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ON METABOLITES OF ACETYLDIGOXIN-β IN THE HEART MUSCLE AND BLOOD OF RATS AFTER ORAL ADMINISTRATION OF GLYCOSIDE

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Abstract—To trace metabolites of acetylated glycosides in the heart muscle and blood of rats after application, a sensitive TLC method of assay for acetyldigoxin-β was developed which allows the separation of the acetylated glycoside from the non-acetylated metabolites in a single analytical step. After oral administration of 50–100 mg/kg of acetyldigoxin-β and periods of reaction of 30 min up to 8 hr, acetyldigoxin-β and non-acetylated decomposition products, including digoxigenin, were recovered in experiments in which acetyldigoxin-β and digoxin were the main constituents. Digoxigenin in the heart muscle was found in 4 out of 8 series of tests, digoxigenin-*bis*-digitoxosid in none, digoxigeninmonodigitoxosid only in one series of tests.

ACYLATED GLYCOSIDES are available as the following sugar-acetylated glycosides: acetyldigitoxin-α[1, 2] ("Acylanid"), acetyldigoxin-β[3–5] ("Novodigal"), the lanatosides A ("Aglunat", Adigal") and C ("Ceglunat", "Cedilanid"), as well as polyacyl derivatives: pentaacetylgitoxin[6–10] ("Pentagit") and pentaformylgitoxin[11, 12] ("Formiloxin"). Acyl groups may also be substituted for secondary hydroxyl groups of genin. To these may be added gitaloxin[13–17] and verodoxin[18] as constituents of "verodigens" as well as 16-acetylgitoxin (=oleandrigenintridigitoxosid[19–21]). Megges and Repke[22–23] detected acetylated metabolites as traces only in the blood and therefore supposed the acetyl groups to have a purely "Gleitschienen-Wirkung" (i.e. to act as a vehicle) in resorbing acetylated gitoxin derivatives.

In the present study the metabolism of the acetyl groups in the organism was followed by examination by means of TLC. Acetyldigoxin-β was given as glycoside which is well resorbed after being administered orally.

METHODS

At first we met great difficulties with the TLC separation of acetyldigoxin-β from its not acetylated metabolites (mono-, *bis*- and trioside of digoxigenin). From paper-chromatographic investigations with acetylated gitoxins we knew that acetylated gitoxins yield higher R_f-values than gitoxigenin or its mono-, *bis*- or triosides (Fig. 1) in the system xylol-methyl-ethylketone 1:1 or 2:1, saturated with formamide, on formamide- (20% formamide dissolved in acetone) impregnated paper. First we tried to separate all metabolites with the same solvent system on TLC's. We did not succeed in doing so because the mono- or biosides of gitoxigenin had about

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the same R_f-value, as the monoacetylgitoxins. The separation of acetylated gitoxins was also not successful with the solvent system of isopropyl ether-methanol-water 80:18:2 (Fig. 2) or different modifications of this solvent system which enables the separation of non-acetylated metabolites of gitoxin. On the assumption that the

