Journal of Chromatography, 115 (1975) 117–121

CHROM. 8464

BEHAVIOUR OF CARDIAC GLYCOSIDES AND CARDENOLIDES RELATED TO DIGITOXIGENIN ON SEPHADEX LH-20*

G. ZÜLLICH, K. H. DAMM and B. P. LISBOA**
Universitäts-Frauenklinik Eppendorf, Hamburg (G.F.R.)
(Received June 4th, 1975)

SUMMARY

The behaviour of six cardenolides and eight cardiac glycosides related to digitoxigenin during column chromatography on Sephadex LH-20 gel has been investigated. Complete resolution was obtained for mixtures of digitoxigenin, gitoxigenin and digoxigenin, but not for those of the 3-epimeric cardenolides. It was possible to achieve a group separation of cardenolides and their glycosides of the digitoxigenin series from those of the digoxigenin or gitoxigenin series.

INTRODUCTION

In a study of the metabolism of cardiac glycosides, the group separation of substances with different aglycones, as glycosides belonging to the digitoxigenin (3β,14β-dihydroxy-5β-carden-20,22-olide; DT) series from those with 12β- or 16β-hydroxylation, became very important. Both chromatography on formamide-impregnated paper[1–3] and multiple thin-layer chromatography (TLC)[4,5] have been used for such a group separation.

In order to minimize losses resulting from elution steps, we attempted the use of column chromatography on Sephadex LH-20 gel for the purification and preliminary separation of cardiac glycosides in biological material. This fractionation procedure has been successfully applied, with a high recovery rate, for the rapid separation of mixtures of steroids into groups according to their substituents[6–8].

The present paper deals with the behaviour of cardiac glycosides and aglycones related to DT on Sephadex LH-20 in a number of specially developed systems. The biological application of this method in association with over-run TLC[9] for the separation and identification of digitoxin (digitoxigenin tridigitoxoside; DT-tris-S) metabolites in rat bile will be described elsewhere[10].

* Taken in part from a dissertation by G. Züllich in partial fulfilment of the requirements for the M. D. degree, Medical School, University of Hamburg.
** To whom requests for reprints should be addressed.

EXPERIMENTAL

Reagents

All solvents and reagents were from E. Merck (Darmstadt, G.F.R.). Sephadex LH-20 (particle size 25–100 μm) was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Steroids[1]

The generally labelled cardiac glycosides [G-^{3}H]DT-tris-S and [G-^{3}H]digoxin (3β,12β,14β-trihydroxy-5β-carden-20,22-olide tridigitoxoside; DG-tris-S) were obtained from New England Nuclear Chemicals (Dreieichenhain, G.F.R.) and had specific activities of 25.5 mCi/mg and 10.2 mCi/mg, respectively. The DT, DG-tris-S, gitoxin (3β,14β,16β-trihydroxy-5β-carden-20,22-olide tridigitoxoside; GT-tris-S) and strophanthidin (3β,5β,14β-trihydroxy-19-oxo-5β-carden-20,22-olide) were from E. Merck. *Epi*-digitoxigenin (*epi*-DT), gitoxigenin (GT), digoxigenin (DG), *epi*-digoxigenin (*epi*-DG). DT mono- and bis-digitoxosides (DT-mono-S and DT-bis-S), gitoxigenin monodigitoxoside (GT-mono-S), DG mono- and bis-digitoxosides (DG-mono- and bis-S) were obtained from Boehringer (Mannheim, G.F.R.).

