Combination of phase-solubility method and capillary zone electrophoresis to determine binding constants of cyclodextrins with practically water-insoluble compounds

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Abbreviations: ACE, affinity capillary electrophoresis; BGE, background electrolytes; CD, cyclodextrins; DMSO, dimethyl sulfoxide; EOF, electroosmotic flow; FA, frontal analysis, HD, Hummel–Dreyer method; HP-β-CD, hydroxypropyl-β-cyclodextrin; HP-γ-CD, hydroxypropyl-γ-cyclodextrin; KCE, kinetic capillary electrophoresis; NECEEM, non-equilibrium CE of equilibrium mixtures; PS-CZE, phase-solubility technique and capillary zone electrophoresis; pre-eq CZE, pre-equilibrated CZE; VACE, vacancy affinity CE; VP, vacancy peak.

Abstract

The combined method based on phase-solubility technique and capillary zone electrophoresis (PS-CZE) was suggested for the determination of binding (stability) constants of cyclodextrins (CD) complexes with water-insoluble organic compounds that have no or weak UV chromophores. In this method, the insoluble compounds are agitated at the desired temperature in CD solutions with different concentration up to the attainment of equilibrium and then CZE is used to determine the concentration of the compounds that have passed into the solutions. To avoid precipitation and complex dissociation, the background electrolyte (BGE) for CZE should contain ethanol and, if necessary, cyclodextrin. The samples should be diluted with the BGE without CD so that the CD concentrations in BGE and samples were equal to preclude a baseline shift. Using the suggested approach, the inclusion complexes between betulinic and betulonic acids, pentacyclic lupane-type triterpenes, and hydroxypropyl-β- and γ-cyclodextrins (HP-β-CD and HP-γ-CD) were studied. It was found that solubility of the acids studied in HP-β-CD solutions did not differ from their solubility in pure water. That is, the HP-β-CD complexes of the acids studied were not formed in noticeable amount. At the same time, the acids formed inclusion complexes with HP-γ-CD, what possibly was caused by the greater size of the HP-γ-CD molecule as compared to HP-β-CD. To determine binding constants by Higuchi and Connors method, the acids solubility was determined by CZE after their agitation in the solutions of HP-γ-CD (with 0.6 molar substitution) at 25 °C for 3 days. The dependences of acids solubility on HP-γ-CD concentration deviated from straight line in the range of high concentration (A_N mode). This can be explained by a self-association of HP-γ-CD molecules. Using the linear segment of the solubility dependences on CD concentration, the binding constants were determined. Their logarithms for the HP-γ-CD complexes with betulonic and betulinic acids were 3.88 ± 0.14 and 3.82 ± 0.12, respectively.

Keywords: Inclusion complexes; Betulin derivatives; Hydroxypropyl-γ-cyclodextrin; Kinetically inert complexes; Solubilization; Drug delivery

1. Introduction

Around 40 % of marketed drugs and about 90 % of drugs in their development have solubility-related problems [1]. One of a way to increase the solubility of poorly soluble or water-insoluble compounds is to obtain the inclusion complexes of drugs with cyclodextrins [2]. Cyclodextrins are natural macrocycles built up from residues of α-1,4-bonded D-glucopyranose. The most widespread compounds are CDs with six (α-CD), seven (β-CD), and eight (γ-CD) glucopyranose units. The distinctive speciality of the CD molecules is a shape of the truncated cone. Due to such structure, CDs can form inclusion complexes or host-guest complexes with different compounds, which have a number of benefits. To increase the CD solubility, a variety of selectively functionalized cyclodextrins was obtained, among which are hydroxypropyl derivatives. Most of

articles available in the literature deal with the properties of complexes of natural cyclodextrins. There are fewer articles concerning the complexes of CD derivatives, particularly HP-β-CD and HP-γ-CD, with different compounds [3-8].

Determination of binding constants between cyclodextrins and different compounds is carried out using a number of techniques such as spectroscopic techniques (ultraviolet/visible, circular dichroism, and fluorescence spectroscopy; nuclear magnetic and electron spin resonance), electroanalytical (polarography and voltammetry, potentiometry, electrical conductivity), separation techniques such as high performance liquid chromathography (HPLC) and capillary electrophoresis, as well as polarimetry, isothermal titration calorimetry, etc [9]. Capillary electrophoresis (CE) has such benefits as rapidity, high selectivity, small value of samples, and low-cost of analysis. Kinetic capillary electrophoresis (KCE) studies the complex dissociation under electrophoretic separation [10]. KCE allow us to determine the binding constants and rate constants for the formation and dissociation of the complex. However, KCE is applicable only for the laser induced fluorescence detection of the fluorescently labeled analytes. For the most frequently used UV detection, the difference in slopes of calibration curves for compounds and its complexes don't allow to apply equations deduced for KCE.