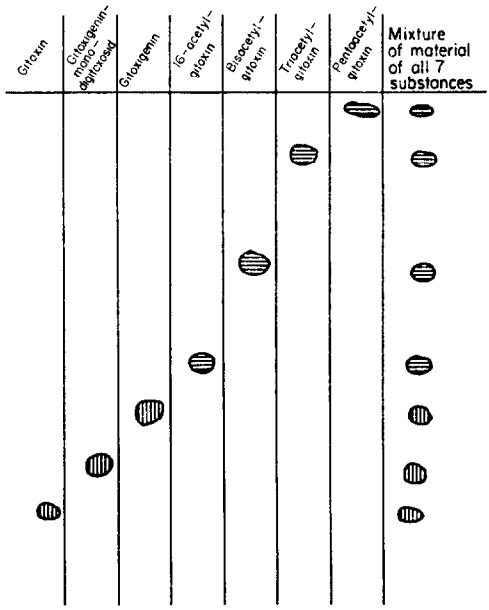

FIG. 1. Paper chromatographic separation of acetylated gitoxin derivatives from gitoxigenin, gitoxigenin-monodigitoxosid and gitoxin in the system xylol-methyl-ethylketone 1:1, saturated with formamide on formamide- (20% formamide dissolved in acetone) impregnated chromatographic paper FN2. 20 μg of each substance were layed-on separately and as mixture of material respectively. Rising chromatography, operating time of 3 hr at a room temperature of 22°. The chromatogram was sprayed with trichloracetic acid-chloramine and evaluated under the u.v. lamp.

higher R_f-values of acetylated gitoxins are due to formamide as a stationary phase we sprayed the silica gel layer with 20% formamide dissolved in acetone and chromatographed the sandwich panels, after being dry, in the solvent system xylol-methyl-ethylketone 1:1, saturated with formamide. Similarly as in the paper-chromatogram the acetylated gitoxins and also the acetyldigoxin-β now yielded higher R_f-values than the corresponding genins (Figs. 3 and 4). This new method, however, was not suitable for detecting acetyldigitoxin-α.

Acetyldigoxin-β combines with dixanthyl urea[24] to form a red colour complex, showing an absorption maximum on the Beckman spectrophotometer within the range of 400–800 nm, investigated at 545 nm (Fig. 5). A calibration curve with dixanthyl urea showed a linear rise in the range of 5–100 μg crossing zero.

As a preliminary experiment for the extraction from biologic material we determined the solubility of acetyldigoxin-β in chloroform, petroleum ether and carbon tetrachloride; we placed 50 μg of glycoside each in 100 ml round bottomed flasks in which there was a solution of 10 ml of 0·9% NaCl with 40 ml of petroleum ether, carbon tetrachloride or chloroform. After having shaken it for 10 min with maximum frequency the extraction substances were separated from the watery phases, compressed

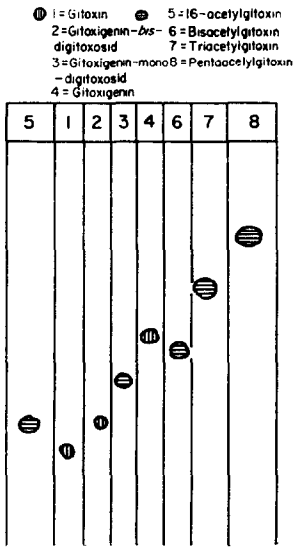

FIG. 2. TLC separation of acetylated gitoxin derivatives in the system isopropyl ether-methanol-water 80:18:2.

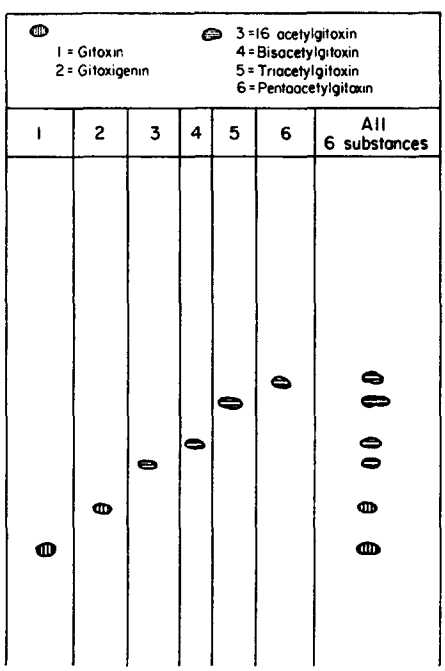

FIG. 3. TLC separation of acetylated gitoxin derivatives in the system xylol-methylethyl-ketone 1:1 saturated with formamide after having the silica gel layer with 20% formamide dissolved in acetone. 5 μg per substance, operating time of 45 min at a room temperature of 23°, thickness of layer 0·25 mm.

to dryness *in vacuo*, and the glycoside content was determined by means of the dixanthyl urea method. In three tests 2–3% of the added acetyldigoxin-β were regained in petroleum ether, 10–15% in carbon tetrachloride and 96–99% in chloroform. Because of the relatively good solubility of acetyldigoxin-β in carbon tetrachloride this solvent was not used in further tests for degreasing the biologic material.