Preparation of labelled DT and DG

An amount (10 μCi) of [G-^{3}H]DT-tris-S (corresponding to 0.39 μg), and of [G-^{3}H]DG-tris-S (corresponding to 0.98 μg), was hydrolysed for 90 min at 35° with 1 ml of 0.1 *N* hydrochloric acid; after neutralization with 100 μl of sodium hydroxide solution, the mixtures were evaporated under nitrogen and the residues were dissolved in chloroform. Purification of the [G-^{3}H]DT and [G-^{3}H]DIG so formed, was carried out by continuous TLC on silica gel as previously described[9]. After 150 min over-run in chloroform–isopropanol (95:5). the zones corresponding to reference substances were scraped off and eluted several times with methanol–chloroform (1:1); the cardenolides so isolated were shown to be pure by TLC in other solvent systems. During this procedure, the radioactive spots on the chromatograms were located by using a thin-layer scanner with flowing methane (Laboratorium Prof. R. Berthold, Wildbad, G.F.R.). The cardenolides chromatographed as markers were located under UV radiation.

Procedure

A glass column (30 × 1 cm) with a PTFE stopcock was filled by gravity with a slurry prepared by allowing 4 to 7 g of dry Sephadex to swell overnight in 50 ml of the solvent system to be employed. The prepared column was equilibrated for 4 h before chromatography. The cardiac glycosides were dissolved in 0.5 ml of the solvent system and applied with a capillary pipette to the surface of the packing. The effluent was collected in 2-ml fractions, and aliquots of each fraction were transferred to silica gel layers, the chromatograms being allowed to develop continuously (ascending technique) in the system described by Züllich *et al.*[9]. The spots were made visible by spraying the plates with an anisaldehyde–sulphuric acid–acetic acid reagent and then heating them at 100° (see ref. 9).

In the experiment carried out with the cardenolides [G-^{3}H]DT, [G-^{3}H]DG and GT, the effluent was collected in 5-ml fractions, 0.5-ml aliquots of each fraction

being used for determining the radioactivity. The rest of each fraction was analysed by measurement of the colour developed with *m*-dinitrobenzene[11].

Solvent systems

The behaviour of eight cardiac glycosides and six cardenolides during chromatography on Sephadex was investigated in the following solvent systems, the amount of Sephadex LH-20 used being shown in parentheses:

S-1 = toluene–chloroform–methanol (82:10:8) (4 g);
S-2 = toluene–chloroform–methanol (84:10:6) (4 g);
S-3 = toluene–chloroform–methanol (80:10:10) (6 g);
S-4 = toluene–cyclohexane–chloroform–methanol (40:40:10:10) (4 g);
S-5 = as S-4, but using 7 g of Sephadex (height of column: 21.5 cm);
S-6 = cyclohexane–chloroform–methanol (80:10:10) (4 g);
S-7 = *n*-hexane–acetone–methanol (70:25:10) (4 g);
S-8 = cyclohexane–chloroform–methanol (80:15:5) (4 g);
S-9 = cyclohexane–chloroform–methanol (80:10:5) (4 g).

Measurement of radioactivity

Radioactivity was measured by using a scintillation spectrometer (Model 3375), with 10 ml of Insta-gel (Packard) as scintillator liquid.

Reaction with m-dinitrobenzene

In the experiment carried out with cardenolides in system S-5, in which labelled DT and DT were chromatographed together with 200 μg of GT, this substance was analysed by the *m*-dinitrobenzene–pyridine reaction: To the dry samples corresponding to 4.5 ml of each fraction were added 2 ml of a 0.5% solution of *m*-dinitrobenzene in pyridine and 0.1 ml of 0.05 *N* sodium hydroxide. After shaking the mixture, the tube was left for 16 min at room temperature, then 0.05 ml of 0.2 *N* boric acid solution in pyridine were added; after 15 min, the absorbance was measured at 585 nm.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a mixture of DT (about 20 ng), DG (about 40 ng) and GT (200 μg) on 7 g of Sephadex LH-20 in system S-5 (height of column 21.5 cm). Under these conditions, it was possible to separate the three aglycones completely. The resolution of the last two aglycones was only partial when shorter columns were used (4 g of Sephadex, system S-4, see Table I). It should be pointed out that DG and GT have very similar polarities and are difficult to separate by TLC[9,12,13].

