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Separation of Cardiac Glycosides by Thin-Layer Chromatography

B. J. White[1] and Dennis Oeth

Abstract. Two structurally similar cardiac glycosides, digoxin and digitoxin, and their aglycones, digoxigenin and digitoxigenin, were separated by thin-layer chromatography using glass plates coated with silica gel H. Of 50 solvent systems tried, 15 were found to effect excellent separation of digoxin and digoxigenin. Separation of digoxin and digitoxin and their respective aglycones was achieved with three solvent systems. Spots were visualized with *meta*-dinitrobenzene sodium hydroxide sprays. The lower limit of detection was 10 micrograms of glycoside per square centimeter. This method of separation should prove significant in identifying drug metabolites and determining toxicity levels of cardiac glycosides.

History

The cardiac glycosides, known commonly as digitalis, are steroids with sugar components that give them an unique effect on the heart of living organisms. These potent structures are important medically as some of the most respected and useful drugs in the medical armament against disease.

Cardiac glycosides have long been used, both for their beneficial and toxic effects. As early as 1500 B.C., there have been accounts of the Egyptians using these compounds for herb medicines. The Romans knew of their diuretic and emetic qualities, and the potent cardiac active drugs have been notoriously used as arrow poisons by natives throughout the ages.

Not until William Withering, an English physician, published his text on the effects of digitalis in 1785 was much scientifically known about these compounds (1). His work, called *An Account of the Foxglove and Some of Its Medicinal Uses: With practical remarks on dropsy and other diseases,* contained standard preparations for digitalis and discussed its effects on such diseases as dropsy and edema. He was not sure that it worked specifically on the heart muscle, but Withering was well aware of its effects on problems stemming from congestive heart failure.

Today, these drugs stand out in the physician's repertoire of

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medicines. These pharmacologically dynamic steriods give strength to the weak or failing heart, improve its tonus and contractility power, and regularize its rhythm. Congestive heart failure problems, such as edema, excessive accumulation of bodily fluids in the lower extremities, irregular heart rhythms, and even complete arrest can be treated (2).

One must remember, however, that digitalis is a potent drug, and if not properly administered it can be very toxic and poisonous. Knowledge of precise dosage amounts, the duration of toxic effects, and the individual patient's tolerance to these drugs is still inconclusive. The pharmacological researcher is thus concerned with ascertaining rates of metabolism of the injected drug and correlating the percentage of recovery of unaltered drug with toxic effects.

Source

The word "digitalis" comes from the two plant sources of these drugs, *Digitalis lanata* and *Digitalis purpurea* (purple foxglove). There are four main digitalis derivatives: digoxin, digitoxin, gitoxin, and gitalin. Digitoxin and gitoxin can be extracted from either plant source, while gitalin can be extracted only from *D. purpurea* and digoxin can only be extracted from *D. lanata.* The drugs are prepared from their raw state by mild alkaline hydrolysis to remove acetyl groups and enzymatic cleavage to remove glucose sugars. By successive alcoholic extractions, the drugs can be obtained in pure crystalline form for drug usage (3).

Structure

These digitalis derivatives are basically a 4-ringed steroid structure with an unsaturated 5-membered lactone ring. A sugar moiety consisting of three digitoxose sugars is connected by a glycosidic linkage to the steroid at the C-3 position. Digitoxose sugar is a 2, 6'-dideoxy aldohexose. All of the digitalis derivatives have methyl groups at C-10 and C-13. Digitoxin has a hydroxy group at C-14, digoxin has an hydroxy group at C-12 and C-14, and gitoxin has a hydroxy group at C-14 and C-16. Gitalin is a mixture of inert ballast substances and the other steroids.

The cardiac glycosides, so called because of their action on the heart by a sugar-bearing compound, can be reduced to their aglycone form (without sugar) by either acid hydrolysis or biological metabolism. The liver and heart are important organs of detoxification. The more sugar moieties the drug has, the more potent or toxic it is. As it successively loses one, two, or all three of its sugars, it is rendered less potent.

