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# Application of thin layer chromatography in the analysis of efavirenz

**Aim.** To carry out the integrated study of visualization conditions of efavirenz on TLC-plates with aplication of standard and particular colored reagents, and the chromatographic behavior of efavirenz using standard mobile phases.

**Results and discussion.** It has been shown that such widely used color reagents as UV-light, Erdmann reagent, Froehde reagent, Liebermann reagent, sulfuric acid, Marquis reagent, Mandelin reagent, acidified iodoplatinate solution, iodine vapor can be used for efavirenz detection on chromatographic plates. Efavirenz gives the positive detection results with reagents used in the TLC-screening of extracts from the biological material for substances of basic, acid and neutral nature. The chromatographic mobility of efavirenz has been studied in 17 solvents systems; the systems are used as standard mobile phases according to the recommendations of the International Association of Forensic Toxicologists for TLC-screening of organic compounds of acid, neutral and basic nature, and as well as in the general TLC-screening of organic substances in the Ukrainian forensic toxicological laboratories.

**Experimental part.** The chromatographic plates Sorbfil® PTLC-IIH-UV and Merck® TLC Silica gel 60G were used as thin layers.

**Conclusions.** The behavior of efavirenz when developing on TLC-plates with two types of a substrate (plastic and glass) and with/without a luminophor with commonly used colored reagents has been studied. The $R_f$ values of efavirenz under chromatographing conditions in the standard solvent systems used for TLC-screening of organic compounds of acid, neutral and basic nature have been set.

***Key words:*** efavirenz; thin layer chromatography; color tests

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## Застосування тонкошарової хроматографії в аналізі ефавіренцу

**Мета.** Провести комплексне дослідження умов візуалізації ефавіренцу на ТШХ-пластинах із застосуванням стандартних і окремих кольорових реагентів, а також хроматографічної поведінки ефавіренцу з використанням стандартних рухомих фаз.

**Результати та їх обговорення.** Показано, що для виявлення ефавіренцу на хроматографічних пластинах можна використовувати такі широко відомі проявники, як УФ-світло, реактив Ердмана, реактив Фреде, реактив Лібермана, сульфатна кислота, реактив Маркі, реактив Манделіна, підкислений розчин йодоплатинату, пари йоду. Ефавіренц дає позитивні результати виявлення з реактивами, що використовують в ТШХ-скринінгу витягів з біологічного матеріалу на речовини основного, кислого і нейтрального характеру. Хроматографічну рухливість ефавіренцу досліджено в 17 системах розчинників, серед яких рухомі фази, що застосовують як стандартні згідно з рекомендаціями Міжнародного комітету з систематичного токсикологічного аналізу Міжнародної асоціації судових токсикологів для ТШХ-скринінгу органічних речовин кислого, нейтрального та основного характеру, а також у загальному ТШХ-скринінгу органічних речовин у судово-токсикологічних лабораторіях України.

**Експериментальна частина.** Як тонкі шари використовували пластини Sorbfil ПТСХ-ІІВ-УФ та Merck TLC Silica gel 60G.

**Висновки.** Досліджено поведінку ефавіренцу при проявленні загальноприйнятими хромогенними реагентами на пластинах для ТШХ з двома типами підложки (пластик та скло) та з УФ-індикатором і без нього. Встановлено значення $R_f$ ефавіренцу за умов хроматографування в стандартних системах розчинників, що використовуються для ТШХ-скринінгу речовин кислого, нейтрального та основного характеру.

***Ключові слова:*** ефавіренц; тонкошарова хроматографія; кольорові реакції

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## Применение тонкослойной хроматографии в анализе эфавиренца

**Цель.** Провести комплексное исследование условий визуализации эфавиренца на ТСХ-пластинах с применением стандартных и частных цветных реагентов, а также хроматографического поведения эфавиренца с использованием стандартных подвижных фаз.

**Результаты и их обсуждение.** Показано, что для обнаружения эфавиренца на хроматографических пластинах можно использовать такие широко применяемые проявители, как УФ-свет, реактив Эрдманна, реактив Фреде, реактив Либермана, серная кислота, реактив Марки, реактив Манделина, подкисленный раствор йодоплатината, пары йода. Эфавиренц дает положительные результаты обнаружения с реактивами, используемыми в ходе ТСХ-скрининга извлечений из биологического материала на вещества основного, кислого и нейтрального характера. Хроматографическая подвижность эфавиренца исследована в 17 системах растворителей, среди которых подвижные фазы, применяемые в качестве стандартных согласно рекомендациям Международного комитета по систематическому токсикологическому анализу Международной ассоциации судебных токсикологов для ТСХ-скрининга органических веществ кислого, нейтрального и основного характера, а также в общем ТСХ-скрининге органических веществ в судебно-токсикологических лабораториях Украины.