Table I shows the behaviour of the cardiac glycosides and cardenolides in the solvent systems tested. From the data on the column effluent summarized in this table, the following conclusions can be drawn:

(a) It is possible to separate cardenolides having different functional groups, but not the epimeric pairs. DT, strophanthidin (5β-hydroxy-19-oxo-DT) and DG (12β-hydroxy-DT) are well resolved in system S-2, whereas DT is separable in all the systems from DG or GT (16-hydroxy-DT). In systems S-3 and S-4, GT is less

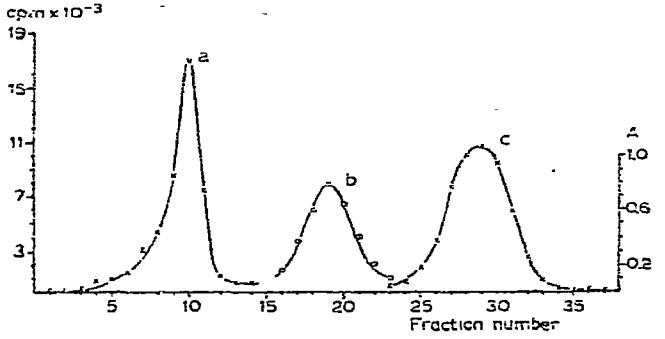

Fig. 1. Gel column chromatography of a mixture of (a) [G-^{3}H]DT (about 450,000 cpm); (c) [G-^{3}H]-DG (about 550,000 cpm); and (b) 200 μg of GT. Column (21.5 × 1 cm). 7 g of Sephadex LH-20; eluent, toluene–cyclohexane–chloroform–methanol (40:40:10:10). Of each 5-ml fraction, 0.5 ml was used for measurement of radioactivity; the rest was used for determining GT with *m*-dinitrobenzene–pyridine.

polar than DG, and its resolution can only be achieved in long columns, as indicated in Fig. 1. The pairs DT–*epi*-DT and DG–*epi*-DG (differing by epimerism at C-3) remain unresolved; identical behaviour of epimeric compounds has also reported for steroids of the androstane (testosterone–*epi*-testosterone) and oestrogen (oestradiol-17β–oestradiol-17α) series[7,8].

(b) As with the cardenolides, a group separation of the cardiac glycosides can also be obtained on Sephadex. This is possible because the influence of the carbohydrate part of the cardiac glycoside is less pronounced than that of the functional

TABLE I

BEHAVIOUR OF CARDIAC GLYCOSIDES AND CARDENOLIDES RELATED TO DIGITOXIGENIN DURING COLUMN CHROMATOGRAPHY ON SEPHADEX LH-20

Elution volumes (in ml) are given for columns of size 21.5 × 1.2 cm; flow-rate, 25 ml/h; volume of fractions, 2 ml.

Glycoside or cardenolide	*Solvent system*								
	S-1	*S-2*	*S-3*	*S-4*	*S-5*	*S-6*	*S-7*	*S-8*	*S-9*
DT-tris-S	18– 30	18– 38	18– 36	18– 38	32– 58	52– 72	40– 66	76–130	104–138
DT-bis-S	—	—	—	—	—	48– 66	32– 56	64–120	90–120
DT-mono-S	24– 36	30– 44	26– 44	24– 42	42– 62	—	—	—	—
DT	20– 30	20– 34	22– 38	22– 38	36– 58	40– 60	26– 46	48– 76	66– 96
Epi-DT	—	—	22– 36	22– 38	34– 58	—	—	—	—
Strophanthidin	38– 52	52– 80	40– 62	—	78–102	—	—	—	—
GT-tris-S	44– 58	58– 88	34– 64	40– 56	90–120	—	—	—	—
GT-mono-S	48– 76	76–128	—	—	108–140	—	—	—	—
GT	—	—	44– 80	44– 74	—	—	—	—	—
DG-tris-S	56– 88	—	52– 84	56– 94	114–144	—	108–160	300–400	300–450
DG-bis-S	64–104	—	66– 94	72–106	128–160	—	—	—	240–352
DG-mono-S	—	—	80–110	80–110	—	136–172	86–136	272–360	—
DG	—	96–152	66–100	66–100	—	112–148	70–100	196–280	180–240
Epi-DG	—	—	66– 96	60– 88	120–148	—	—	—	—