In affinity capillary electrophoresis (ACE or sometimes named as mobility shift assay) to study complexation, several electropherograms of analyte are recorded using BGEs with varying content of ligand, and on the base of effective electrophoretic mobilities and ligand concentration the stability, association or binding constant is calculated [6,11-23]. Sometimes, analyte is added to the BGE, and ligand is injected as a sample. To use ACE for practically water-insoluble compounds, it is necessary that these compounds may be recorded in electropherograms when their concentration is low. This is applicable to the compounds having chromophores, for example, benzene ring. ACE has been used to study complexation of CDs with such water-insoluble compounds as alkylnaphthalene derivatives [22] and erlotinib [23]. But there are water-insoluble compounds with no or weak chromophores such as betulin and its derivatives (Figs. 1a, b). When samples of the compounds solutions are injected, peaks may not appear in electropherograms, that is, the peak intensity is lower than the signal-to-noise ratio. For ACE, the on-line concentration approach with large sample injection (stacking) used for the determination of concentration is not applicable because changing the migration times is observed for large sample injection and it is difficult to separate the effects of complexation and stacking on the measured values of effective mobility [24].

For the practically water-insoluble compounds, the binding constants can be determined using phase-solubility method with the following determination of concentration by capillary zone electrophoresis (CZE). In CZE, a sample can be injected in a large amount as compared to ACE. In

addition, because of the incubation (agitation in order to obtain equilibrium mixture), the approach is suitable for slowly forming complexes to which the complexes of the practically water-insoluble compounds with cyclodextrins can be attributed. The aim of this study was to suggest the combined method based on phase-solubility technique and capillary zone electrophoresis (PS-CZE) for the determination of binding constants of cyclodextrins complexes with water-insoluble organic compounds. The method was tested by example of the complexes between betulin derivatives (pentacyclic lupane-type triterpenes having pharmacological activity [25]), betulonic and betulonic acids, and hydroxypropyl-β- and γ-cyclodextrins (Fig. 1).

2. Theory

To study the CD complexes of practically water-insoluble compounds using phase-solubility method, the suspensions of a compound in CD solutions with different concentration are prepared. Then the suspensions are agitated at the desired temperature up to the attainment of equilibrium. The attainment of equilibrium may take a long time (as a rule≤ 1 week). For 1:1 complex, the process is described as follows:

$$D + CD \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} D/CD, K = \frac{[D/CD]}{[D][CD]} = \frac{k_{on}}{k_{off}} \quad (1)$$

where D is the compound studied, D/CD is the inclusion complex between D and CD; [D/CD], [D], [CD] are the equilibrium concentrations of appropriate species, K is the equilibrium constant for complexation (also named binding, stability, formation or association constant), k_{on} and k_{off} are the rate constants for the formation and dissociation of D/CD, respectively. The solubility S of compound D in the CD solutions can be described using Eq. (2):

$$S = [D] + [D/CD] = S^0(1 + K[CD]) \quad (2)$$

where S^0 is the solubility of the compound in water, [D/CD] = K[D][CD] and [D] = S^0. The binding constant is determined by Higuchi and Connors method [3]. According to this method, the dependence of compound solubility in CD solutions, determined by appropriate analytical techniques, on total CD concentration is constructed. High selective separation techniques such as HPLC and CE have advantages over poorly selective spectrophotometry because the compound may be decomposed or transformed under long-term agitation. In turn, CE has higher efficiency and less cost of analysis as compared to HPLC. For linear mode of the dependence (A_L mode), the binding constant is calculated using Eq. (3):

$$K = \frac{b}{(1-b)S^0} \quad (3)$$

where b is the slope of the dependence of solubility on total CD concentration, C_{CD}. For positive deviation of the dependence from straight line (A_p mode), the formation of 1:2 complexes or self-assembly of the complexes is taken into account. The negative deviation of the dependence from straight line (A_N mode) can be explained by a self-association of CD molecules [26]. In addition, the

dependences can have a plateau at low (B_I mode) or moderate concentration of CD (B_S mode), what relates to the limited solubility of complexes.

3. Experimental

3.1. Instrumentation

The study was carried out using a capillary electrophoresis system with a diode-array detector Agilent ^{3D}CE G1600A (Agilent Technologies, Waldbronn, Germany) of the Krasnoyarsk Regional Center of Research Equipment, Federal Research Center "Krasnoyarsk Science Center SB RAS". Untreated fused silica capillaries with 50 μm id and the total/effective lengths of 64.5/56 cm and 80.5/72 cm were used (Agilent Technologies). The capillary temperature was kept constant at 25.00 ± 0.04 ºC. The data acquisition and processing were performed with the computer program ChemStation Rev.A.10.02. The separation was achieved by applying a voltage of + 30 kV. The positive voltage was applied to the capillary inlet. The direct detection was made at 200, 210, 220, 250, and 275 nm with the bandwidth of 6-10 nm. For indirect detection, the signal wavelength was at 350 nm with the bandwidth of 80 nm and the 5 above-listed values were used as references.

