POLYMERIC ADSORBENTS OF THE AFFINITY TYPE IN THE INVESTIGATION OF PHYSIOLOGICALLY ACTIVE SUBSTANCES

II. NEW APPROCHES TO THE SEPARATION, ISOLATION, AND PURIFICATION OF HYDROXYCOUMARINS

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A comparison has been made of the chromatographic behaviors of the hydroxycoumarins and their derivatives on polymeric adsorbents of the affinity type with phenolic and polyphenolic ligands and with unmodified supports. It has been shown that it is desirable to study adsorbents in both the analytical and in the microsemipreparative regimes of liquid chromatography.

Particular interest is caused by chromatographic methods of investigating physiologically active substances (PASs), including plant biologically active substances (pBASs) in the area of obtaining ultrapure compounds [1]. This area, intermediate between the regimes of preparative liquid chromatography (PLC) and analytical high-performance chromatography (HPLC) has been studied inadequately.

It has recently been shown [2] that the efficiency of the liquid column chromatography (LCC) of the coumarins in the medium-pressure regime is not inferior to HPLC and is even superior to it in terms of economy. However, the adsorbents used in the LCC of the coumarins and other pBASs (polyamide (FA), Sephadex (SPD), silica gel (SG), and its reversed-phase analogs (rfSGs)) being poorly effective in aqueous media, are used in large amounts and require the employment of organic eluents, while SG and its analogs are unstable at pH > 8 [3-6].

An analysis of the latest nontraditional methods of LCC has shown that the most important prospects of their application are connected with the uses of polymeric adsorbents of the affinity type (AATs) obtained by the strategy of synthesizing affinity adsorbents [7, 8].

For the hydroxycoumarins (HCMs), investigations in the field of nonclassical affinity chromatography (NAC) [7] are all the more interesting since the formation of specific complexes of the HCMs (derivatives of 4-hydroxycoumarin) not only with the fibrinolysis enzymes and other widely distributed proteins (bovine serum albumin etc.) but also with some synthetic polymers (polyvinylpyrrolidone, polymethacrylate is known) [9-11].

The aim of the present work was to investigate the AATs described previously [12, 13] for the separation, isolation, and purification of some HCMs and their derivatives. The results are given of a comparative characterization of the chromatographic behaviors of the HCMs on AATs and a number of unmodified polymeric supports, and also results in connection with the use of the strategy of affinity synthesis on adsorbents partially modified by p-aminophenyl-containing inserts.

The AATs were obtained by a known modification of Campbell's method [14] from such supports as agarose (AG) and Toyopearl (TP) epoxy-activated by the method of Axen et al. [13]. As inserts we used p-nitroaniline and p-nitrophenyl-containing hydrazides [14, 15] upon which we then immobilized ligands of phenolic and nonphenolic nature – resorcinol (RS), hexylresorcinol (h-RS), estradiol (EST), derivatives of benzo-1,4-pyran (apigenin – APG; quercetin – QCN; morin – MRN; rutin – RTN, and catechin – CTC) tanning substances (tannin – TN; and extracts of oak, acacia, and bilberry – OE, AE, and BLE, respectively) [12]. These ligands were immobilized similarly on Spheron-Ara-1000 (SPR-A-1000).

To investigate chromatographic behaviors and to compare model mixtures we used the following HCMs: coumarin (I), 4-methylumbelliferone (II), scopoletin (III), esculetin (IV),

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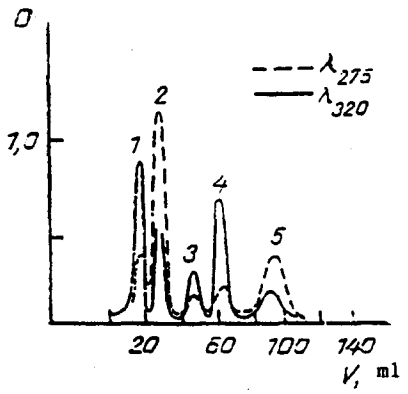