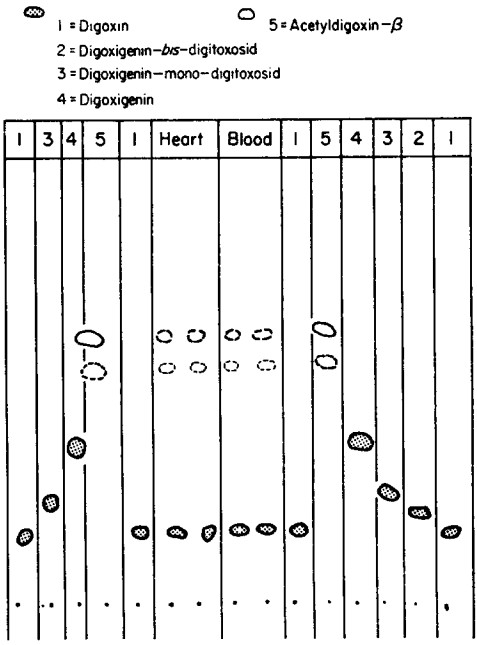

FIG. 4. Proof of acetyldigoxin-β in the heart muscle and blood of rats which received 50 mg/kg of acetyldigoxin-β orally by means of a probang and which were killed 8 hr later. By way of comparison acetyldigoxin-β, digoxigenin, digoxigenin-*bis*-, monodigitoxosid and digoxin were chromatographed simultaneously (as in Fig. 3).

The chromatographic plates layered with silica gel G (Merck) were placed in a gastight sandwich chamber, modified according to Stahl,[25] sprayed with trichloracetic acid-chloramine-reagent and read in u.v. light. Acetyldigoxin-β fades within about ½–1 min so that the identification of the spots must be done very quickly. The sensitivity in glycosides on the thin layer chromatogram was about 200 ng, being ten times more sensitive than by paper chromatography.

Male Wistar rats were used of 120–130 g in weight when from a colony kept for breeding which had not been fed for 24 hr. Acetyldigoxin-β* dissolved in 1·5% pyridine and 20% ethyl alcohol was administered to 10–15 rats each in dosages of 50 or 100 mg/kg, by means of a probang. Between 30 min and 8 hr the animals were killed by a blow in the neck, their blood, obtained by decapitation, was collected in heparin solution, the hearts, removed from the body, were freed from the rest of blood in NaCl-solution and heart as well as blood were frozen in dry-ice. Before the homogenization 10 ml of physiologic salt solution were added, the homogenates shaken

* We would like to thank Fa. Beiersdorf AG, Hamburg, for leaving us the amounts of glycoside.

for 10 min by a mechanical shaker in a 500 ml round-bottomed flask with four times the volume of petroleum ether and after that centrifuged at 4000 *g*. The centrifuged material was shaken two times for 20 min with double the volume of chloroform and centrifuged for 15 min at 4000 *g*. Impurities, contained in attached drops of water were removed by freezing out for 12 hr at −15°, the deep-frozen chloroform was filtered on wadding purified with chloroform and compressed to dryness *in vacuo*.

