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CHROMATOGRAPHIC ANALYSIS OF DIGITALIS GLYCOSIDES AND THEIR RELATION TO SOLUBILIZATION OF MUSCARINIC RECEPTOR

(Presented by O. Kirret)

Introduction

Glycosides from *Digitalis purpurea* are known as physiologically active compounds and several of them are used as therapeutic agents [1]. Besides that some glycosides from this group have been used as effective tools for solubilization of protein components of biological membranes [2]. Moreover, in some cases these compounds seem to present unique possibility to extract particular membrane proteins in the active form [3]. Muscarinic receptor belongs to the latter group of proteins and it retains ligand binding properties if solubilized by digitonin preparations [4–10]. Several other substances were also applied for this purpose but they have all been found less effective [6, 8, 10–13].

It has been shown by several authors that the effectiveness of solubilization of muscarinic receptor depends on the particular lot of digitonin preparation applied [10]. The latter fact clearly points both to the complex nature of the solubilization process and to heterogeneity of the commercially available digitonin preparations.

An attempt has been made by H. Repke and H. Matthies to analyze the dependence between digitonin and gitonin content of the detergent preparations and the effectiveness of solubilization of muscarinic receptor from rat brain membranes [14]. Both these components were considered important for the receptor solubilization giving the maximal yield of the solubilized receptor at digitonin/gitonin ratio 3 : 2 [15]. However, other components of the commercial preparation of digitonin, which can be detected by HPLC or HSCCC (high-speed centrifugal countercurrent chromatography) [16], were not analyzed in this study. Moreover, the possible dependence of the effectiveness of the receptor solubilization upon the ratio of protein-detergent concentration has not been taken into account by these authors [14]. Thus, neither the role of different components in the commercial digitonin preparations nor optimal conditions for the receptor solubilization are still not clear.

In the present study a complex TLC and HPLC analysis of nine different digitonin preparations from Merck, Fluka and Sigma was carried out and the composition of the solutions of these samples was correlated with the yield of receptor solubilization. Four components were separated by both TLC and HPLC methods in all samples investigated. Two major peaks (C_1 and C_2) followed with two minor peaks (C_3 and C_4) were eluted from Separon SGX C18 column with methanol-water (70/30 v/v) (Fig. 1a) on Zorbax ODS column with acetonitrile-water (40/60 v/v) (Fig. 1b). The location of the same components was identified on the TLC

Table 1

Composition of commercial digitonin preparation

Sample number	Origin of samples and lot number	Components, %			
		1	2	3	4
1	Merck 3832317	66.1	15.7	12.5	5.6
2	Merck 3193823	41.9	36.9	8.4	12.9
3	Sigma 33F-0061	72.5	3.1	17.8	6.6
4	Merck 3934961	54.6	21.3	14.5	9.4
5	Ockardt Munchen	48.6	23.0	17.2	11.1
6	Merck 7430254	56.9	27.5	9.2	6.4
7	Fluka 125136 49K	83.9	1.5	10.7	3.8
8	Sigma D-5628	50.7	21.8	15.8	12.2
9	Merck 31114898	84.3	1.5	11.0	3.2

plates after their preparative separation. Proceeding from the R_f values for digitonin (C_1) and gitonin (C_2) these compounds were identified as the first and the second main components using methanol/water mixture (Fig. 1a) and the first and the third main components using acetonitrile/water mixture (Fig. 1b). The chemical structure of the following components was not characterized, except the fact that the fourth compound (C_4) separated by HPLC involves the lactone group, identified by a specific colour reaction [17] and by the IR spectrum of the lyophilized fraction of this compound isolated by preparative HPLC. The data of the relative content of these four components in the commercial preparations of digitonin are listed in Table 1. Our assumption was that the variable composition of these samples is responsible for the different properties of these preparations, i. e. different solubility and effectiveness of protein and muscarinic receptor solubilization. These putative interrelationships between the composition of the samples and these properties are discussed in the following analysis.

Table 2

The average composition of the commercial preparations, saturated water solution and precipitates for the studied digitonin preparations

Component	Commercial preparation	Solution	Precipitate
C_1	62±14	65±10	44±11
C_2	17±11	19±8	26±5
C_3	13±3	12±4	14±3
C_4	8±3	5±3	15±8

The solubility of different components can be characterized by the changes in their relative content in the initial samples, in their saturated water solutions and in precipitations as illustrated in Table 2. It can be seen that in the water phase digitonin (C_1) prevails, while in the precipitate the amounts of the components C_2 and C_4 have markedly risen. On an average a crude commercial digitonin preparation consists of 80% water-soluble material. Digitonin solutions with concentrations up to 0.75% can be obtained (Table 3). Simultaneously other components remain in the precipitate.

