water available in the chloroform extract was frozen out in the refrigerator at −15°. Afterwards, the homogenate was quickly decanted and the chloroform filtered over cottonwool previously washed by cooled chloroform.[3] The extract of chloroform was boiled down at room temperature *in vacuo*. The small residue was absorbed three times, each time by means of 2 m[1] 30 per cent aqueous methanol, and the resultant milky turbid solution centrifuged at 3000 *g* for 30 min. The still dimly turbid, supernatant liquid was absorbed, evaporated *in vacuo* to 2 ml and brought on the Sephadex column.

Sephadex G 200 intended for gel filtration was allowed to swell in aqueous methanol for 24 hr, before it was slowly cast into a column of 10 mm diameter and 150 cm length which is enclosed by a thin G 3 frit and partly filled with 30 per cent aqueous methanol. For a proper separation the basic requirement of a homogeneous column was filled free from air bubbles which has a low dead volume at the column exit. The ready-for-use column is thoroughly washed by means of a 30 per cent aqueous methanol and fed by the glycoside-containing solution. Elution was also effected by means of

30 per cent aqueous methanol. A turbid solution was obtained as the first fraction which did not contain any glycosides or genins and was therefore rejected. The subsequent glycoside-containing clear elutriate (80–100 ml) which had to be properly separated from the first turbid elutriate in order to avoid losses of glycoside, was collected and evaporated to dryness *in vacuo* at room temperature. The residue, however, absorbed in 0·1 ml methanol, was applied to thin-film chromatographic plates together with silica gel G (Merck) according to Stahl or with silica gel D intended for thin-film chromatography of the VEB Chemiewerk Greiz-Dölau and subjected to as ascending chromatographic treatment within the isopropyl ether methanol 9:1 system for 2–3 hr at room temperature. After drying, the plates were treated with trichloracetic acid and chloramine according to Kaiser.[4] In u.v. light digitoxin and its derivatives fluoresce were seen to be yellow, whereas digoxin and its metabolites fluoresce were seen to be blue.

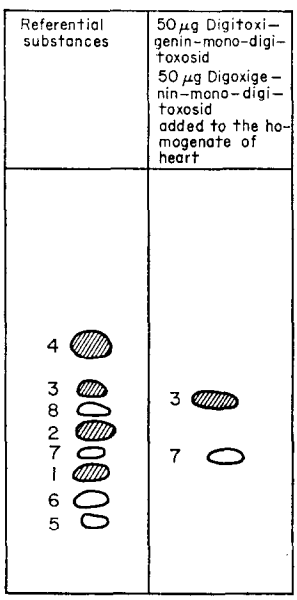


FIG. 1. Thin-film chromatographic test reaction of digitoxigenin-mono-digitoxoside and digoxigenin-mono-digitoxoside which are added in quantities of each 50 µg to the homogenate of 20 rat hearts. In the course of reconditioning no genins were formed.

1 = digitoxin; 2 = digitoxigenin-*bis*-digitoxosid; 3 = digitoxigenin-mono-digitoxosid; 4 = digitoxigenin; 5 = digoxin; 6 = digoxigenin-*bis*-digitoxosid; 7 = digoxigenin-mono-digitoxosid; 8 = digoxigenin.

*Reconditioning of blood*

After decapitation of the animals the resultant blood is collected in a beaker containing heparin solution, frozen on dry-ice or in a refrigerator at −20° and homogenized with several milliliters of physiological saline solution. Further reconditioning was done analogous to the method adopted for the tissues. The haemoglobin either

deposited after centrifuging with petroleum ether or conglomerated with carbon tetrachloride in the separating layer between carbon tetrachloride and aqueous blood is rejected only after extraction with chloroform.

## RESULTS

The fat-removing and extraction method should avoid any metabolization of the glycosides. To control this requirement repeatedly individual glycosides were added to the heart homogenate and it was attempted to find out whether genins are formed during the process of chemical reconditioning. Such a control test is shown in Fig. 1: each 50 μg of digitoxigenin-mono-digitoxoside and digoxigenin-mono-digitoxoside were added to the heart homogenate. As seen from the chromatogram, no genins were formed in the course of reconditioning.

For the quantitative determination of the recovery quota, six tests were carried out, each with 2 guinea-pigs' hearts. 100 μg each of digitoxin and digoxin were each added to the heart homogenate, the heart homogenate was conditioned as described above, and after having been cleaned on an aluminium oxide column, determined by means of the Xanthydrol reaction the following recovery quotas: 84, 86, 84, 72, 69 and 68 per cent. In one test with the homogenate of 20 guinea-pigs to which 15 μg of digitoxin had been added, 86 per cent was found, and in a test with the homogenate from 20 rats' hearts, to which 30 μg was added, 80 per cent of the glycoside added was recovered. The conditioning and cleaning of the Sephadex column described above was controlled by six control tests each time the collected blood and heart homogenate of 20 rats was used. Two tests were carried out each with 50, 25 and 12 μg of digitoxin and, by the dixanthyl urea methods the following recovery quotas were determined:

| Digitoxin (Merck) μg | Recovery quota in heart homogenate of 20 rats in each case μg | Blood of 20 rats in each case μg |
|---|---|---|
| 50 | 68 | 73 |
| 50 | 66 | 70 |
| 25 | 77 | 70 |
| 25 | 72 | 84 |
| 12 | 73 | 77 |
| 12 | 71 | 75 |

The reaction sensitivity of the method was moreover checked by the fact that in several test series each 20, 5, 2 and ultimately only each 1 μg of all digitoxin metabolites were added to the heart homogenate and to the blood obtained from 20 rats. It was digitoxin, digitoxigenin-*bis*-digitoxoside, digitoxigenin-mono-digitoxoside, digitoxigenin, digoxin, digoxigenin-*bis*-digitoxoside, digoxigenin-mono-digitoxoside and digoxigenin. On the basis of the above-mentioned approach all compounds

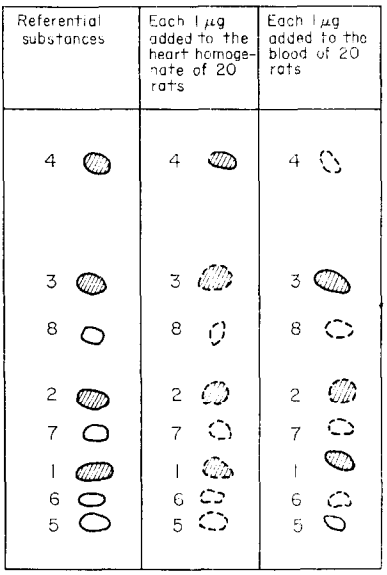


FIG. 2. Thin-film chromatographic test reaction of the metabolites of digitoxin which are added to the heart homogenate and blood of 20 rats in quantities of each 1 μg.
1 = digitoxin; 2 = digitoxigenin-*bis*-digitoxosid; 3 = digitoxigenin-mono-digitoxosid;
4 = digitoxigenin; 5 = digoxin; 6 = digoxigenin-*bis*-digitoxosid;
7 = digoxigenin-mono-digitoxosid; 8 = digoxigenin.

could be recognized chromatographically (Fig. 2). Since 0·5 μg/stain was still discernible, the lower limit of detectability for the group of 20 rats was fixed at about 25 ng/rat heart.

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