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The Effect of Various Cardenolides and Bufadienolides with Different Cardiac Activity on the ^{86}Rubidium Uptake of Human Erythrocytes

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Summary. The inhibitory effect of 23 cardiac glycosides, genins and derivatives on the ^{86}Rb-uptake of human erythrocytes was measured. Proscillaridin and its acetates, methyl ethers and β-epoxide were the most active inhibitors of ^{86}Rb-uptake, followed by ouabain, digitoxin and digoxin. Inhibition induced by genins occurred at concentrations distinctly higher than with the glycosides. Substances with 3-α-configuration were less active than those with 3-β-configuration.

The glycoside concentrations exerting half maximal inhibition of ^{86}Rb-uptake were correlated with the minimum lethal doses in guinea pigs ($r = 0.71$) and cats ($r = 0.75$).

Key words: Cardiac Glycosides — Cation Transport — Plasma Concentrations — ^{86}Rb-Erythrocyte-Assay.

Active uptake by erythrocytes of potassium was demonstrated to be inhibited by cardiac glycosides twenty years ago (Schatzmann, 1953). This effect is due to an inhibition of Na-K-ATP-ase activity (Post *et al.*, 1960; Dunham and Glynn, 1961).

Potassium uptake is inhibited at such low concentrations of glycosides as are present in plasma of patients under digitalis therapy. Therefore Lowenstein (1965) measured plasma concentrations of cardiac glycosides by that way. Potassium had to be replaced by rubidium, since available potassium isotopes are impractical for standard laboratory techniques. ^{86}Rb is taken up similar to potassium (Love and Burch, 1953). Parameter analysis and specificity of the ^{86}Rb-erythrocyte-assay were established previously (Belz *et al.*, 1972; Vollmer *et al.*, 1972; Wissler *et al.*, 1972). The clinical validity of this assay depends upon its ability to measure that part of glycoside activity being responsible for its cardiac action.

The purpose of this investigation was to correlate the inhibitory activity of a series of cardiac glycosides on the ^{86}Rb uptake with their cardiotoxicity, as expressed by their minimum lethal dose. The time-independent minimum lethal dose in cats is considered to be the best

pharmacological parameter of the cardiac glycosides in animals (Lenke and Schneider, 1969). A positive correlation should support the thesis that plasma concentrations determined by the ^{86}Rb-uptake method are representative of the cardioactivity of the glycosides or their metabolites. Special emphasis was laid on the scillarenin glycosides and their derivatives.

Method and Materials

The following substances were kindly supplied by Dr. Kubinyi and Dr. Steidle, Knoll A.G., Ludwigshafen/Rhein: Proscillaridin, -3'acetate, 4'-acetate, -3'-methyl ether, -4'-methyl ether, -β-epoxide, -α-epoxide; 3-β-scillarenin, 3-α-scillarenin, scillarenin 3-β-methyl ether and -3-α-methyl ether; scillarenin-3-β-methyl ether-4-β-5 and -4-α-5-epoxide; canarigenin-3-β-methyl ether and canarigenin -3-β-methyl ether-4-α-5-epoxide.

Digitoxin and lanatoside C were from E. Merck, Darmstadt, digoxin, digoxigenin, -mono-digitoxoside, -bisdigitoxoside and β-methyldigoxin from Boehringer, Mannheim, ^{86}Rb chloride (initial specific activity 5.34 mCi/mg) was from Amersham-Buchler, Braunschweig.

Laboratory work was done with Eppendorf Microliter System, including microliter pipettes, except application of erythrocytes which were measured with a 500 µl tuberculin syringe. A Telefunken γ-counter was used (zero effect 50—60 cpm).

Glycosides were dissolved to 1.05×10^{-3} M in ethanol 70%. The solutions were diluted with sterile saline to the following concentrations: 10^{-10}, 3×10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7} M. For proscillaridin, 3-α-scillarenin, scillarenin-3-α-methyl ether, scillarenin-3-β-methyl ether-4-α-5-epoxide, canarigenin-3-β-methyl ether, canarigenin-3-β-methyl ether-4-α-5-epoxide and digoxigenin concentrations of 10^{-6} and 10^{-5} M were also included. In order to compensate for variations in the erythrocytes from different donors a proscillaridin standard curve was run with each sample of erythrocytes. Each determination of Rb-uptake was done as triplet.

For preparation of the erythrocytes venous blood was taken with 3.8% Na-citrate 10:1 from healthy fasting men. After centrifugation plasma and leucocytes were separated from the erythrocytes by suction. Erythrocytes were washed twice the 5-fold volume of sterile saline. Erythrocytes packed at $1200\times g$ (tip) were used within the same day. Each reaction vessel was filled with 1 ml sterile saline, containing the glycoside and 400 µl of the erythrocytes. The mixture was preincubated for 2 h at 37°C.