Fig. 1

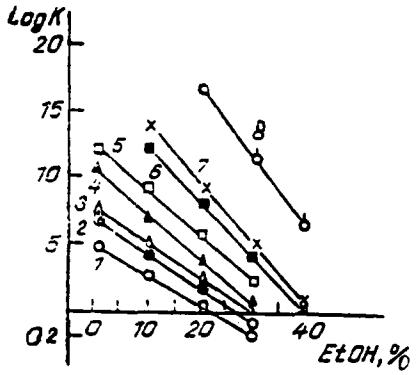

Fig. 2

Fig. 1. Chromatographic separation of a model mixture: 1) esculin (VI); 2) coumarin (I); 3) esculetin (IV); 4) 4-methylumbelliferone (II); 5) xanthotoxol (XV). Adsorbent – CTN-TP; volume of gel 5 ml. MP of type a – distilled water.

Fig. 2. Dependence of log k for 4-methylumbelliferone (II) on the concentration of ethanol in a MP of the ethanol–distilled water type in chromatography on 2 ml of adsorbent (height of the column of adsorbent 55 mm): 1) TP (HW-50); 2) TP (HW-65); 3) TP (HW-40); 4) TP (HW-75); 5) H_2N-SPD; 6) RS-SPD; 7) CTN-SPD; 8) ODS-SG (MP: MeOH–H_2O [16].

herniarin (V), esculin (VI), the β-glucoside of (II) ((VII)), the β-galactoside of (II) ((VIII)), 4-hydroxycoumarin (IX), Varfarin (X), syncumar (XI), phepromaron (XII), dicoumarol (XIII), neodicoumarin (XIV), xanthotoxol (XV), marmesin (XVI), and carbocromen (XVII), and also individual purified fractions of coumarin-containing phytopreparations (PPs) – belladonna extract (BDE) and tincture of wormwood (TW). As the mobile phases (MPs) we used: distilled water (type A), a 2 M solution of sodium chloride (type B), a 4 M solution of urea (type C), a 10% solution of ethanol (vol./vol.) (type D), and a solution of acetic acid or hydrochloric acid with pH 3.5 or a sodium citrate buffer with pH 3.5 (type E).

The results of the primary screening of the AATs based on AG already showed their incapability of separating even the simplest model mixtures [a) (IV) and (VI); b) (II), (IV), and (IX); and c) (IX), (XV), and (XVI)] and they were therefore not studied further.

For the AATs of the TP type, columns containing 2-5 ml of packed absorbent gel proved to be fairly effective from the point of view of rate, quality of the separation process, and amount of substances analyzed (0.1-1.0 mg), successfully combining the speed of the analytical variant of LCC (1-4.5 h) with the possibility of microsemipreparative (MSP) separation and the treatment of the pure components of mixtures in the regime of low-pressure LCC (Fig. 1).

A comparison of unmodified supports actively used in phytochemistry – of the polysaccharide (AG, SPD) and synthetic (PA, TP, SPR) types revealed the following sequence characterizing increasing strength of the inflection of the AATs with the HCMs: AG, SPD << TP < SPR << PA. As was expected, PA and the polysaccharide supports proved to be unsuitable in practice for work under the regimes investigated (analytical and MSP) although their efficacy in the area of PLC is known [3-5]. Nevertheless, when the HCMs were chromatographed on TP gels of different manufacturers' grades (TP HP-40, -50, -65, -75), substantially differing capacity factors k were obtained (Fig. 2).

A comparison of the polymeric matrices of the polyvinyl (TP) and polymethacrylate (SPR) gel types showed their high analytical potential for the HCMs. Possessing the properties of reversed-phase sorbents (RFSs), at the same time they are distinguished by weaker interactions with the HCMs. The HCMs are therefore eluted comparatively rapidly by MPs of type A (distilled water) or MPs of type D (10% aqueous ethanol). The last-mentioned eluent is the most effective for the SPR sorbents and TP HW-75, since on them MPs of type A elute HCMs in the form of broad highly overlapping peaks, and this even with a change in the chromatographic mobilities of some of them, for example (X-XVI). The dependence of log k for 4-methylumbelliferone (II) on the concentration of ethanol in a MP of type D that we have found for the sorbents under investigation in comparison with those of Duren and Diehl [16], obtained on octadecylsilica gel are shown in Fig. 2.

