# MEDICINAL PLANTS

## SPECTROPHOTOMETRIC DETERMINATION OF HYDROXYCYNNAMIC ACID AND RELATED COMPOUNDS IN ECHINACEA PREPARATIONS

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In recent years, preparations based on biologically active substances from various species of *Echinacea Moench* genus have occupy leading positions on the market of drugs prepared from medicinal plants. Echinacea extracts possess immunostimulant, antiinflammatory, wound-healing, and antimicrobial, and antiviral properties [1, 2]. Nevertheless, no simple and reliable analytical methods for monitoring the quality of echinacea preparations have been developed so far.

In most cases, there is no need for identification and quantitative determination of each component in a given phytopreparation, since the biological effect is frequently determined to a considerable extent by the synergistic action of several substances, including unidentified compounds present in trace amounts [3, 4]. For product quality monitoring and standardization, it is expedient to determine one or several compounds from each group of biologically active substances contained in a given plant, which account for the pharmacological effect of a preparation based on this plant.

One of the the main groups of biologically active substances in echinacea includes hydroxycinnamic (caffeic) acid (HCA) and related compounds representing HCA conjugates with sugars, quinic acid, and tartaric acid [1, 3, 4]. HCA possesses antibacterial, antifungal, and antioxidant properties [5]. A key role in the immunostimulant action of HCA preparations is played by chicoric acid [1, 3, 4]. For this reason, methods capable of determining the total content of HCA and its derivatives are commonly used for quality monitoring and standardization of echinacea preparations.

According to a temporal pharmacopoeial article for the echinacea rhizome and root tincture [6], the preparation quality is checked by titration. However, this method determines the total content of carboxylic acids rather than the content of HCA derivatives accounting for the immunostimulant action. A more selective method for determining HCA derivatives (calculated for chicoric acid) is based on the optical absorption in the UV spectral range [7], which is included into a pharmacopoeial article for *Echinacea purpurea* herbs [8] and into a temporal pharmacopoeial article for the echinacea rhizome and roots [9]. Unfortunately, this method is still insufficiently selective and reliable, which is related to the fact that the analytical signal of optical absorption at 330 nm reflects the presence of both HCA derivatives and their oxidation products (quinones) absorbing in the same region [10]. For example, analyses of echinacea extract by the pharmacopoeial method [7] show virtually the same content of HCA derivatives in both fresh preparations and those with expired storage duration, which can hardly be correct. It should also be noted that chicoric acid cannot be used as a reference compound because it does not meet all the requirements of the existing normative documents [11].

Since the immunomodulant action of echinacea is due to the HCA derivatives, it is important to develop a method for their selective determination in the presence of other stained substances contained in a given phytopreparation. In order to increase the selectivity of analysis, it is necessary to find a reagent capable of selectively interacting with HCA derivatives. As is known, HCA and related compounds exhibit the properties of both carboxylic acids and polyphenols. Readily hydrolyzable metal salts form complexes with such compounds but do not react with with their oxidation products [12]. The known method of spectrophotometric determination [13] of a HCA complex with zirconium chloride requires prolonged time and shows unsatisfactory reproducibility of results.

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We have chosen HCA to be the reference compound and studied the interaction of Al(III) with HCA for developing a new spectrophotometric method for the quality control of echinacea preparations.

## EXPERIMENTAL PART

**Materials and instruments**. Analytical-grade HCA (Reakhim) was additionally purified by recrystallization from hot ($T$ = 70°C) distilled water, followed by drying to constant weight at $T$ = 130°C. A HCA solution with a concentration of 0.2 g/liter was obtained by dissolving an accurately weighed amount of the purified compound in distilled water; $AlCl_3$ (0.50 M) and $NH_4Cl$ (10%) solutions were prepared by dissolving analytical-grade compounds (Reakhim) in 0.01 M HCl and water, respectively.

The optical absorption measurements were performed on a Specord M-40 (Carl Zeiss Jena, Germany) and KFK-3 (LOMO, Russia) spectrophotometers in 1-cm optical cells. Acidity (pH) of the sample solutions was monitored with an EV-74 ionometer equipped with a glass electrode.