A new capillary was first flushed with 1 M NaOH for 10 min, then with ultra pure water for 10 min. At the beginning of each day, the capillaries were first flushed with 0.1 M NaOH for 5 min, twice with ultra pure water for 10 min and with running BGE for 15 min. Between the runs the capillaries were flushed with BGE for 5 min.

All pH measurements were made using a calibrated precise pH instrument «Expert-001-1» (Econix-Expert, Moscow, Russia) with a precision of 0.005 pH units. For phase solubility experiments, a liquid thermostat VT10-2 with a submersible circulating block for temperature adjustment «M01» (TERMEX, Russia, Tomsk) and an overhead stirrer with the fixing for 10 ml tubes were used.

3.2. Chemicals

The used reagents were analytical grade purity. (2-hydroxypropyl)-γ- and β-cyclodextrins with extents of labeling 0.6 molar substitution (average molecular weight 1580) and 1 (average molecular weight 1540), respectively, were purchased in Sigma-Aldrich (Moscow, Russia). CDs were dissolved in BGEs. The solution of 0.05 % dimethyl sulfoxide (DMSO) was used as an electroosmotic flow (EOF) marker. BGEs were filtered through 0.45 μm filters. Deionized water with electrical conductivity less than $0.1 \cdot 10^{-6}$ $S \cdot cm^{-1}$ from a water purification system Direct-Q3 (Millipore, France) was used for the solution preparation.

Betulonic and betulinic acids were synthesized in Institute of Chemistry and Chemical Technology SB RAS, Federal Research Center "Krasnoyarsk Science Center SB RAS" as described in articles [20,27]. Stock solutions of the acids with a concentration of 1 g/l were prepared by

dissolution of accurate weights in ethanol. Samples were prepared by dilution of the stock solutions with BGEs before electrophoretic separation.

3.3. Phase-solubility study and separation conditions

The solids of betulonic and betulinic acids were placed in 10 ml polypropylene tubes with tightly closed caps. In each tube, 3 ml of pure water or HP-CD solution was poured. Then the tubes were closed and placed in overhead stirrer to equilibrate. The agitation was carried out in liquid thermostat at 25 °C.

The suspensions after the attainment of equilibrium were centrifugated and filtered through 0.45 μm filters. The samples contained HP-CDs were injected hydrodynamically for 5-20 sec at a pressure of 50 mbar. The samples without HP-CDs were injected hydrodynamically for 100 sec at a pressure of 50 mbar. The concentration of betulonic and betulinic acids was determined from calibration curves which be constructed as the dependences of electrophoretic peak areas on acid concentration (5, 15, 30, and 50 mg/l). The solubility of betulonic and betulinic acids in pure water was determined using the BGE without HP-γ-CD from calibration curves which be constructed as the dependences of electrophoretic peak areas on acid concentration (0.5, 1, 2 and 3 mg/l). All experiments were repeated 2–3 times.

The effective electrophoretic mobility from experimental data was calculated as follows:

$$\mu = \frac{l \cdot l_{eff}}{U}\left(\frac{1}{t} - \frac{1}{t_{eof}}\right) \quad (4)$$

where l and l_{eff} are the total and effective capillary lengths, respectively, U is the voltage, t is the migration time measured at the top of peak, t_{eof} is the migration time of EOF marker.

The factor allowing the corrections for viscosity change, υ, for each BGE was calculated as follows [20]:

$$\upsilon = t^{'}/t^{0} \quad (5)$$

where t' and t^0 are the times for DMSO migration at voltage of 0 kV and hydrodynamic pressure of 50 mbar in the BGEs with the HP-CD addition and without it, respectively.

4. Results and discussion

4.1. Concept of the combined method based on phase-solubility technique and capillary zone electrophoresis

Increase of solubility of water-insoluble compounds in CD solutions occurs as a result of the formation of inclusion complexes of the compounds with CD (Eq. (1)). To quantify the solubility by CZE, it is necessary to select such conditions for electrophoretic separation under which the complex will be practically undissociated or, on the contrary, fully dissociated. Since the compounds studied are water-insoluble, the investigation of complex dissociation should be conducted using the BGEs in which the compound would not precipitate. For this purpose we

suggest BGEs with the addition of 10 % ethanol (vol.). If the complex is not fully dissociated under electrophoretic separation then, to prevent the complex dissociation, CDs should be added in BGEs.