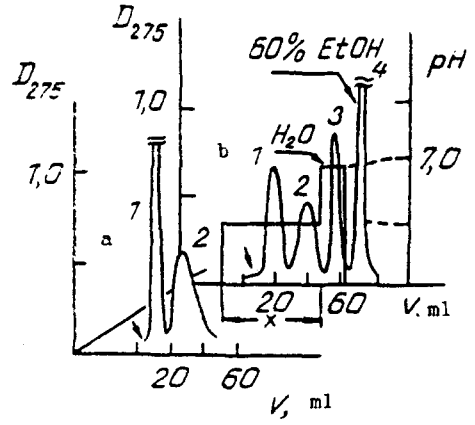

Fig. 3. Chromatographic separation of HCMs (coumarin (I), 4-hydroxycoumarin (IX), neodicoumarin (XIV), and syncumar (XI) on the adsorbent CTN-TP (2 ml of gel): a) MP – distilled water: peak 1 – mixture of (I), (IX), and (XIV); peak 2 – (XI); b) zone X (MP of type E – 0.1 M sodium citrate, pH 3.5): peak 1 – (I); peak 2 – (IX); peak 3 – (XIV); peak 4 – (XI).

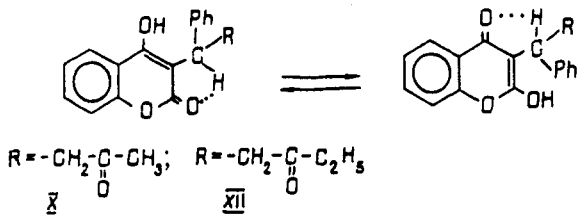

Fig. 4. Conformations of 3-substituted 4-hydroxycoumarins stabilized by intramolecular hydrogen bonds.

Conversely, adsorbents based on TP gels (of the HW-40, -50, and -65 types) are fairly selective in MPs of type A, and the resolving capacity of the sorbents increases substantially with a lengthening of the chromatographic column. For this reason, the AATs synthesized from the matrix TP HW-50 were used as the main ones for the investigation of the HCMs.

The influence of the immobilization of phenolic and polyphenolic ligands (as compared with unmodified TP and amino-TP gels) on the process of separation of the model mixture b with ligands of the OE, AE, and RTN types is apparently poorly effective although, like other AATs, they separated mixtures c well. However, in this process an unsatisfactory separation of (IX) and (XVI) in mixture c on a TN sorbent was observed. It is interesting that the screening of the CTN-, APG-, and SN-containing AATs with different types of insert does not lead to appreciable improvements for adsorbents with an insert elongated by a pentamethylene fragment [$(-CH_2)_5-$].

Great interest is presented by the mechanism of the chromatographic behavior of the HCMs investigated on the AATs. Thus, for all the HCMs except (XIII) in MPs of type A there is no sorption effect, and they are eluted by water in a definite sequence characterized by stable retention volumes V_{sp}. It is known that, according to the mechanism of partition chromatography [17], the values of k decrease with the replacement of an MP of type A by an aqueous organic one (type D). We confirmed this conclusion by using such a dehydrating agent as a 2 M solution of sodium chloride, which led to an increase in the values of k for the HCMs investigated, probably because of an increase in the polarity of the MPs [17]. It was found that the most resistant to such changes in the MPs were the coumarins (I) and (XVI) having no polar groups, HO and H_3CO, in their structure. Thus, the presence of these groups plays an important role in the processes of interaction of the HCMs with the surface of an AAT, apparently through the formation of hydrogen bonds (HBs), since the cleavage of HBs by a 4 M solution of urea (an MP of type C) sharply lowers the values of k for all the HCMs.