**Analytical procedure**. The optical absorption of the complex of aluminum(III) and HCA was studied as a function of pH. The sample solutions were prepared by mixing 1.0 ml of a 0.2 g/liter HCA solution (or 0.3 ml of echinacea tincture), 2.3 ml of a 0.5 M $AlCl_3$ solution, and the necessary amount of a 10% $NH_4Cl$ solution (to obtain the desired pH) in a 25-ml measuring flask. The optical density of each solution was measured at a wavelength of 335 nm ($A_{335}$).

Then, the optical density was studied as a function of the Al(III) concentration in solution. The samples were prepared by mixing 0.25 ml of a 0.2 g/liter HCA solution, 0.08 – 6.0 ml of a 0.5 M $AlCl_3$ solution, and a 10% $NH_4Cl$ solution (to adjust pH 4.8). The optical density of each solution was measured at a wavelength of 335 nm ($A_{335}$).

The calibration curve was constructed using solutions prepared in 25-ml measuring flasks from 0.1 – 10.0 ml of a 0.02 g/liter HCA solution, 2.3 ml of a 0.5 M $AlCl_3$ solution, and 10% $NH_4Cl$ solution (to adjust to pH 4.8). The optical density of each solution was measured in a 1-cm cell at a wavelength of 335 nm ($A_{335}$).

## RESULTS AND DISCUSSION

The standard substance has to be chosen so as to meet two requirements: it should be readily available and possess properties specific of the object of analysis. In the case under consideration, such properties are the values of characteristic optical absorption in the UV and visible spectral regions. The spectra of cinnamic and chlorogenic acids (HCA derivatives more readily available than chicoric acid) differ significantly from those of echinacea extracts [4]. As can be seen from Fig. 1, the absorption spectrum of an HCA solution (curve *1*) is generally analogous to the spectra of chicoric acid (curve *2*) [10] and echinacea extract (curve *3*). This is evidence that the optical absorption of echinacea preparations in this spectral region is mostly due to chicoric acid and other HCA derivatives.

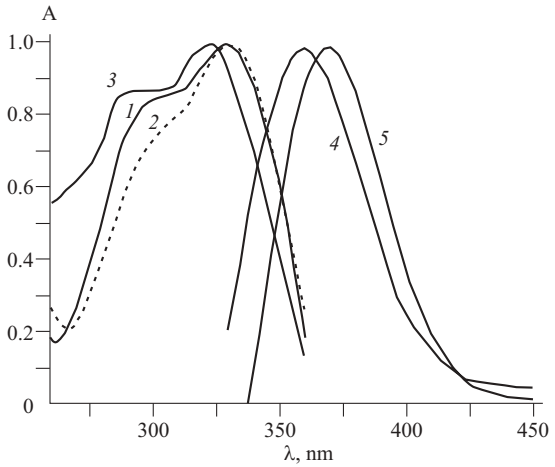


**Fig. 1.** Normalized optical absorption spectra of the aqueous solutions of (*1*, *4*) HCA, (*2*) chicoric acid [10], and (*3*, *5*) echinacea tincture: (*1 – 3*) without Al(III); (*4*, *5*) in the presence of Al(III) ($C_{Al(III)}$ = 0.045 mole/liter).

In the presence of Al(III), the spectra of an HCA solution and an aqueous echinacea extract (Fig. 1, curves *4* and *5*, respectively) also virtually completely coincide. The bathochromic shift of the absorption peak observed upon introduction of the metal ions is related to the formation of complexes between HCA derivatives and Al(III) in solution. Based on the analogous properties and close absorption spectra of HCA and chicoric acid in solutions with and without Al(III), and taking into account that HCA is readily available and can be purified by recrystallization from aqueous solutions, we have chosen HCA as a reference compound for the spectrophotometric determination of the total content of HCA and its derivatives in echinacea preparations. The analyses were performed at a wavelength of 355 nm corresponding to the maximum absorption of the HCA – Al(III) complex.