Table 3

Composition of saturated solutions of commercial digitonin preparations and the yields of protein and receptor solubilization

Sample number	Total concentration of sample in solution	Concentration of components in solution				Yield of solubilization		
		C_1	C_2	C_3	C_4	protein	receptor	
1	0.71	0.49	0.11	0.09	0.02	59.7	28.5	21.5
2	0.38	0.20	0.14	0.03	0.01	42.2	13.8	10.0
3	0.97	0.75	0.11	0.09	0.02	59.3	23.5	19.4
4	0.53	0.34	0.10	0.07	0.02	65.7	21.5	14.6
5	0.79	0.36	0.16	0.15	0.12	62.0	31.4	24.3
6	0.76	0.42	0.22	0.08	0.04	67.5	18.0	18.0
7	0.90	0.64	0.07	0.13	0.05	68.4	42.1	34.1
8	0.60	0.40	0.11	0.08	0.01	25.0	10.9	10.9
9	0.96	0.74	0.11	0.09	0.02	37.0	53,9	53.9

In all solubilization experiments the same protein concentration was used (0.6 mg per ml). Thus the results can be compared by making use of the values of the solubilization yields given in per cent. The appropriate data are listed in Table 3. It can be seen from these data that there is no correlation between the overall concentration of the solubility of commercial preparation of detergent and the yield of solubilization of the membrane proteins ($R=0.05$) (Table 4). On the other hand, the correlation between the yield of the solubilized muscarinic receptor and the overall concentration of the detergent in solution is more significant ($R=0.64$), pointing to different nature of these processes (Table 4, Fig. 2).

Table 4

Correlation between the yield of protein and muscarinic receptor solubilization and concentration of components of saturated solutions of digitonin

Components	Correlation coefficient	
	protein	receptor
Crude digitonin	0.05	0.64
fraction C_1	0.10	0.63
fraction C_2	0.19	0.33
fraction C_3	0.26	0.48
fraction C_4	0.42	0.18

Further we analyzed the correlation between the yield of solubilization of muscarinic receptor and the concentration of particular components of the commercial samples of detergent, in phosphate buffer used for treatment of brain membranes (Table 3). The results obtained are listed in Table 4. It can be seen that there is no correlation between the content of any fractions and the yield of total protein solubilization. On the other hand, for the receptor solubilization the most significant correlation appears in the case of digitonin (C_1) while the variation of the amount of gitonin (C_2) in the mixture has remarkably less effect.

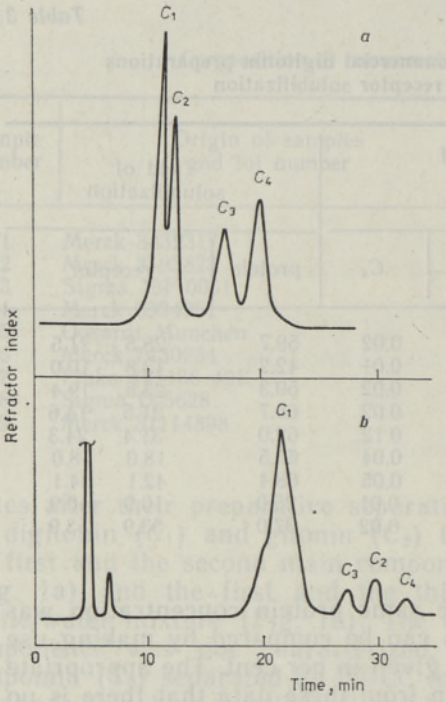

Fig. 1 HPLC of the digitonin preparation on the Separon SGX C18 column with methanol-water (70/30 v/v) (*a*) and Zorbax ODS column with acetonitrile-water (40/60 v/v) (*b*).

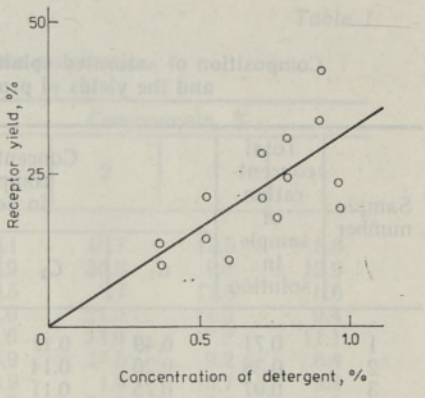

Fig. 2 Dependence of rat brain muscarinic receptor solubilization yield from the solubility of the digitonin used.