It is interesting that, in contrast to other HCMs, the nitrogen-containing coumarin (XVII) was readily sorbed (in a MP of type A) on all the AATs investigated, including those of the agarose type, being eluted with a change in their ionic background (2 M solution of

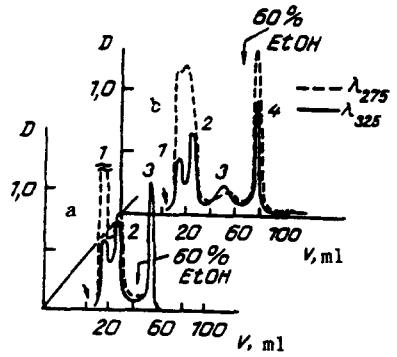

Fig. 5. Chromatography of extracts of PPs: a) hydrolysate of an ethyl acetate fraction of TW; b) ethereal extract of BDE; adsorbent – CTN-TP with a volume of 2 ml; a) peaks 1 and 3 are unidentified minor components; peak 2 is a mixture of coumarins; b) peaks 1, 3, and 4 are unidentified minor components of coumarin nature; peak 2 is scopoletin (III).

sodium chloride). These results agree completely with our investigations of nitrogen-containing pBASs and drugs in the area of NAC [7, 8]. We must also mention specific differences in the chromatography of (IX) and its 3-substituted analogs (X)-(XIV) which are anticoagulants with an indirect action. Thus, the adsorption of the latter took place in MPs of types E and B (beginning from a concentration of their components of 5 mM for E and 20 mM for B), while for (IX) an increase in k (3.5-fold) was observed only in an MP of type C.

The conditions for the elution of these compounds, characterizing the force of their interaction with the adsorbent, are also different. For example, while (XV) was eluted with a change from MPs of types B and E to water (a type A MP), compounds (X)-(XIII) were eluted with 60% aqueous ethanol, which enables their mixtures to be successfully separated (Fig. 3).

The adsorption of (XIII) likewise in an aqueous MP of type A is obviously connected with its pronounced lipophilic properties – the capacity of the molecule for existing in a specific conformation stabilized by an intramolecular HB (particularly in an acid medium) as proposed long ago by Knobloch and Procházka [18] to explain the characteristics of the paper chromatography of (XIII). It is possible that the reason for such effects which we were the first to find for compounds (X), (XI), and (XII) is the formation of specific conformers stabilized by intramolecular HBs (Fig. 4).

At the same time, only for the neodicoumarin (XIV), having the highest possibility for the creation of a specific spatial conformation, was the effect of a two- to threefold increase in k on an AAT when bivalent metal ions (Ca^{2+}, Mg^{2+}, etc.) were added to the MP observed. It was assumed that the reason for this anomalous behavior of (XIV) is the formation of stable conformations with intramolecular HBs creating a resemblance to the so-called "crown cell" [19] capable of actively including metal ions.

Under the given conditions, no changes in k were observed for the other HCMs (II-IX), which is explained by the impossibility, in our opinion, of the formation of stable conformations with intramolecular HBs.

One of the most effective sorbents according to the results of the screening of the AATs – CTN-TP – has been used for the analysis of coumarin-containing phytopreparations – BDE and TW. On the chromatography of an ethereal extract of BDE a satisfactory separation of scopoletin (III) and unidentified compounds of coumarin nature from accompanying substances took place (Fig. 5). From an acid hydrolysate of TW about 10 compounds of coumarin nature were isolated, and their study is continuing.

Thus, the interaction of HCMs with the AATs synthesized is a fairly complex multifactorial process realizing mechanisms of retention on adsorbents that require further study. The results that we have obtained will promote the establishment in the area of NAC of a new direction polyphenol ligand chromatography (PPLC) as a promising variety of LCC [20, 21].

EXPERIMENTAL

We used: PA and AG (4%) of domestic (SSSR) production; TP HW-40, -50, -65, and -75 from Japan; SPR LC-300 and Ara-1000 from Czechoslovakia; and AATs with immobilized ligands of the

phenolic type – RS, h-RS, CTN, APG, MRN, QCN, and EST – and a number of polyphenolic ligands –OE, AE, BLE, and TW. The AATs were synthesized as described in [12, 14]. The samples of OE were isolated as described in [12], and the AE and BLE were obtained by known methods involving chromatography on SPD G-50 from extracts of Acacia catechi, and Vaccinium vitisideae. A sample of TN was obtained by purifying a commercial preparation on an AAT of the CTN-TP type.