In order to determine the optimum conditions for determining HCA derivatives, we have studied the optical absorption of HCA solutions in the presence of Al(III) as a function of pH, aluminum chloride content, and HCA concentration in

**TABLE 1.** Determining Hydroxycinnamic Acid in Standard Solutions ($n$ = 5, $P$ = 0.95)

| HCA concentration, mg/liter | |
|---|---|
| Introduced | Found ($x \pm \Delta x$) |
| 0.20 | 0.20 ± 0.02 |
| 1.20 | 1.20 ± 0.02 |
| 4.0 | 4.03 ± 0.04 |
| 8.0 | 8.0 ± 0.1 |

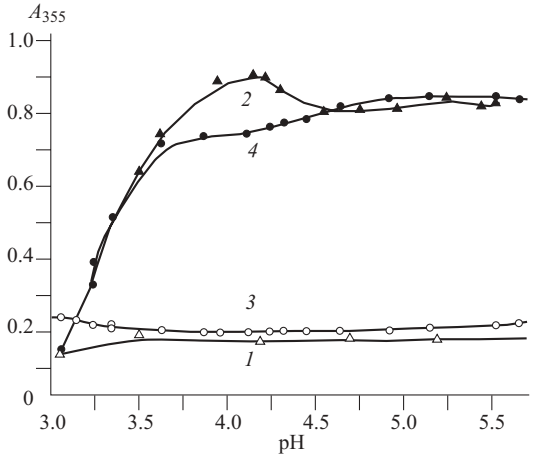


**Fig. 2.** Plots of the optical density ($l$ = 1 cm) at λ = 355 nm versus pH for the aqueous solutions of (*1*, *2*) HCA and (*3*, *4*) echinacea tincture (ET): (*1*, *3*) without Al(III); (*2*, *4*) in the presence of Al(III) ($C_{HCA}$ = 8.0 mg/liter; pH 4.8 ± 0.1).

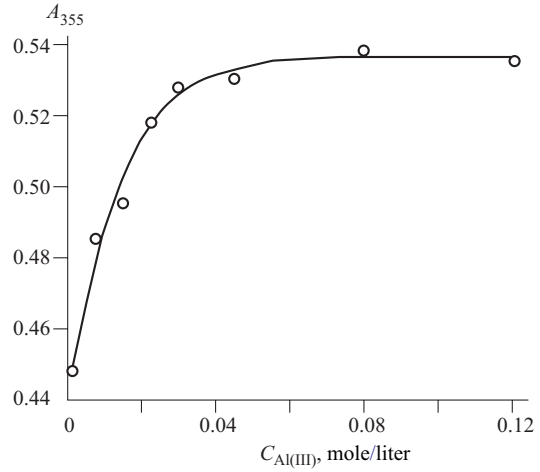


**Fig. 3**. The optical density ($l$ = 3 cm) of the aqueous solutions of HCA in the presence of various concentrations of Al(III) ($C_{HCA}$ = 2.0 mg/liter; pH 4.8 ± 0.1).

solution. It was established that the maximum optical density of HCA solutions in the presence of Al(III) is observed at pH 3.7 – 5.5 (Fig. 2, curve *2*). The optimum pH interval for the determination of HCA and its derivatives is from 4.5 to 5.5: under these conditions, the absorption is virtually independent of pH, which must improve the reproducibility of analyses. This pH interval also features a plateau in the absorption of echinacea tincture in the presence of $AlCl_3$ (Fig. 2, curve *4*). Subsequent experiments were performed with solutions adjusted at pH 4.8 ± 0.1 by adding $NH_4Cl$ solution.

Investigation of the dependence of the analytical response on the Al(III) content in solution showed that the optimum concentration of triply charged aluminum ions is 0.045 mole/liter (Fig. 3). The best Al(III) compounds are chloride and sulfate, whereas the use of $Al(NO_3)_3$ is undesirable because nitrate ions exhibit strong oxidative properties.

Under the optimum conditions, the interaction of Al(III) with HCA and its derivatives in a mixed solution immediately produces coloration that is stable for at least ~1.5 h.