Thereafter 1 µCi ^{86}Rb-activity and 1 mg of glucose in 50 µl of saline was added and incubated for 2 h at 37°C. After centrifugation the supernatant was discarded. The remaining erythrocytes were washed twice with 1 ml of saline at 0°C in order to remove the extracellular radioactivity. After the last suctioning ^{86}Rb-γ-activity incorporated by the erythrocytes was directly counted for 1 min. Impulse rates ranged from 1500 to 3500 cpm.

Calculations. The median of three determinations without glycosides were defined to be 100% ^{86}Rb-uptake, and the percentage of ^{86}Rb-uptake of the remaining samples was calculated.

From the 13 single proscillaridin standard curves a mean standard curve was calculated. This mean standard curve is shown in Fig. 1.

The glycosides concentration having half maximal effect are called C_{50}. The C_{50} values were obtained by graphical logit regression. The C_{50} value of the mean proscillaridin standard curve was used as reference for all the other curves by adjusting the C_{50} points of the daily proscillaridin standard curve to this point. Thus individual differences of the various erythrocyte charges could be compensated for. The minimal ^{86}Rb-uptake also varied with the erythrocyte donor. These values were not compensated for.

Minimum Lethal Doses of the Cardiac Glycosides. The cardiotoxic effects of the cardiac glycosides (except digoxigenin, -mono-, and -bis-digitoxide and canarigenins) were examined in guinea pigs in urethane anesthesia (1.5 g/kg subcutaneously), and in cats in chloralose anesthesia (70 mg/kg by intraperitoneal route). The glycosides in solution with ethanol (1.25 to 50% v/v) were given intravenously by continuous infusion until cardiac arrest occurred. This was verified by ECG recordings (electrical base line). 3 to 9 different rates of infusion velocities were used for each substance with 2 to 5 animals for each infusion velocity. The time-independent lethal dose was calculated from the individual lethal doses of each experiment using the method of Lenke and Schneider (1969, 1970).

Results

None of the substances tested inhibits ^{86}Rb-uptake of the erythrocytes completely. The maximal inhibition depends on the individual erythrocytes and not on the substances used.

Proscillaridin, Proscillaridin-3'-Methyl Ether, Proscillaridin-4'-Methyl Ether, Proscillaridin-3'-Acetate and Proscillaridin-4'-Acetate

Fig. 1 presents the mean standard curve of proscillaridin. Inhibition of ^{86}Rb-uptake starts at concentration as small as 10^{-10} M. Half maximal inhibition is observed at 1.6×10^{-9} M (range from 0.7 to 3.6×10^{-9} M). A maximal effect occurs at concentrations of 3×10^{-8} M.

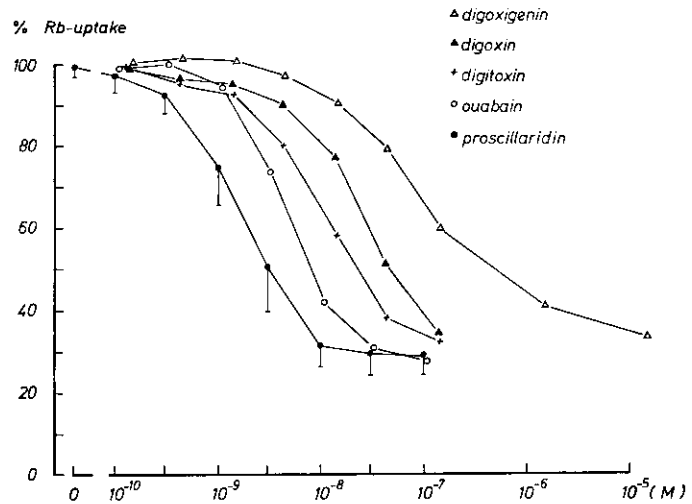

Fig. 1. Inhibition of ^{86}Rb-uptake. The proscillaridin curve is calculated from 13 single curves, mean values and standard deviation are shown. For the remaining substances median values or triplets are given

Introduction of a methyl or acetate group in position 3′ or 4′ to the rhamnose does not change the activity of these substances as compared with proscillaridin. These results well correspond to the results of pharmacological experiments in cats.

3-β-Scillarenin, 3-α-Scillarenin, Scillarenin-3-β-Methyl Ether and Scillarenin-3-α-Methyl Ether

Inhibition of ^{86}Rb-uptake induced by the genins of proscillaridin and by their methyl ethers occurs at distinctly higher concentrations than with proscillaridin. Substances with 3-α-configuration are again less active than the ones with 3-β-configuration. These observations are in accordance with the corresponding pharmacological data for these genins.