Samples of the HCMs (I-XVII) were chromatographed on a glass column (0.5 × 15 cm) (Pharmacia, Sweden) in the low-pressure regime (Unipan peristaltic pump, type 304, Poland). The issuing eluate was fractionated in volumes of 2-3 ml in a Diafrak 002 fraction collector (USSR), detection being performed on a SF-26 instrument (USSR). The samples of HCMs were kindly provided by O. Grigor'ev, A. Khabarov, I. Kozlova, and G. Vysochin. The neocoumarin, phepromaron and syrcumar were isolated from tabletted medicinal forms (Pelentan, Fepromaron, and Sinkumar) by extraction. The other reagents and preparations were of ChDA ["pure for analysis"] grade or corresponded to the requirements of the State Pharmacopeia, Xth edition.

To prepare model mixtures we used 0.01% solutions of HCMs containing 4-8% of dimethyl sulfoxide ("Dimeksid", USSR).

The capacity factors k and the other chromatographic parameters were calculated in accordance with Schoenmakers' recommendations [17].

Typical Procedure for the Chromatography of the HCMs. A column was filled with 2-5 ml of packed adsorbent gel, and this was washed with 1.5 ml of 65% aqueous ethanol and then with 40 ml of distilled water and 10 ml of the MP of the required type. Then a solution of HCM (model mixture) was deposited on the column and chromatography was carried out at a rate of flow of 1.3-2.5 ml/min. The volume of the fractions was 2-2.5 ml, with UV detection in the interval of 250-345 mm.

The identification of the HMCs isolated and monitoring of the completeness of separation were carried out by UV spectroscopy (including the use of ionizing additives), and also by the method of TLC with markers [22].

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PHENYLPROPANOIDS OF A CALLUS CULTURE OF Rhodiola rosea

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Using 11 phenylpropanoids isolated from the biomass of a callus culture of roseroot stonecrop, the component compositions in the biomasses of callus and suspension cultures have been studied and their triandrin contents have been determined by the HPLC method.

We have previously [1, 2] reported the isolation of phenylpropanoids from the biomass of a tissue culture of roseroot stonecrop (Rhodiola rosea L.) possessing stimulating properties [3].

In the present paper we give experimental results confirming the structures of the compounds isolated (I-XIII). In addition, we have determined the component compositions of the biomasses of tissue and cell cultures of roseroot stonecrop by the method of high-performance liquid chromatography (HPLC) and have developed a method for the quantitative determination of triandrin (III) – one of the main biologically active substances of the biomass [4].

CHEMICAL STUDY

In the course of an investigation of the chemical composition of the biomass of a callus culture of roseroot stonecrop we isolated compounds having a phenylpropane skeleton that were derivatives of p-hydroxycinnamyl alcohol (I-IV), of p-coumaric acid (V)-(VII), and of caffeic acid (VIII and IX), or belonged to the lignan group (X and XI). Among the accompanying substances, β-sitosterol (XII) and its glucoside daucosterol (XIII) were identified.

In a study of the structures of the compounds isolated we used the results of chemical investigations [acetylation, methylation, enzymatic and acid hydrolysis, and the qualitative reaction with diazotized sulfanilic acid (DSA)], and also a comparison of physicochemical constants and spectral characteristics with those given in the literature; in some cases a comparison with authentic samples was used.

On methylation with diazomethane, triandrin (III) is converted into vimalin (IV). Their enzymatic hydrolysis with β-gluosidase gave, respectively, p-coumaryl (I) and p-methoxycinnamyl (II) alcohols, which were isolated in the free form. The acetylation of triandrin (III) gave a pentaacetate including an aromatic acetoxy group (δ 2.30 in the PMR spectrum) which showed the glycosylation of the alcohol group of the p-coumaryl alcohol. Doublets with J = 16 Hz in the PMR spectra characterized compounds (I-IV) as derivatives of trans-cinnamyl alcohol.

Triandrin and vimalin with the cis configuration of the double bond, isolated from willow bark (Salix trianda and S. viminalis), have been described in the literature [5]. We repeated this experiment. The substances isolated from the willow bark had constants corresponding to this given in the literature [5] but they had the trans configuration of the double bond, like compounds (III) and (IV). In view of this, the structures of triandrin and vimalin given in the literature [5] must be corrected.

β-Glucosidase readily hydrolyzed compounds (VI) and (VII) to p-coumaric acid (V), and compound (IX) to caffeic acid (VIII), and these were also isolated from the biomass. The

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