Comparison of the suggested approach (PS-CZE) with the existing methods for the estimation of stability constants (binding constants) on the basis of capillary electrophoresis is shown in Table 1. The approach is suitable for the complexes with slow and moderate kinetics when the time needed for equilibration, t_{eq}, is comparable to the CE time scale, t_{CE}. Unlike pre-equilibrated CZE (pre-eq CZE), in PS-CZE the studied compound is agitated into incubation solutions, after that, the solid phase is separated by centrifugation and filtration. The second distinction is that the organic solvent is added into BGE to prevent the precipitation of the water-insoluble compound as a result of mixing the sample with BGE. Ligand should be added in BGE when $t_{eq} \sim t_{CE}$. Even if a complex is fully dissociated under electrophoretic separation, the method can be used because the compound studied is water-insoluble and the increase of solubility in the presence of HP-γ-CD is caused by the complexation.

4.2. Application to studying CD complexes of betulin derivatives

Below, the approach is considered using the study of the inclusion complexes between betulinic and betulonic acids and hydroxypropyl-β- and γ-cyclodextrins as an example.

4.2.1. Optimization of conditions for electrophoretic separation

At first, such BGE was chosen which allows one to record the acids anions with the largest sensitivity. A number of BGEs for indirect and direct detection was studied. The compounds with weak chromophores such as betulin and its derivatives (Figs. 1a, b) are usually detected in CZE using indirect UV detection. In such mode of detection, BGE contained UV absorbing co-ions is used and the decrease of co-ion concentration in analyte zone is recorded [19]. According to the literature data, pKa of betulinic acid is 5.5 [7], therefore the pH of BGE should not be less than 4.5, so that the acids would be in the ionic form [28]. The following BGEs for indirect detection were studied:

1) 90 % (10.0 mM benzoic acid, 9.1 mM LiOH, pH 5.3) + 10 % ethanol,

2) 90 % (10.0 mM picolinic acid, 6.7 mM LiOH, pH 5.7) + 10 % ethanol.

No peak of betulonic or betulinic acids appeared in electropherograms recorded using these BGEs even when the acids with a concentration of 1 g/L were injected. The decrease of absorbance as a result of the BGE ions decrease in the sample zone is possibly compensated by the absorbance of the studied acids and so the peak intensity is lower than the signal-to-noise ratio.

Then the following BGEs for direct detection were studied (i.e. detection of the inherent absorbance of analytes):

1) 90 % (10.0 mM acetic acid, 5.3 mM LiOH, pH 4.8) + 10 % ethanol,

2) 90 % (10.0 mM phosphoric acid, 16.5 mM LiOH, pH 7.2) + 10 % ethanol,

3) 90 % (10 mM borax, pH 9.3) + 10 % ethanol.

When using acetic BGE, no peak appeared in electropherograms even for high acid concentration. The peaks recorded using phosphate BGE were low-intensity. Only borate BGE allows to record peaks with acceptable signal-to-noise ratios with the optimal detection wavelength of 200 nm. It was found that the capillary with length of 80.5 cm allows us to better separate the acids peaks from the peak of EOF marker as compared to the capillary with length of 64.5 cm, which agrees with the results in articles [28,29].

Using borate BGE with the addition of 10 % ethanol, electropherograms of betulonic acid (BA) with different content of HP-γ-CD in samples were recorded (Figs. 2a-d). As can be seen in Fig 2b, c, the complex between BA and HP-γ-CD was partially dissociated when HP-γ-CD concentration in samples was 0.5 and 1 mM. This indicates that the on-off kinetics is moderate. A peak of the complex was recorded in electropherograms when the HP-γ-CD concentration in samples was 5 mM (Fig. 2d), but the baseline was distorted and a small additional peak occurred at the right shoulder of the complex peak. The occurrence of the peak is possibly caused by moderate kinetics of dissociation of the complexes for high HP-γ-CD concentration. Thus, for the compounds studied, it is impossible to fully decompose the complex even as the HP-γ-CD concentration in the sample is low. To prevent the complex dissociation under electrophoretic separation, HP-γ-CD should be added in BGE.

Figs. 2 e, f shows electropherograms of betulonic acids obtained with using the BGEs with the addition of 2 and 10 mM HP-γ-CD. The effective electrophoretic mobilities of the complex peak calculated for BGEs with the addition of 2 and 10 mM HP-γ-CD were practically identical, taking into account viscosity correcting factor ($\mu \cdot \nu = 6.15 \pm 0.05 \cdot 10^{-9}$ $m^2V^{-1}s^{-1}$). For the BGEs with the HP-γ-CD addition and for the case when the HP-γ-CD concentration in samples was 5 mM, a trace peak appeared in electropherograms (Fig. 2 d-f). It is possible that this is an impurity in BA. But the ratio of the complex peak area to the peak area of the impurity was more than 30, this is less than 3.3% of impurity in BA. Thus, the inaccuracy in calculating the BA concentration in the standard solution due to the impurity is less than the random error in the peak area measurement (5-7 %). For subsequent separations, borate BGE with the addition of 2 mM HP-γ-CD was chosen. The calibration curves obtained on the basis of this BGE were linear in the concentration range from 5 to 50 mg/l for the sample injection with hydrodynamic pressure of 50 mbar in 20 sec and in the concentration range 0.5-3 mg/l for 50 mbar*100 sec. The similar picture was observed for betulinic acid.