Proscillaridin-β-Epoxide and Proscillaridin-α-Epoxide Scillarenin-3-β-Methyl Ether-4-β-5-Epoxide and Scillarenin-3-β-Methyl Ether-4-α-5-Epoxide

The epoxides are characterized by an oxygen bridge at the C-atoms in position 4 and 5 of the genin. Proscillaridin-β-epoxide is as effective as proscillaridin in the ^{86}Rb-erythrocyte assay. The corresponding α-epoxide is much less active.

The same is true for the epoxides of scillarenin-3-β-methyl ether. These in vitro results are in accordance with the experiments in cats.

Canarigenin-3-β-Methyl Ether and Canarigenin-3-β-Methyl Ether-4-α-5-Epoxide

Canarigenin is a genin of the cardenolide family, differing from scillarenin only at position 17. To produce the same inhibition of ^{86}Rb-uptake, 20 times higher concentrations of the cardenolides than of the bufadienolides are needed. Pharmacological data for the canarigenin derivatives are not available. The results are, however, in accordance with the general observation, that bufadienolides are more active than the analogous cardenolides.

Digoxigenin, Digoxigenin-Mono-Digitoxoside, Digoxigenin-Bis-Digitoxoside, Digoxin, β-Methyl Digoxin and Lanatoside C

Digoxigenin is the weakest, digoxigenin-mono-digitoxoside the strongest member of the family. The activity decreases from the monoside to the bioside and the trioside. The tetroside lanatoside C is positioned between the mono- and the bioside. β-methyl digoxin shows an activity nearly identical to digoxin.

The sequence of activity observed here is analogous to the sequence in the animal experiment (Kronenberg, 1959; Chen 1970; Schaumann and Wegerle, 1971; Böttcher *et al.*, 1973).

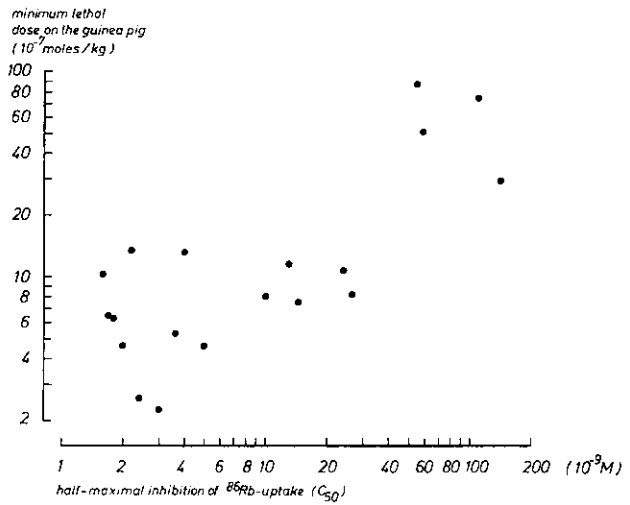

Fig. 2. Correlation between glycoside concentration at half maximal inhibition of ^{86}Rb-uptake in human erythrocytes and minimum lethal dose in guinea pigs ($r = 0.71$, $P < 0.001$)

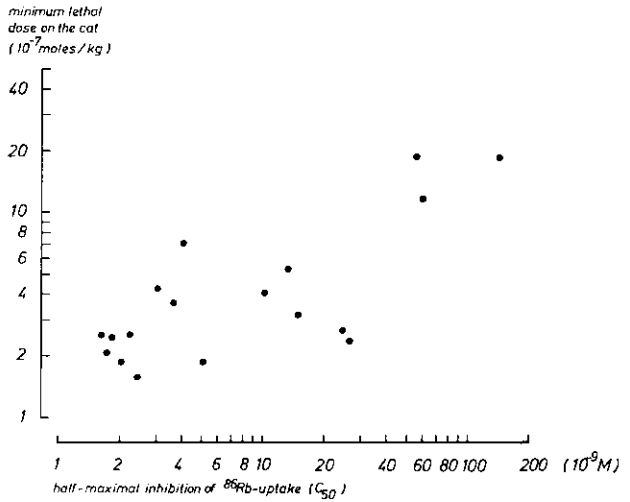

Fig. 3. Correlation between glycoside concentration at half inhibition of ^{86}Rb-uptake in human erythrocytes and minimum lethal dose in cats ($r = 0.75$, $P < 0.001$)

Ouabain and Digitoxin

Ouabain shows the highest molar activity of all cardenolides tested, corresponding well to the animal experiments. Digitoxin is less active than ouabain.