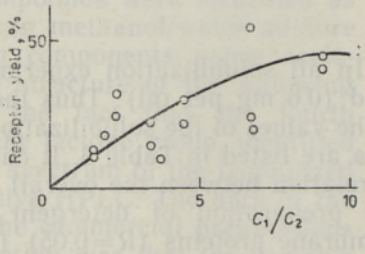

Fig. 3 Dependence of rat brain receptor solubilization yield from the ratio of digitonin concentration (C_1) to the gitonin concentration (C_2) in solution.

The dependence of the yield of the receptor solubilization upon the ratio of digitonin and gitonin concentrations is shown in Fig. 3. It can be seen that there is no optimal composition of the detergent mixture for the receptor solubilization. Thus the present analysis does not confirm the previous data published in [15] about the optimal digitonin and gitonin ratio in buffer used for solubilization of muscarinic receptor.

In summary, digitonin seems to be the most important component for solubilization of the brain muscarinic receptor and the yield of solubilization seems to be determined mainly by the concentration of this component in the solution. However, the content of digitonin in several commercial preparations was different and therefore their solubilization capability was also different. On the other hand, only a part of the membrane-bound muscarinic receptor can be solubilized by digitonin solutions [9], while the repeating extraction of membranes with new amount of the detergent did not increase the solubilization yield. Thus the latter fact seems to be connected with some structural peculiarities of the membrane-bound protein in its complex with lipids or other proteins in biomembrane. These facts as well as the selective solubilizing effect of digitonin among other closely related digitalis glycosides point to the importance of further studies on the structure of the receptor-lipid complex.

Experimental

Commercial digitonin preparations were obtained from Merck, Fluka, Sigma and Ockardt and their lot numbers are listed in Table 1. 1% solution of these preparations in K-phosphate buffer (pH 7.4) was prepared

at 80 °C and stayed overnight at 4 °C. The pellet formed during this time was separated by filtration and the obtained solution was used for the following solubilization experiments. Thin layer chromatography (TLC) was carried out on Silufol UV 254 (Kavalier) and Kiselgel 60F254 (Merck) pre-coated plates. The plates were eluated with mixtures of chloroform-methanol-water (65/35/5 v/v) or with acetonitrile-water (40/60 v/v) and the chromatograms were revealed with anisaldehyde according to [18].

The separation of digitonin by HPLC was carried out on the columns Separon SGX C18 (3.3×150 mm) (Laborotorni Pristroje) and Zorbax ODS C18 (0.4×250) (Dupont) using «Laborotorni Pristroje» (Czechoslovakia) HPLC system. The samples were eluated with mixtures methanol-water (70/30 v/v) or acetonitrile-water (40/60 v/v) at flow rate 0.4 ml/min at room temperature. The peaks were registrated with differential refractometer and the fractions were collected and further analyzed with TLC.

Concentration of the rat brain muscarinic receptor was determined by the specific binding of L-[^{3}H]quinuclidinyl benzilate, measured by the common filtration assay in the case of membranes [13] or by the gel-filtration assay in the case of solubilized receptor [9]. Protein concentration was determined by the modified Lowry method [19] using bovine serum albumine as a standard.

IR spectra were measured on the spectrometer IKS 29 (Lomo, USSR).

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DIGITAALISE GLÜKOSIIDIDE KROMATOGRAAFILINE ANALÜÜS JA NENDE MÕJU MUSKARIINSE RETSEPTORI SOLUBILISEERIMISELE

On uuritud üheksat erinevat digitoniinipreparaati firmadest «Merck», «Fluka», «Sigma» ja «Ockardt» kõrgrõhu vedeliku- ja õhukese kihi kromatograafiaga ning korreleeritud digitoniinipreparaatide küllastatud vesilahuse komponentide sisaldust muskariinse retseptori solubiliseerimise saagisega ja üldvalgu sisaldusega ekstraktis. On näidatud, et muskariinse retseptori solubiliseerimise maksimaalse saagise saamiseks ei ole vaja digitoniini ja gitoniini suhet 3 : 2, nagu eelnevalt kirjanduses viidatud.

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ХРОМАТОГРАФИЧЕСКИЙ АНАЛИЗ ГЛИКОЗИДОВ ДИГИТАЛИСА И ИХ СВЯЗЬ С СОЛЮБИЛИЗАЦИЕЙ МУСКАРИНОВОГО РЕЦЕПТОРА

С помощью высокоэффективной жидкостной и тонкослойной хроматографии изучено девять препаратов дигитонина фирм «Merck», «Sigma», «Fluka» и «Ockardt». Содержание компонентов насыщенных водных растворов этих препаратов скоррелировано с выходом солюбилизации мускаринового рецептора. Показано, что для достижения максимального выхода солюбилизации последнего не требуется соотношения дигитонина и гитонина, равного 3/2, как ранее отмечалось в литературе.