4.2.2. Determining binding constants of betulin derivatives with HP-β-CD and HP-γ-CD

The solubility of betulonic and betulinic acids in solutions of HP-β-CD and HP-γ-CD was studied by PS-CZE. It was found that the acids solubility in the HP-β-CD solutions, obtained after

acids agitation for 7 days in 10 mM HP-β-CD, did not differ from their solubility in pure water (Fig. 3a), that is, complexes of the acids with HP-β-CD under such conditions were not formed. It is possible that the hydroxypropyl groups precluded the complex formation because previously it was shown that the solubility of the acids in the presence of β-CD increased owing to complexation [20].

The solubility of betulonic and betulinic acids was found to increase in the presence of HP-γ-CD (Figs. 3b, c), that attests the complex formation between the acids and these CD. This may be due to the greater molecule size of HP-γ-CD as compared with HP-β-CD. In electropherograms of the samples obtained after the agitation of the solid compounds in HP-γ-CD, another peak designated as sp is present (Figs. 3a, b). Its occurrence is possibly caused by the ethanol absence in the samples because the system peak was more low-intensity when the samples were diluted with BGE (with the addition of ethanol) as compared to the samples diluted with water. But the presence or absence of this peak did not influence the peak area of the complexes and so the samples may be diluted by water.

To find the time needed for equilibration, the solubility of betulonic and betulinic acids was studied in HP-γ-CD solutions after different agitation times (Fig. 4). As shown in Fig 4, the equilibrium was reached after 3 days of agitation, irrespective of HP-γ-CD concentration. It is significantly longer than 2 hour needed for equilibration of the β-CD complexes with these acids [20].

To evaluate the binding constants by Higuchi and Connors method, the solubility of betulonic and betulinic acids at 25 °C in HP-γ-CD solutions with different concentration after 3 day agitation was determined by PS-CZE. It was shown that in order to eliminate the baseline shift, the samples with larger HP-γ-CD content as compared to BGE should be diluted with BGE without HP-γ-CD so that the CD concentrations were equal in BGE and samples. In Fig. 5, solubility diagrams for the studied system were shown. As can be seen from Fig. 5, they are not classical linear dependences. They deviate from linear dependences in the range of high concentration (higher than 20 mM), that is A_N mode of dependences. The similar dependences were observed for other water-insoluble compounds, such as curcumin [3] and tolbutamide [8] in HP-γ-CD solutions. This can be explained a self-association of HP-γ-CD molecules [26].

Using the linear segment of the solubility dependences on CD concentration, binding constants were determined. The values of experimental solubility in water and binding constants (Eq. (3)) are shown in Table 2. It is worth noting that the experimentally obtained value of betulinic acid solubility agrees with the literature value obtained by HPLC, 1.6 ± 0.7 mg/L [30] or 3.5 ± 1.5 10^{-6} M (taking into account of molecular weight, 457). In addition, the obtained results on solubility were confirmed by HPLC (Supplementary data). Taking into consideration the very low solubility

of the compounds studied and their incomplete dissociation, the values of *K* can be equated to the thermodynamic binding constants at null ionic strength. The betulonic and betulinic acids have similar binding constants within the error. Thus, the small change of the structure of a voluminous molecule does not effect on binding constants.

The obtained value of binding constant of betulinic acid with HP-γ-CD agrees in principle with the value determined by HPLC in 55 % acetonitrile medium, 3470 [7], taking into account the fact that the addition of organic solvent generally results in the decrease of binding constants for inclusion complexes [31]. At the same time, the obtained values of binding constant significantly differ from the value of 23 M^{-1} obtained by spectrophotometry [5]. In addition in the study [5], the experimentally obtained value of betulinic acid solubility was higher by a factor of a hundred. This possibly relates to the fact that betulinic acid is a low absorbing compound and spectrophotometry is not a suitable technique for such compounds.

The obtained values of binding constants are larger by an order of magnitude than the values for these acids with β-CD: 250 ± 20 M^{-1} for the complex with betulinic acid and 300 ± 20 M^{-1} for the complex with betulonic acid [20]. It is possible that the presence of the hydroxypropyl groups not only inhibits the kinetics of complex formation but also results in higher binding constants. But the presence of the hydroxypropyl groups in HP-β-CD is likely to preclude the complex formation under studied conditions.