Table 1. Activity of the glycosides and genins in ^{86}Rb-erythrocyte-assay and in vivo. In vitro activity of the substances is defined by that concentration which produces a half maximal inhibition of ^{86}Rb-uptake (C_{50}) in human erythrocytes. Effects of the same substances on the heart of guinea pigs and cats is indicated by their minimum lethal dose independent of time (Lenke and Schneider, 1969)

Substance	Half maximal inhibition of human erythrocytes ^{86}Rb-uptake (C_{50}) (10^{-9} M)	Minimum lethal dose independent of time (D)				Ratio $\frac{D}{C_{50}}$	
		cat		guinea pig		cat	guinea pig
		µg/kg	10^{-7} moles/kg	µg/kg	10^{-7} moles/kg		
Proscillaridin	1.6	134	2.52	545	10.3	1.58	6.44
Proscillaridin-3′-acetate	1.7	119	2.08	374	6.53	1.22	3.84
Proscillaridin-4′-acetate	1.8	139	2.43	357	6.23	1.35	3.46
Proscillaridin-3′-methyl ether	2.0	101	1.86	252	4.6	0.93	2.30
Proscillaridin-4′-methyl ether	2.2	138	2.53	735	13.5	1.15	6.14
Proscillaridin-β-epoxide	2.4	87	1.59	141	2.58	0.66	1.08
3-β-scillarenin	3.0	164	4.27	860	2.24	1.42	0.75
Proscillaridin-α-epoxide	3.6	195	3.57	294	5.38	0.99	1.49
Scillarenin-3-β-methyl ether	4.0	285	7.15	529	13.27	1.79	3.32
Ouabain	5.0	109	1.86	270	4.61	0.37	0.92
Digoxigenin-mono-digitoxoside	8.5						
Digitoxin	10.0	310	4.05	618	8.08	0.40	0.81
Scillarenin-3-β-methyl ether-4-β-5-epoxide	13.0	221	5.34	476	11.5	0.41	0.89
Lanatoside C	14.5	312	3.17	737	7.48	0.22	0.52
Digoxigenin-bis digitoxoside	15.0						
Digoxin	24.0	210	2.69	837	10.72	0.11	0.45
β-methyl digoxin	26.5	190	2.39	653	8.21	0.09	0.31
Scillarenin-3-α-methyl ether	55	762	19.12	3468	87.03	0.35	1.58
3-α-scillarenin	59	446	11.6	1966	51.13	0.20	0.87
Canarigenin-3-β-methyl ether	110			2953	74.48		0.68
Digoxigenin	140						
Scillarenin-3-β-methyl ether-4-α-5-epoxide	140	772	18.62	1200	28.95	0.13	0.21
Canarigenin-3-β-methyl ether-4-α-5-epoxide	7500						

Half Maximal Inhibition of the ^{86}Rb-Uptake and Minimum Lethal Doses

The C_{50} values of the 24 glycosides tested are compiled in Table 1 together with the minimum lethal doses.

The ratio between minimum lethal doses and C_{50} with cats and guinea pigs decreases from the highly active to the less active substances.

We observe good correlation between the logarithms of C_{50} values of the glycosides on the one hand and the logarithms of the values for the minimum lethal dose (molar) obtained for guinea pigs on the other hand ($r = 0.71$, $P < 0.001$) (Fig. 2). A slightly better correlation is found between the C_{50} values and the minimum lethal doses measured in cats (Fig. 3; $r = 0.75$; $P < 0.001$).

Discussion

A correlation between the inhibition of active potassium uptake and the cardiac activity of various glycosides had already been shown in former experiments using "Kälteerythrocyten" (Kahn and Acheson, 1955; Solomon *et al.*, 1956; Glynn, 1957; Machova, 1960; Grobecker *et al.*, 1963; Greeff and Schlieper, 1967). Comparison of glycosides with and without cardiac activity indicated a specificity of the rubidium method for the cardiac activity of glycosides (Vollmer *et al.*, 1972). This specificity was questioned by Somogyi *et al.* (1972) on the finding of one case of intoxication with high plasma levels but no toxic signs ten days after ingestion of digitoxin.

Considering the good correlations between the activity of the cardiac glycosides in our assay and the cardioactivity one should be aware of two facts: First we correlate values obtained from different species (man—guinea pig, resp. cat), second the values of toxicity analysis in vivo are modified by parameters like distribution, elimination etc. in various degrees for the different glycosides and derivatives.

After application in man, cardiac glycosides may undergo biotransformation (Repke, 1963; Marks *et al.*, 1964; Marcus *et al.*, 1966a, b, c; Wilson, 1969; Somogyi *et al.*, 1972; Selden and Smith, 1972). Thus both original glycosides and its metabolites may be present in the plasma samples. Our observations demonstrate that the cardiac effects of glycosides as well as of their facultative metabolites show a correlation with their activity in the ^{86}Rb-assay, and that the ratio between the doses effective in vivo and in vitro decreases with decreasing absolute activity of the substances.

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