**TABLE 2.** Determining the Total Content of Hydroxycinnamic Acid and Its Derivatives (Calculated for HCA, mg/liter) in *Echinacea Purpurea* Rhizome and Root Tincture (Batch 14.05.99) Using Intrinsic Absorption Method [7] and the Proposed Procedure ($n$ = 3, $P$ = 0.95)

| Storage duration *, months | HCA found, mg/liter ($x \pm \Delta x$) | |
|---|---|---|
| | method [7] | proposed method |
| 10 | 1120 ± 11 | 601 ± 10 |
| 30 | 642 ± 5 | 123 ± 3 |

* Maximum storage time according to manufacturer's instruction, 24 months.

The analytical signal intensity is a linear function of the HCA concentration in the interval from 0.1 to 8.0 mg/liter, in which the calibration plot is described by the equation

$$A_{355} = (0.002 \pm 0.001) + (0.0841 \pm 0.0005)C_{HCA}\ [mg/ml]$$

for $r$ = 0.9998. The detection threshold for HCA is 0.1 mg/ml.

Metrological characteristics of the proposed method were determined by analyzing a series of standard HCA solutions. The results summarized in Table 1 show that the spectrophotometric procedure provides for satisfactory reliability and reproducibility of the results.

An analysis of echinacea preparations should be performed with allowance for the absorption of components (e.g., the products of oxidation of HCA and its derivatives) not forming complexes with Al(III). This "background" can be subtracted by measuring the spectra with reference to a solution containing all the same components except for Al(III) salt.

Table 2 presents the results of statistical processing of the experimental data on the content of HCA derivatives (recalculated for HCA) in echinacea tinctures with various expiry dates. As can be seen, oxidation and decomposition of the complex of biologically active substances (including HCA derivatives) in the plant extract in the course of storage leads to a general decrease in the optical absorption signal intensity. The presence of other colored compounds is responsible for the absence of a direct proportionality between $\Delta A_{355}$ and $\Delta C_{HCA}$, which is not taken into account by the method [7] based on the measurement of the intrinsic optical absorption of echinacea tinctures. For example, the results of analyses using the proposed method with allowance for the intrinsic absorption showed that the content of HCA and its derivatives (and, hence, the immunomodulant activity)

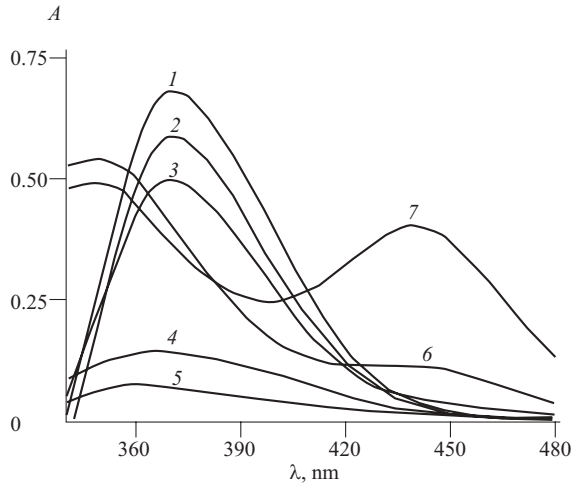


**Fig. 4.** The optical absorption spectra of (*1 – 5*) echinacea preparations and (*6*, *7*) model HCA – quercetin 2 : 1 and 10 : 1 solutions, respectively, in the presence of Al(III). Solution concentrations (vol.%): (*1*) *Echinacea purpurea* extract (Lubnyfarm, batch 21100), 0.48; (*2*, *5*) *Echinacea Purpurea* rhizome and root tincture (Kiev, batches 03.02.01 and 05.03.96, respectively), 1.2; (*3*, *4*) immunal (Lek, batches 1102810B and 43045009A, respectively), 1.2; $C_{HCA} = 3.5 \times 10^{-5}$ M; $C_{quercetin} = 1.75 \times 10^{-5}$ (*6*), $3.5 \times 10^{-6}$ M (*7*); $C_{Al(III)} = 0.045$ M; pH 4.8 ± 0.1; $l = 1$ cm.

dropped by a factor of almost 5, while the existing method [7] showed the decrease to be only by a factor of about 1.7.