5. Conclusions

Thus, the method combined phase-solubility technique and capillary zone electrophoresis (PS-CZE) was suggested for the determination of the binding constants of cyclodextrins complexes with water-insoluble organic compounds that have no or weak UV chromophores. In this method, the insoluble compounds are agitated at the desired temperature in CD solutions with different concentration up to the attainment of equilibrium and then CZE is used to determine the concentration of the compounds transferred to solution. To avoid precipitation and complex dissociation, the background electrolyte should contain ethanol and, if necessary, cyclodextrin. The samples with larger CD content as compared to BGE should be diluted with the BGE without CD so that the CD concentrations in BGE and samples were equal to preclude a baseline shift. Using the suggested approach, the complexes of betulinic and betulonic acids with HP-β-CD and HP-γ-CD were studied. It was found that the HP-β-CD complexes of the studied acids were not formed in noticeable amount. The logarithms of stability constants for the HP-γ-CD complexes with betulonic and betulinic acids were 3.88 ± 0.14 and 3.82 ± 0.12, respectively. The results were confirmed by HPLC, compared with which the PS-CZE approach has less cost of analysis.

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Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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Tables

Table 1

Comparison of the suggested approach with the existing methods for the estimation of stability constants (binding constants) on the basis of capillary electrophoresis [10,12-16].

On-off kinetics[a]	Method	Variation of content	Sample	Additive in BGE	Quantitative foundation	Analyte and complex mobilities	Ligand and complex mobilities
Slow or moderate	PS-CZE	ligand in incubation solutions	analyte+ligand	organic solvent + ligand for $t_{eq} \sim t_{CE}$[a]	peak area	different	different
Slow	pre-eq CZE[b]		analyte+ligand	-	peak area or height	different	different
Moderate	KCE[c] (NECEEM)	-	analyte+ligand	-	peak area	different	different
Fast	ACE	additive in BGE	analyte	ligand	mobility	different	any
			ligand	analyte		any	different
	VP	ligand in BGE	neat buffer	analyte+ligand	peak area or height	different	equal
	VACE		neat buffer	analyte+ligand	mobility	different	any
Fast or slow[d]	HD	ligand in samples	analyte +ligand	analyte	peak area or height	different	equal
	FA	ligand in samples	analyte+ligand	-	plateau height	different	equal

[a] On-off kinetics is moderate when t_{eq}, the time needed for equilibration, is comparable to t_{CE}, the CE time scale. If $t_{eq} >> t_{CE}$, that is slow on-off kinetics. And for $t_{eq} << t_{CE}$, on-off kinetics is fast.

[b] Other names are direct separation method of free and complexed analyte, pre-incubated CZE

[c] For only laser induced fluorescence detection of the fluorescently labeled analytes

[d] For slow on-off kinetics, it is necessary pre-equilibration.

Table 2

Experimental solubility of the acids in deionized water and binding constants for the complexes between betulin derivatives and HP-γ-CD (with 0.6 molar substitution) at 25 ºC.

Parameter	Betulonic acid	Betulinic acid
S^0, 10^{-6} M	2.6 ± 0.6	3.0 ± 0.7
log K	3.88 ± 0.14	3.82 ± 0.12

Figure captions

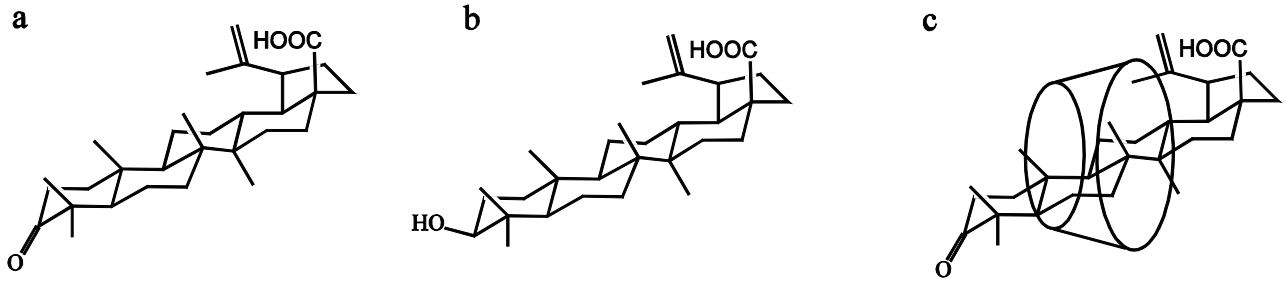

Fig. 1. Structural formulas of (a) betulonic and (b) betulinic acids and (c) possible scheme of the inclusion complex between betulonic acid and cyclodextrin.

