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POLYMERIC ADSORBENTS OF THE AFFINITY TYPE IN THE INVESTIGATION OF PHYSIOLOGICALLY ACTIVE SUBSTANCES. VII. CHROMATOGRAPHIC SEPARATION OF FLAVONOIDS GLYCOSIDES DERIVED FROM QUERCETIN AND LUTEOLIN

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The chromatographic behavior of some glycosides of quercetin and luteolin on polymeric adsorbents of the affinity type has been investigated by low-pressure column chromatography. The separation of the glycosides according to the total polarity of their molecules has been shown — first the triosides are eluted, then the biosides, and, finally the monosides. The prospects of developing nonclassical affinity chromatography in the study of flavonoids are discussed.

As reported previously [1], the field of obtaining particularly pure physiologically active substances, including those of plant origin, which is intermediate between the analytical and preparative variants of liquid column chromatography (LCC), has been studied inadequately. One of the new approaches to the separation and purification of plant biologically active substances (pBASs) such as coumarins, tanning substances, etc., that we are actively developing in this field is nonclassical affinity chromatography (NAFC) — in particular, polyphenol ligand chromatography on polymeric adsorbents of the affinity type (AAFTS) [2, 3].

At the present time, the majority of investigations by the LCC method of the separation, isolation, and purification of flavonoids (FLs) and their derivatives is conducted on polyamide and polysaccharide supports (Sephadexes, etc.), silica supports being used more rarely [4, 5]. Paper or preparative thin-layer chromatography [4, 6] or high-performance LCC [4, 7-11] is used for the further study of the FL-containing fractions isolated. In the last-mentioned method, in addition to known modifiers of the silica surface — cellulose triacetate and tri(phenylcarbamate) [5, 7, 8] — poly(triphenylmethyl methacrylate) [9] and poly(diphenyl-α-pyridylmethyl methacrylate) [11] have also been used. In these cases, the isolation of individual FLs in the milligram amounts necessary for the detailed study of the structures of new derivatives is a laborious process. Definite complications also arise in the study of impurities and trace amounts of FLs by concentrating their dilute solutions.

We have previously reported the development of a new method of isolating the aglycons of FLs and their glycosides which also permits the sorption from dilute solutions on polymeric AAFTs of aglycons, including those obtained by the partial hydrolysis of FL glycosides (0.4 M hydrochloric acid, 10-20 min) [3, 12, 13]. On the other hand, it has been shown [13] the capacity of the AAFTs studied, of both the agarose (AG) and the Toyopearl (TP) types synthesized by the epoxy activation method, for aglycons of FLs is approximately an order of magnitude higher than the capacity of unmodified AG. In their turn, TP analogs of AAFTs have a capacity substantially greater than their AG derivatives (up to 6 mg of FL aglycon/ml of adsorbent gel) [13]. The unusually high capacity for this type of FLs of the benzoquinone-activated analogs (more than 10 mg/ml) requires further study. It is interesting that, under the conditions investigated — with the mobile phases (MPs) distilled water and 5-20% aqueous solutions of ethanol — FL glycosides readily issue with the free volume, while sorbed FL aglycons are most fully eluted by 50-70% aqueous ethanol. The best AAFT for the separation of FL 3- and 8-O-glycosides and also of

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TABLE 1. Separation of FL Glycosides on Polymeric AAFTs

Type of AAFT; (volume of adsorbent — 1 ml)	Main model mixtures of FL glycosides									
	1	2	3	4	5	6	7	8	9	10
MRN–ACH–TP	–	–	n/s	–	n/s	±	n/s	n/s	n/s	n/s
MRN–NBH–TP	–	–	n/s	+	±	±(I)	n/s	n/s	n/s	±
MRN–SP	+	–	±	+	+	+	–	+	–	+

n/s) separation not studied; –) absence of separation; ±) partial separation; +) complete separation; (I) — complete separation achieved on 2 ml of sorbent, which also applies to mixture 13 (LT-7-g/LT-7-dg).

biosides from monosides [rutin (RUT) and isoquercitrin (IQCR)] proved to be ones with morin (MRN) and catechin (CC) as the immobilized ligands [3, 13].

The aim of the present work was to investigate the use of the AAFTs that we had obtained previously [13, 14] for the separation of glycosides of some FLs [quercetin (QT), luteolin (LT), etc.] differing in the total polarities of their molecules because of the different numbers of carbohydrate components in them.

The AAFTs were synthesized from epoxy- and benzoquinone-activated supports (AG, TP) and also from the *p*-aminophenylsulfonyl derivative of Spheron (SP). The ligands were immobilized on the *p*-aminophenyl-containing supports by the azo coupling reaction [3, 14]. The spacers were *p*-nitrobenzhydrazide (NBH) and the *p*-nitrobenzoyl derivative of ε-aminocaproic acid hydrazide (ACH). The ligands used were resorcinol (RC), MRN and CC.