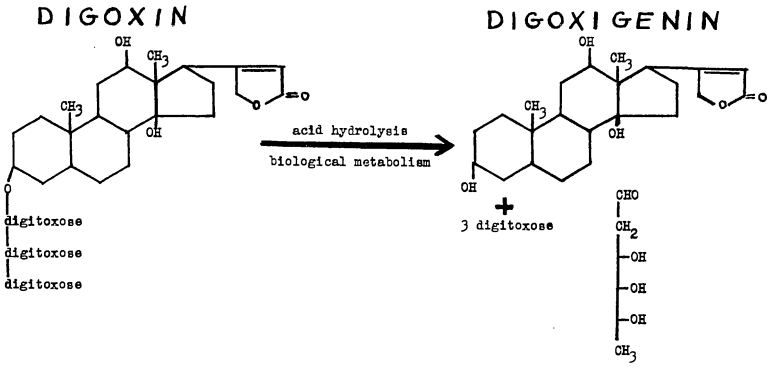

Figure 1. Detoxification of digoxin to its aglycone, digoxigenin.

Current Research

The problem of the pharmacologist is to accurately separate the glycoside from its metabolite in the aglycone form. This glycoside to aglycone ratio is helpful in determining the rate of metabolism, the toxicity of the drug, and the duration of its effects. By applying this practically to medicine, proper dosage ratios can be adjusted for every patient's tolerance.

The difficulty in separation arises from the similarity of molecular weights, chemical structure, and the fact that work is being done on very small quantities of biological substances. The researcher needs a special investigative tool for quick separation and identification.

Thin-layer chromatography can serve as the answer to this problem. It has been shown to be useful for many steroid separations; it is fast, convenient, inexpensive, and it works particularly well on small quantities of recovered compounds.

Experimental Procedure

In this study, 50 different solvent systems in varying concentrations were compared to establish which solvent systems might serve best for thin-layer chromatography of the cardiac glycosides. Some of these solvents were reported in the literature, others have been known to work well on other steroid systems (4), and others were first attempts (5).

The two drugs, digoxin and digoxigenin, were used primarily for this investigation. (Drugs were supplied through the courtesy of Burroughs Wellcome & Company Pharmaceuticals, Tuckahoe, N.Y., and from Dr. James L. Spratt, Department of Pharmacology, College of Medicine, University of Iowa). A standard solution was prepared with 1 mg of drug to 1 ml of absolute

ethanol and stored in glass-stoppered flasks to prevent evaporation or uptake of water.

The chromatography equipment consisted of six cylindrical jars, about 30 cm high and 7 cm in diameter; these jars are of the type used to display biology specimens. Each jar had a ground glass rim that sealed well when covered with a plate glass top. A large glass block-type chamber was also used.

The two types of chromatography plates were 3.8 mm thick, 20 cm high, and 5 cm and 20 cm wide; they are found with the popular Desaga chromotography sets. A Desaga spreader was used for coating the plates.

The plates were thoroughly cleaned and flushed with distilled water before use. The plates were then coated at a thickness of .25 mm with silica gel H according to the procedure of Stahl, and stored in a cabinet with dessicant. No special activation or deactivation steps were taken.

The drugs were spotted on the prepared plates with B-D Hypak disposable syringes (2½cc 22 G 1½), one drop at a time. The spots were kept as small as possible to minimize the spreading during chromatography. Three separate spots were made on each plate: one 25 microliter spot of the 1 mg/ml standard solution of pure digoxin, one mixed spot of 25 microliters each of digoxin and digozigenin, and one pure spot of 25 microliters of digoxigenin. In this manner the central mixed spot could be observed for separation while the outer pure spots provided comparison. The spots were allowed to dry thoroughly before starting chromatography.

The dry plates were set upright in a jar, 25 ml of solvent was added by pouring down the side of a stirring rod, and the jars were quickly covered. No prior equilibration time was allowed and no filter paper linings were considered to be necessary due to the small volume of the jars. After the solvent front had reached the top of the plate (generally one to two hours), the plate was removed and allowed to air dry.

Meta-dinitrobenzene (10 grams in 200 ml benzene) and 16% NaOH were successively sprayed on the plates to develop the drugs. Spots were usually blue, brown, or orange and faded quickly. This spray is specific for the lactone ring in the steroid structure. R_f values of all three spots, their dimensions and other characteristics were noted immediately.