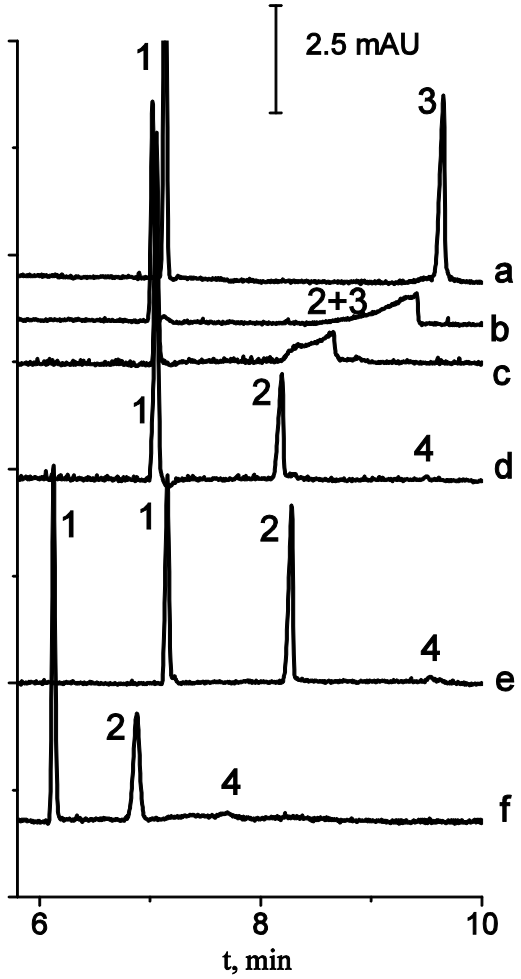

Fig. 2. Electropherograms of the standard solutions of betulonic acid (100 mg/l) (a) without HP-γ-CD, with the HP-γ-CD addition in samples with a concentration of (b) 0.5 mM, (c) 1 mM, (d) 5 mM, with the HP-γ-CD addition in BGE with a concentration of (e) 2 mM and (f) 10 mM. BGE: 9 mM $Na_2B_4O_7$, 10 % ethanol, and 0 mM HP-γ-CD if another was not stated. Detection was at 200 nm. Samples were injected hydrodynamically for 5 sec at a pressure of 50 mbar. Peaks: 1 – EOF marker, 2 – complex between HP-γ-CD and betulonic acid, 3 – betulonic acid, 4 - trace impurity peak

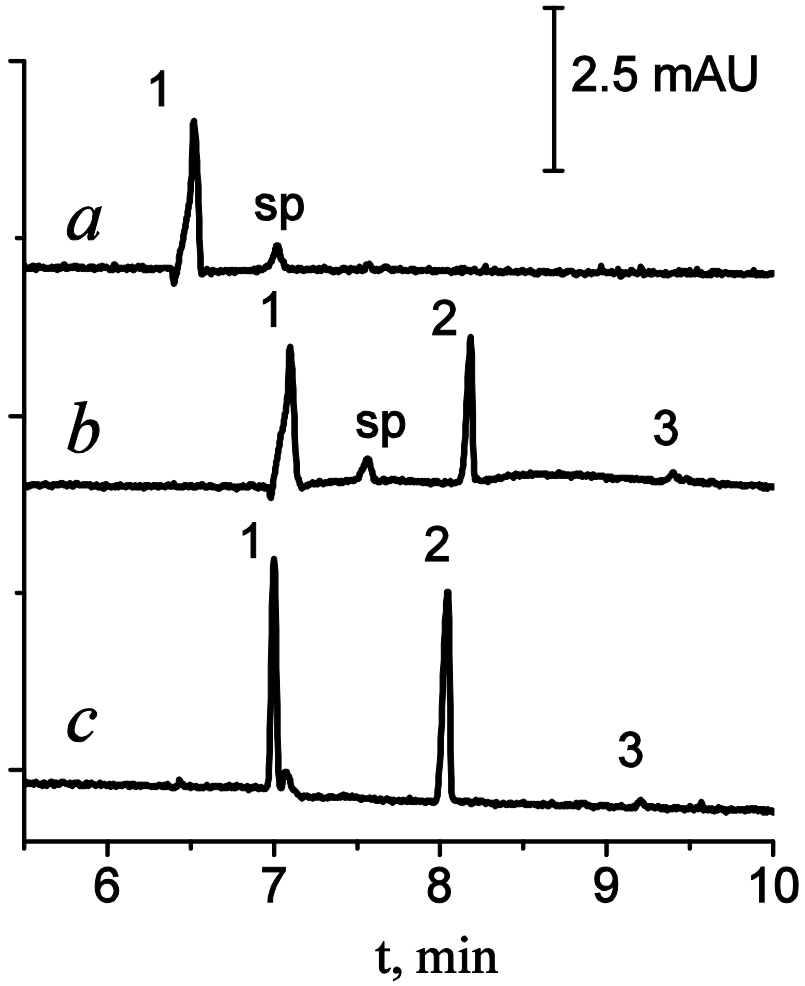

Fig. 3. Electropherograms of the solutions obtained after agitation of betulonic acid for 7 days in 10 mM solutions of (a) HP-β-CD and (b) HP-γ-CD, and (c) the standard solution of betulonic acid (100 mg/l). BGE: 9 mM $Na_2B_4O_7$, 10 % ethanol, (a) 10 mM HP-β-CD or (b, c) 2 mM HP-γ-CD. Samples were injected hydrodynamically for 5 sec at a pressure of 50 mbar. Detection was at 200 nm. Peaks: 1 – EOF marker (neutral compound, dimethyl sulfoxide), 2 – complex between HP-γ-CD and betulonic acid, 3 - trace impurity peak, sp is the system peak (see text).