In order to study the chromatographic behaviors of FLs on the AAFTs obtained we used two- and three-component (model) mixtures of FLs (aqueous alcoholic solutions with FL concentrations of $1.6\text{-}5.0 \cdot 10^{-2}$%). The following were used as FLs: RUT, IQCR, isorhamnetin 3-glucoside (IRM-3-g), quercitrin (QCR), luteolin 7-glucoside (LT-7-g), luteolin 7-diglucoside (LT-7-dg), luteolin 7-xyloglucoside (LT-7-xg), luteolin 7-rutinoside (LT-7-rt), and luteolin 7-rutinoside 4′-glucoside (LT-7-rt-4′g).

The first screening of the AAFTs investigated permitted the selection for further work of only adsorbents based on TP and SP, with immobilized MRN. Other products — MRN-epoxy-AG and MRN-benzoquinone-TP and also RC- and CC-containing AAFTs — proved to be less effective. Experimental results on the separation of the main model mixtures of FLs (for their compositions, see the Experimental part) on the AAFTs investigated are given in Table 1.

It may be concluded from the results in Table 1 that an AAFT based on TP is less effective than its SP analog. The introduction into the structure of an AAFT of a more hydrophobic spacer — ACH, differing from the spacer NBH by a pentamethylene ($-(CH_2)_5-$) fragment — is less effective. It is interesting that a study of the chromatographic behaviors of analogous AAFTs in the separation of coumarins and their derivatives also revealed only a slight improvement in the separation of this type of pBASs on a more hydrophobic AAFT [1, 15], while the sorption of nitrogen-containing drugs (alkaloids, vitamins, etc.) on them was 1.5-2 times greater [3].

As was assumed, the quality of the separation of FL glycosides on MRN–NBH–TP could be raised by using a larger volume of adsorbent gel (see Table 1, model mixtures 6 and 13), which will permit the effective use of a broader spectrum of synthesized AAFTs (Fig. 1).

It is possible that the high separating capacity of the Spheron AAFTs that we have detected and have found previously [3, 15] for coumarins and their derivatives and for derivatives of purine and of pyrazole is due to such parameters of the Spheron AAFTs as a high polarity of the spacers and a relatively high capacity for the ligand [14].

The following is the main conclusion derived from the experimental results (see Table 1, model mixtures 6, 8, 9, and 10): the AAFTs investigated separate FL monoglycosides from di- and triglycosides fairly effectively, the order of their elution coinciding with the decrease in the total polarity of the FL molecule — the more polar triglycosides are eluted first, then the diglycosides, and finally the monoglycosides (Fig. 2).

An analogous pattern was observed on the chromatographic separation of two-component model mixtures containing a diglycoside (RUT) paired with a monoglycoside (LT-7-g, model mixture 4) or with a triglycoside (LT-7-rt-4′-g), model mixture 14 (Fig. 3).

The subsequent analysis of the results on the separation of mixtures of diglycosides (QT and LT derivatives) substituted, respectively, in the 3- and 7- positions of the FL molecule showed that it might be complete (the pair RUT/LT-7-rt, model mixture 1), partial (RUT/LT-7-dg, model mixture 3) or absent (RUT/LT-7-dg, model mixture 2) (see Table 1). It is possible

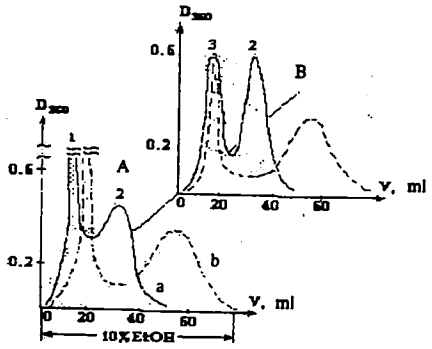

Fig. 1. Chromatographic profiles of the separation of FL glycosides on MRN–NBH–TP with various volumes of the gel. a) 1 ml; b) 2 ml. Model mixtures 6 (A) and 13 (B) were used. FL peaks: 1) LT-7-rt; 2) LT-7-g: 3) LT-7-dg. Volume of each mixture) 1 ml.

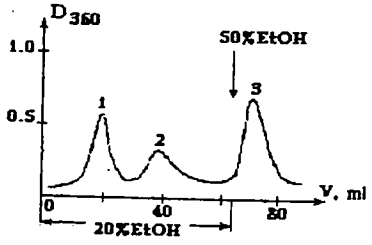

Fig. 2. Chromatographic profile of the separation on MRN–SP (gel volume — 1 ml) of model mixture 9 (sample volume — 1 ml). Peaks of the FLs: 1) LT-7-rt–4′-g (trioside); 2) LT-7-rt (bioside); 3) LT-7-g (monoside).

that the total polarity of the molecule in a rutinoside falls somewhat because of the presence in the disaccharide residue of the less polar (than glucose) rhamnose, which is a 6-deoxy monosaccharide, and this, in its turn, levels out differences in the polarities of the aglycons (QT is more polar than LT because of the additional 3-OH group) and of the molecule as a whole. The first pair (rutinosides) is therefore sharply separated (the difference in the polarities of the aglycons has its effect) and the last is not separated at all, while in the pair RUT/LT-7-xg the leveling out of the polarities of the aglycons by the sugar residue apparently takes place only partially. It is interesting to note that similar chromatographic effects (aglycons are sorbed more strongly than glycosides; free hydroxy groups actually play an important role) are fairly well known in the chromatography of FLs on polyamide, which is widely used in large-scale GLC [16].