As is known [1], echinacea ethanol extract contains, in addition to HCA and its derivatives, comparable amounts of flavonoids (glycosides of apigenin, luteolin, kaempferol, quercetin, etc.) representing another class of natural polyphenols and exhibiting significant optical absorption in the wavelength range of λ = 320 – 380 nm [10]. We have used quercetin to study the effect of flavonoids on the results of determination of HCA and its derivatives in echinacea preparations. It was found that quercetin, as well as HCA, forms a complex with Al(III), but the peak of absorption of this complex in the UV – VIS spectral range occurs at 433 nm [14]. Figure 4 shows the absorption spectra of model solutions containing HCA and quercetin in different ratios (curves *6* and *7*) in comparison to the spectra of echinacea preparations (curves *1 – 5*) in the presence of Al(III). As can be seen from this figure, even the spectrum of a mixed solution with an HCA/quercetin ratio of 10 : 1 (curve *7*) clearly reveals a peak related to the Al(III) – quercetin complex formation. At the same time, the spectra of echinacea preparations in the presence of Al(III) exhibit no absorption peak in this region, which is evidence of the absence of quercetin and other flavonoids in solution. This can be related to a significant (80-fold) dilution of the initial echinacea extract in the course of the sample preparation. As a result, the concentration of ethanol decreases from 40 to 0.5% and the flavonoids (poorly soluble in water) exhibit precipitation. Therefore, the flavonoids present in echinacea preparations do not interfere with the proposed spectrophotometric analysis for HCA and its derivatives in water – alcohol based phytopreparations.

**TABLE 3.** Determining the Total Content of Hydroxycinnamic Acid and Its Derivatives (Calculated for HCA, mg/liter) in *Echinacea Purpurea* Rhizome and Root Tincture (Samples 1 – 6), *Echinacea Purpurea* Extract (Sample 7), and Immunal Preparation (Samples 8 and 9) by the Proposed Method ($n = 3$, $P = 0.95$)

| Sample No. | Manufacturer (Batch) | Expiry date | [HCA], mg/liter ($x \pm \Delta x$) |
|---|---|---|---|
| 1 | Kiev, 05.03.96 | III 1997 | 95 ± 6 |
| 2 | Kiev, 14.05.99 | V 2001 | 123 ± 3 |
| 3 | Kiev, 21.10.00 | X 2002 | 65 ± 5 |
| 4 | Kiev, 03.02.01 | II 2003 | 407 ± 9 |
| 5 | Ternopol, 25.10.01 | XI 2003 | 229 ± 7 |
| 6 | Ternopol, 18.08.01 | IX 2003 | 361 ± 4 |
| 7 | Lubnyfarm, 21100 | XII 2002 | 1800 ± 20 |
| 8 | Lek (Ljubljana, Slovenia), 4304509A | IX 2001 | 170 ± 9 |
| 9 | Lek (Ljubljana, Slovenia) 1102810B | X 2002 | 369 ± 8 |

**Determining HCA and its derivatives in echinacea preparations**. To 2.5 ml of a water – alcohol based echinacea preparation in 25-ml measuring flask was added distilled water to the mark and the mixture was thoroughly stirred and filtered through a paper filter. To 1-ml aliquot of the filtrate in a 25-ml measuring flask was added 2.3 ml of 0.5 M $AlCl_3$ or $Al_2(SO_4)_3$ solution and the mixture was adjusted at pH 4.8 ± 0.1 with the aid of a 10% $NH_4Cl$ solution, after which the flask was filled with distilled water to the mark. The solution was thoroughly stirred and characterized with respect to the optical density at λ = 355 nm in a spectrophotometer (or photoelectrocolorimeter) in a 1-cm optical cell relative to reference solutions containing the same components except for Al(III) salt. The content of HCA and its derivatives is determined using the $A_{355} - C_{HCA}$ calibration plot.

Table 3 presents the results of analyses for a series of water – alcohol based echinacea preparations and the complex preparation immunal from various manufacturers. The correctness of these results was confirmed by the method of standard additives. As can be seen, the content of HCA and its derivatives (and, hence, the immunomodulant activity) varies within broad limits even in products from the same manufacturer. This scatter confirms the need for standardization of echinacea preparations.

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