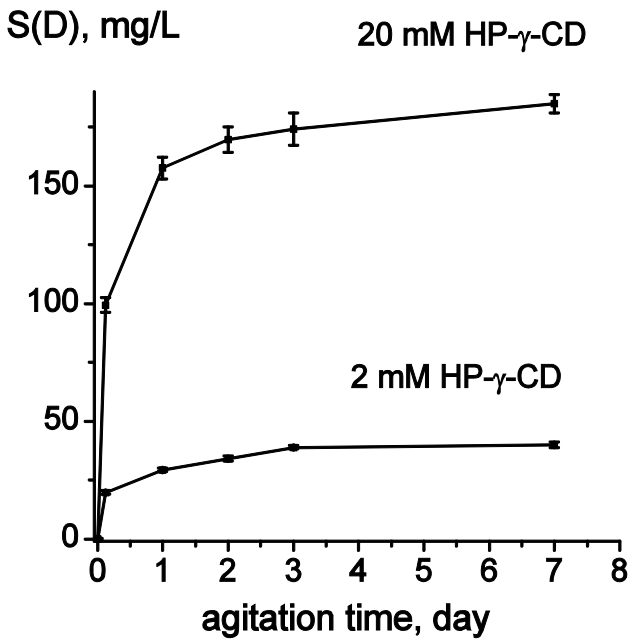

Fig. 4. Dependences of solubility of betulonic acid in 2 and 20 mM HP-γ-CD solutions on the agitation time

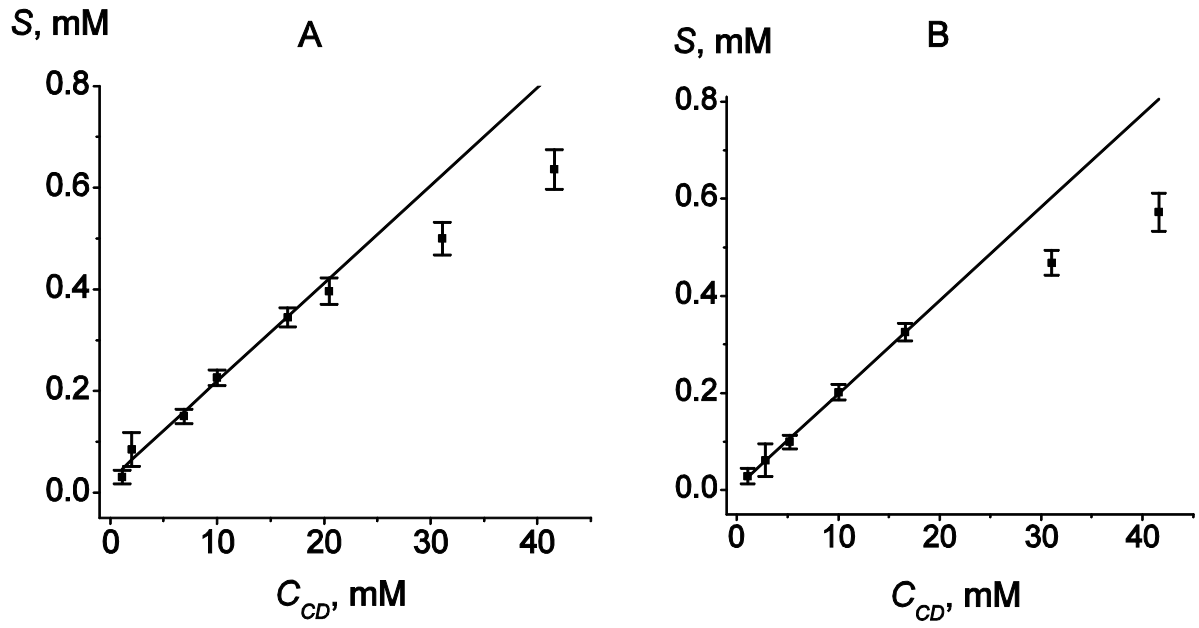

Fig. 5. Solubility diagrams of betulonic (A) and betulinic (B) acid in HP-γ-CD solutions. The straight lines were constructed by the least-squares method using all the points below 20 mM.