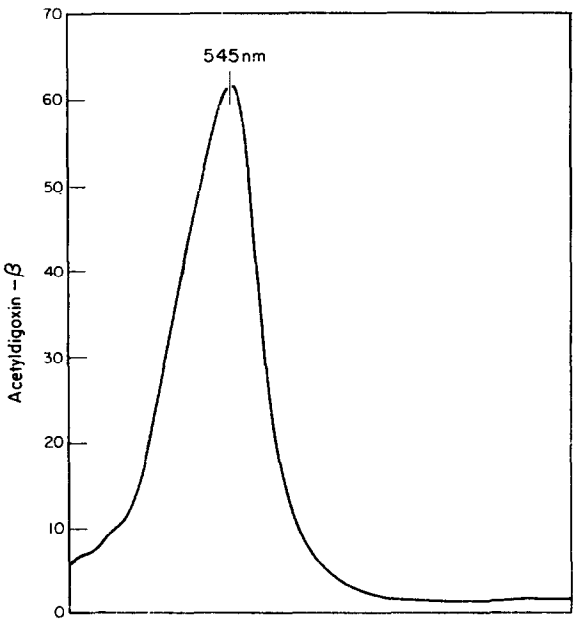

FIG. 5. Spectrum of absorption of acetyldigoxin-β: 50 μg of acetyldigoxin-β were tempered with 5 ml of dixanthyl urea reagent for 10 min at 40°, heated for 3 min in a boiling water-bath and cooled in ice-water for 5 min. The continuous spectrum of the red colour complex was recorded on the Beckman spectrophotometer at a thickness of layer of 1 cm in the range of 400–800 nm.

The oily brown residue was shaken 5 times with 0·6 ml of pure methanol, centrifuged for 40 min, the excessing methanol compressed in vacuum and the residue taken up with 1 ml of 30% methanol. The whitish but still muddy liquid was passed through a Sephadex G 200-column for further purification. The elution followed ascendingly with 30% methanol with a hydrostatic pressure of 60 cm column of water and a dropping velocity of about 3 drops/min. A muddy eluate, obtained at first, is free of glycoside. The investigation of the subsequent eluate showed that the glycosides are in fractions which correspond about the 15th–20th ml of the limpid eluate. The glycoside containing eluate was used after its compression for the dixanthyl urea-reaction or for the TLC.

In order to control the loss from the biologic material we added 25 μg acetyldigoxin-β each to the heart homogenate and blood respectively of 15 rats each and detected 72, 72 and 80 per cent of the added amounts of glycoside in the heart homogenate and 76, 80 and 80 per cent in the blood. In a further test we were able to demonstrate distinctly both glycosides on the TLC after having added 5 μg digoxin and acetyldigoxin-β each to the heart homogenate and blood of the 15 rats.

Because quantitative recovery of the separated metabolites by TLC was only possible with great loss, we were satisfied with a semi-quantitative evaluation of the intensity of the different spots (× till ××××).

RESULTS

The results of 8 single tests are summarized in Table 1. Digoxin was found in the heart muscle and blood in all series of tests. Acetyldigoxin-β was recovered in the blood in all experiments and in the heart muscle in 7 out of 8 experiments. In one series only small amounts of digoxin were detected and all other glycosides were absent; in blood, however, we found higher amounts of digoxin and acetyldigoxin-β.

TABLE 1. SUMMARY OF THE EXPERIMENTS OF RATS WITH ORAL ADMINISTRATION OF 50–100 mg/kg OF ACETYLDIGOXIN-β.

The animals were killed 30 min up to 8 hr after the administration of glycoside. The intensity of the fluorescence, becoming visible on the TLC under the u.v. lamp after trichloracetic acidchloramine reagent was evaluated semi-quantitatively.