**Экспериментальная часть.** В качестве тонких слоев использовали пластины Sorbfil ПТСХ-IIВ-УФ и Merck TLC Silica gel 60G.

**Выводы.** Исследовано поведение эфавиренца при проявлении общепринятыми хромогенными реагентами на пластинах для ТСХ с двумя типами подложки (пластик и стекло) и с УФ-индикатором и без него. Установлены значения $R_f$ эфавиренца в условиях хроматографирования в стандартных системах растворителей, используемых для ТСХ-скрининга веществ кислого, нейтрального и основного характера.

***Ключевые слова:*** эфавиренц; тонкослойная хроматография; цветные реакции

HIV infection is usually treated with drug combinations consisting of at least three different antiretroviral medicines. Essential components of this highly active antiretroviral therapy are non-nucleoside and nucleoside reverse transcriptase inhibitors [1, 2]. The group of non-nucleoside reverse transcriptase inhibitors are currently presented by five medicines (nevirapine, delavirdine, efavirenz, etravirine and rilpivirine) approved by the Food and Drug Administration; four of them (nevirapine, efavirenz, etravirine and rilpivirine) have been approved by the European Union [3–5].

Efavirenz is recommended currently as a preferred first-line medicine with a low pill burden, once daily dosing, a long half-life allowing for relatively stable plasma concentrations and some forgiveness for doses not taken exactly on the schedule [6, 7].

Efavirenz therapy accompanies with quite a number of side effects showed by psychiatric symptoms, including insomnia, nightmares, memory loss, depression, and anxiety. Efavirenz is characterized by definite neuropsychological symptoms in 50% of cases; its neurotoxicity exceeds other antiretroviral medicines [8–10]. The studies of efavirenz showed that in 20–50% of cases the toxic concentrations of the medicine in the blood were fixed [11–12]. There are cases of acute poisoning due to administration of efavirenz, including cases of suicide attempts [13]. Therefore, in our opinion, efavirenz may be approved as a potential toxic compound in forensic toxicology.

The method of thin layer chromatography (TLC) is widely used in the process of forensic toxicological examinations for screening and confirming investigations – with the purpose of analyte detection and identification, respectively [14, 15]. The main focus is the chromatographic behavior of the substances using standard mobile phases (or solvents systems), as well as the conditions of analyte spots visualization using standard colored reagents [14, 15].

The aim of our work was the integrated study of visualization conditions of efavirenz on TLC-plates using standard and particular colored reagents, as well as the chromatographic behavior of the substances using standard mobile phases.

To fix the results of visualization of the substances to be analyzed 4 developing modes of TLC-plates with two types of a substrate (plastic and glass) and with/without a luminophor (or UV-indicator) were chosen:

1) immediately after processing the substances with a reagent and after drying the plates at the ambient temperature;

2) in UV-light at two wavelengths – 254 nm and 365 nm;

3) after heating the plates for 15 min at 110°C (the plates are covered with glass);

4) in UV-light at two wavelengths – 254 nm and 365 nm – after heating.

A number of the reagents studied (hydrogen peroxide solution, Nessler reagent, perchloric acid**,** FPN reagent, hydrochloric acid vapor, 1% $H_8[Si(W_2O_7)_6]$ solution, 1% $H_7[P(Mo_2O_7)_6]$ solution, 1% $H_7[P(W_2O_7)_6]$ solution, 1% $H_8[Si(Mo_2O_7)_6]$ solution, formaldehyde/sulfuric acid, Forrest reagent, Dragendorff reagent, 5% ferric chloride solution, iodoplatinate solution) do not color efavirenz either before or after heating the plates, and also quench the initial fluorescence both at 254 nm and at 365 nm.

The total results of efavirenz visualization on chromatographic plates are presented in Table 1.

Such commonly applied developers as UV-light, Erdmann reagent, Froehde reagent, Liebermann reagent, sulfuric acid, Marquis reagent, Mandelin reagent, acidified iodoplatinate solution, iodine vapor can be used for the detection of efavirenz on chromatographic plates.