substituents in the aglycone. It is possible to achieve complete separation of compounds of the DT series (DT and its glycosides DT-mono-S, DT-bis-S and DT-tris-S) from those of the DG series (DG and its glycosides DG-mono-S, DG-bis-S and DG-tris-S) or GT (GT, GT-mono-S and GT-tris-S). Separation between compounds of the DG- and GT-series is only partial, owing the incomplete resolution of DG-tris-S from GT-mono-S.

(c) In each group of glycosides the polarity of the genin-tris-digitoxoside is less than that of the genin-bis-digitoxoside, which is in turn less than that of the genin-mono-digitoxoside for systems in which toluene is the principal component. An inversion of this sequence occurs for systems in which an aliphatic hydrocarbon (cyclohexane or *n*-hexane) is the principal component. In this last group of solvent systems (S-6 to S-9), the aglycones are less polar than the corresponding cardiac glycosides.

For qualitative purposes, very good separation of substances belonging to the DT-, DG- and GT-classes was obtained on formamide-impregnated paper when chloroform[2], ethyl methyl ketone–xylene (1:1) (with 3% of formamide) or ethyl acetate–benzene–*n*-heptane (3:2:1) [with 0.6% of formamide–water (1:1)][3] was used as mobile phase. Further, a group separation between substances of the DT- and DG-series was achieved on silica gel layers by multiple runs in systems of the cyclohexane–acetone–acetic acid type[4,5,14]. However, none of these methods is acceptable for quantitative work, for which a high yield is necessary, particularly if the metabolites to be isolated are present in very different amounts, *e.g.*, in biological material.

The fractionation procedure described involving gel chromatography was useful for initial separation of the cardiac glycoside groups, the groups being further fractionated quantitatively by continuous TLC.

REFERENCES

1 F. Kaiser, *Chem. Ber.*, 88 (1955) 556.
2 E. Watson, P. Tramell and S. M. Kalman, *J. Chromatogr.*, 69 (1972) 157.
3 L. Nover, G. Baumgarten and M. Luckner, *J. Chromatogr.*, 32 (1968) 93.
4 W. E. Wilson, S. A. Johnson, W. H. Perkins and R. E. Ripley, *Anal. Chem.*, 39 (1967) 40.
5 M. L. Carvalhas and M. A. Figueira, *J. Chromatogr.*, 86 (1973) 254.
6 B. E. P. Murphy, *Nature (London), New Biol.*, 232 (1971) 21.
7 B. P. Lisboa and M. Strassner, *J. Chromatogr.*, 111 (1975) 159.
8 U. Hoffmann, H. Maass and B. P. Lisboa, *Eur. J. Biochem.*, (1975) in press.
9 G. Züllich, W. Braun and B. P. Lisboa, *J. Chromatogr.*, 103 (1975) 396.
10 G. Züllich, K. H. Damm, W. Braun and B. P. Lisboa, *Arch. Int. Pharmacodyn. Ther.*, 215 (1975) 160.
11 W. Wendland and K. Lohmann, *Biochem. Z.*, 328 (1957) 420.
12 L. Nover, G. Baumgarten and M. Luckner, *J. Chromatogr.*, 39 (1969) 450.
13 W. H. Bulger, R. E. Talcott and S. J. Stohs, *J. Chromatogr.*, 70 (1972) 187.
14 M. J. Frey and G. M. Jacobsohn, *Anal. Biochem.*, 36 (1970) 78.