However, even today, where polymeric AAFTs are used for separating and purifying FLs, the following advantages of them (as compared with polyamide) are being traced: adequate rapidity of the method (in the investigations, the small volumes of adsorbent gel made themselves felt), there is practically no absorption of FL glycosides (the higher the percentage of ethanol in the MP, the faster do they issue with the free volume), the separation processes take place effectively under fairly mild conditions (the MPs are distilled water and aqueous solutions of ethanol) [12], and, finally, it is very important that the strategy of synthesizing the affinity adsorbents permits not only a variation of the capacity parameters (in terms of ligand) of the AAFTs within wide limits but also a substantial variation in their hydrophilic–hydrophobic status as a whole through the diversity not only of the immobilized ligands but also of the spacers [2, 3]. A study of the influence of "chemical crownization", recently discovered for AAFTs [2, 15] on the processes involved in the separation and purification of FLs appears extremely interesting.

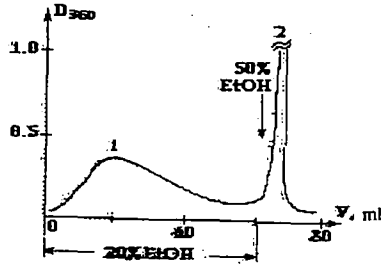

Fig. 3. Chromatographic profile of the separation on MRN–SP (gel volume — 1 ml) of model mixture 14 (sample volume — 1 ml). FL peaks: 1) LT-7-rt–4′-g; 2) RUT.

In conclusion we may note that the new approaches that we have suggested for the investigation of FLs on polymeric AAFTs is very highly promising, above all, in our view, in the investigation of the fine structure of new types of FLs, since the loading of chromatographic columns with milligram amounts of the samples under study is sufficient for the analysis of the products concerned by modern complex spectral methods. Moreover, the chromatography of FLs using polymeric AAFTs is also extremely effective in the group separation of pBASs in composite phytopreparations (of the "éskuzan" type), where it permits a substantial concentration of trace amounts of FL impurities not determined by the TLC method under the usual conditions [3].

Thus, the results of the investigations performed permit the use of polymeric AAFTs for the separation and purification of FLs to be regarded as a new direction in the phytochemistry of pBASs within the general run of NAFC [2, 3, 15].

EXPERIMENTAL

We used AG (CIS), TP–HW–65 (Japan), and SP–Ara–1000 (Czech Republic) for the synthesis of AAFTs with immobilized ligands of the phenol type — RC, CC, and MRN — obtained as in [14, 15]. The samples of FL glycosides investigated were chromatographically pure substances from the collection of the Department of Pharmacognosy, St. Petersburg Institute of Pharmaceutical Chemistry (head of the department Prof. G. P. Yakovlev). The other reagents and materials were of ch.d.a. [pure for analysis] grade or corresponded to the Xth edition of the State Pharmacopeia.

The following model mixtures of FLs were used for investigation: 1) RUT/LT-7-rt; 2) RUT/LT-7-dg; 3) RUT/LT-7-xg; 4) RUT/LT-7-g; 5) RUT/QCR; 6) LT-7-g/LT-7-rt; 8) LT-7-rt/LT-7-rt–4′-g; 9) LT-7-rt–4′-g/LT-7-rt; 10)RUT/IQCR; 11) IRM–3-g/IQCR; 12) QCR/IQCR; 13) LT-7-g/LT-7-dg; 14) RUT/LT-7-rt–4′-g; in concentrations of $1.6\text{-}5.0 \cdot 10^{-2}$% (20-50% aqueous alcohol).

Typical Procedure for the Chromatographic Separation of FL Glycosides. A chromatographic column was filled with 1 ml (or, where necessary, 2 ml) of packed adsorbent gel and was washed with 2-3 ml of 50% aqueous ethanol, followed by 20-30 ml of distilled water and 10 ml of MP of the required type. Then a solution (1-2 ml) of FL glycosides (model mixture) was deposited on the column, and chromatography was carried out at the rate of 1-3 ml/min. The fraction volume was 4 ml, and detection was achieved in the UV region at 360 nm (SF-26, CIS).

The FLs isolated were identified spectrophotometrically and also by paper chromatography with markers.

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