Orally administered acetyldigoxin-β		Intensity of fluorescence of the isolated and identified substances of rats' hearts			
reaction (hr)	dosage (mg/kg)	Digoxin	Digoxigenin-mono-digitoxosid	Digoxigenin	Acetyldigoxin-β
½	50	×××	×	×	××
1	50	×			
1	100	××××		××	××
2	100	××××			×××
4	50	××××		×	×××
4	100	××××		××	×××
8	50	×××			××
8	50	×××			×
½	50	×××		×	××
1	50	××××			××
1	100	××××		×	××
2	100	××××			×××
4	50	××××		×	×××
4	100	××××		××	×××
8	50	××××			××
8	50	×××			×

Digoxigeninmonodigitoxosid could be demonstrated only once, whereas digoxigenin was found in 4 out of 8 tests in the heart and blood. Generally the fluorescence of acetyldigoxin-β was weaker than that of digoxin. There was no time-dependent relationship of the amounts of digoxin within the period of action under investigation. On the other hand acetyldigoxin-β was found in the heart muscle and blood in a greater quantity after 2–4 hr of reaction than after 30–60 min or after 8 hr.

DISCUSSION

The investigations under consideration were carried out to learn the behaviour of acetylated glycosides in the organism. The biologic significance of an acetyl substitution in heart glycosides cannot yet be estimated generally. The acetylation of the C_{16}-hydroxyl group alters basically the biologic behaviour. Gitoxin being very insoluble and not resorbed is converted to the well soluble 16-acetylgitoxin

of which 90 per cent is absorbed.[21, 26] Acetyldigitoxin-α and acetyldigoxin-α offer no advantages over their not acetylated derivatives in animal tests,[21, 27] whereas the oral toxicity of higher acetylated digitoxin derivates diminishes with an increasing number of acetyl groups.[21] The fact is known from the clinic that the enteral share of resorption of acetyldigitoxin-α, being acetylated at the C_3-atom of the last digitoxose molecule, amounts to 70–80 per cent as opposed to 80–100 per cent with digitoxin. The acetylation at the C_4-atom of the third digitoxose molecule (β-acetyl-) gives in gitoxin and digitoxin enterally ineffective products,[29] while the β-acetylation of digoxin increases the enteral resorption according to references.[5] Lingner *et al.*[30] analysed using fewer synthetic products of different glycosides. No improvement of the enteral resorption in the cat by acetylation of cymarin, digoxin, K-strophanthosid and convallatoxin was noticed. This bibliographic survey show the diverse biologic behaviour of different acetylated glycosides. An exceptional position is taken by the acetylation of the 16-hydroxyl group and an explanation of this divergent behaviour cannot be given at present.

Contrary to the behaviour after oral administration the toxicity of acetylated glycosides alters homogeneously after i.v. injection. In proportion to the number of the acetyl groups the toxicity of highly acetylated glycosides (pentaacetylgitoxin, tetraacetyldigitoxin, diacetylcymarol) is much smaller than that of their not acetylated starting products.[31, 32] Obviously the acetyl groups are split off very slowly after i.v. injection, so that acetylated glycosides with a sufficient enteral resorption such as 16-acetylgitoxin may require a higher dosage given i.v. clinically than given orally.[26] The slow splitting off of the acetyl groups is probably explained by the smaller share of decay of pentaacetylgitoxin (10%) in comparison with 16-acetylgitoxin (20–25%).

Our results demonstrate that the splitting off of the acetyl group of acetyldigoxin-β does not occur in a quantitative way during the enteral resorption. Acetyldigoxin-β could be shown in the blood and heart muscle of rats 8 hr after oral administration but its amount decreases with time. On the other hand we found digoxin in the rat's heart and blood only 30 min after oral administration, i.e. a partly de-acetylation starts during or soon after the resorption of glycoside. Similar results were obtained with other glycosides (16-acetylgitoxin, pentaacetylgitoxin).[33] After oral administration of pentaacetylgitoxin we found the different acetylated (tetra-, tri-, *bis*- and monoacetyl derivatives) as well as the non-acetylated decomposition-products of gitoxin, coexisting simultaneously in the blood and heart muscle of rats. As in previous tests[33, 34] after administration of digitoxin digoxigenin could be shown to be a metabolite in the blood and heart muscle in several series of tests also after application of acetyldigoxin-β.

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