Results

Of approximately 50 solvent systems, 15 were found to give excellent separation with an R_f difference between the digoxin and the digoxigenin greater than .10. Listed below are the better

ten of these solvents, in order of decreasing R_f difference. The optimum solvent mixtures are given.

Table 1. TLC of Cardiac Glycosides

SOLVENT SYSTEM	OPTIMUM MIXTURE	Rf Digoxin	Rf Digoxigenin	DIFFERENCE $Rf_G - Rf_D$
$CHCl_3$ / MeCOEt	(1:4)	.22	.46	.24
BuOH / EtOAc	(1:20)	.17	.39	.22
EtOAc / MeOH	(4:1)	.30	.51	.21
EtOAc / benzene / H_2O upper phase	(26.4:15)	.10	.30	.20
$CHCl_3$ / BuOH	(1:4)	.62	.81	.19
cyclohexane / EtOAc	(2:8)	.08	.27	.19
ether / $HCONMe_2$	(20:1)	.38	.57	.19
ether / MeOH	(20:1)	.32	.51	.19
EtOAc	pure	.08	.27	.19
ether	pure	.13	.30	.17

The smallest amount of digoxin that could be visually detected was 5.5 microliters of the 1 mg/ml standard solution, or an amount corresponding to 5.5 micrograms of pure digoxin. A more positive limit for successful detection was 7.4 microliters or 7.4 micrograms of digoxin. This work was done with an EtOAc/MeOH (4:1) solvent system and the metadinitrobenzene/NaOH spray.

Digitoxin and its aglycone, digitoxigenin, were also successfully separated using some of the previous solvents. By preparing these drugs in a 1 mg/ml solution of absolute alcohol and spotting on the larger 20 cm by 20 cm plates, simultaneous separations of both digoxin, digitoxin, and their aglycones were achieved. Note that digoxin differs from digitoxin only by the presence of an extra hydroxy group at C-12 as do their respective aglycones, thus making them difficult to separate by other means. The three solvent systems below were found to be exceptionally good for these drug separations.

Table 2. Comparative TLC of Cardiac Glycosides

	approximate Rf values			
	digoxin	digoxigenin	digitoxin	digitoxigenin
$CHCl_3$ / MeCOEt (1:4)	.30	.62	.54	.89
$CHCl_3$ / acetone (7.3)	.08	.30	.17	.69
BuOH / EtOAc (1:9)	.40	.62	.54	.69

Although there are several reports in the literature of detecting these steroids by u.v. light, we failed to do so. Lamps of both long and short u.v. wavelengths were used both before and after chromatography, but never were the drugs visible. The silica gel H used as a coating may possibly be blamed for this failure.

Commercial Chromatography Paper

Eastman Chromagram sheets, type K301R2 and K301R with fluorescent indicator supplied by the Eastman Kodak Company

were tested in some of the better solvents. These sheets are precoated plastic and are popular among experimenters because they are convenient and eliminate much of the labor needed to coat glass plates. Fair to good results were obtained in most cases, although the spots were less distinct, harder to visualize, faded quicker, and were subject to more tailing and spreading than their glass counterparts.

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Correlating Data Which Fit the Gompertz Equation of Growth

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Abstract. The Gompertz equation describes the decay of biological systems in which the rate of decrease of the concentration of an organism varies directly with its concentration and inversely with the concentration of an inhibitor. The inhibitor concentration falls exponentially with time. Four methods are compared for correlating data on the destruction of Ascites tumor cells which fit the Gompertz equation: analytical, graphical, analog computer, and digital computer.

The Gompertz equation (1) describes the rate of decay of a substance, where the decay is inhibited by a second substance which is also decaying. A common form of the equation as it is encountered in biology is

$$\frac{dy}{dt} = \frac{-k_1 y}{x} \bullet \qquad (1)$$

Here y is the concentration of the primary substance which is decaying, and x the concentration of the inhibitor. The concentration, x, can be expressed as a falling exponential

$$x = x_o e^{-k_2 t} \qquad (2)$$

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