The positive results were recorded when developing efavirenz with the reagents used in the analysis of barbituric acid derivatives [14] – the mercuric chloride/diphenylcarbazone reagent and cobalt nitrate/ammonia vapor; the spots of various tints of violet color appeared, and they disappeared when heating the plates; in UV-light the spots were not visualized.

The most of reagents used for detection and identification of substances of basic nature, including the so-called “generally alkaloid reagents”, allows to visualize efavirenz on the TLC-plates. It is colored with the concentrated sulfuric acid, Marquis reagent and the mixture of formaldehyde and the concentrated sulfuric acid, Erdmann reagent, Froehde reagent, Liebermann reagent, Mandelin reagent, acidified iodoplatinate solution.

We also processed efavirenz according to the scheme of TLC-screening of the substances of basic nature. Developing the plates with acidified potassium permanganate solution leads to the formation of brown spots; and after heating the plates the spots are brown. Application of ninhydrin solution in traditional modification for TLC-screening (acidified ninhydrin spray) results in brown spots. Overspraying the plates with FPN reagent and Dragendorff reagent followed by acidified iodoplatinate solution does not change the previous results.

**Table 1**

The results of efavirenz visualization on chromatographic plates

| No. | Reagent | Stationary phase* | Spot color/sensitivity, mg in the sample |
|---|---|---|---|
| 1 | **UV-light** (λ = 254 nm) | A | violet/0.1 |
| | | B | – |
| 2 | **UV-light** (λ = 365 nm) | A, B | – |
| 3 | **formaldehyde vapor** (for 5 min in the covered cell) | A | violet (λ = 254 nm)/0.5<br>yellow (λ = 365 nm)/0.5 |
| | | B | – |
| 4 | **phosphoric acid [14, p. 2464]** pour on | A | light blue (λ = 365 nm)/0.5 |
| | | B | – |
| 5 | **Erdmann reagent [14, p. 485]** pour on | A | bright yellow/0.1 |
| | | B | – |
| 6 | **Froehde reagent [14, p. 478]** pour on | A | pink-brown/0.1 |
| | | B | – |
| 7 | **Liebermann reagent [14, p. 480]** pour on | A, B | bright yellow/0.1 |
| 8 | **glacial acetic acid [14, p. 2463]** pour on | A, B | yellow/0.5 |
| 9 | **mercuric chloride – diphenylcarbazone reagent [14, p. 2463]** spray | A, B | violet/0.5 |
| 10 | **cobalt nitrate** spray, dry<br>**+ ammonia vapor** for 5 min in the covered cell | A, B | light violet/0.5 |
| 11 | **sulfuric acid [14, p. 488]** pour on | A, B | light yellow (15 min at 110°C)/0.5 |
| 12 | **Marquis reagent [14, p. 480]** pour on | A | light yellow/0.5<br>light yellow (15 min at 110°C) / 0.5 |
| 13 | **Iodine vapor** for 5 min in the covered cell | A, B | brown/0.1 |
| 14 | **Mandelin reagent [14, p. 480]** pour on | A | light brown/0.1<br>light brown (15 min at 110°C)/0.1 |
| | | B | pink brown/0.1<br>pink brown (15 min at 110°C) / 0.1 |
| 15 | **formaldehyde vapor** for 5 min in the covered cell<br>**+ Mandelin reagent** pour on **[14, p. 612]** | A | light brown/0.1<br>light brown (15 min at 110°C)/0.1 |
| | | B | pink brown/0.1<br>pink brown (15 min at 110°C)/0.1 |
| 16 | **acidified potassium permanganate solution [14, p. 478]** spray | A, B | brown/0.1 |
| 17 | **acidified ninhydrin spray [14, p. 2464]** spray | A, B | light brown (15 min at 110°C)/0.5 |
| 18 | **+ FPN reagent [14, p. 478]** overspray | A, B | light brown/0.1<br>light brown (15 min at 110°C)/0.1 |
| 19 | **+ Dragendorff reagent [14, p. 476]** overspray | A, B | light brown/0.1<br>light brown (15 min at 110°C)/0.1 |
| 20 | **+ acidified iodoplatinate solution [14, p. 2463]** overspray | A, B | light brown/0.1<br>light brown (15 min at 110°C)/0.1 |
| 21 | **Van Urk reagent (acidified *p*-dimethylaminobenzaldehyde solution in ethanol) [14, p. 476]** spray | A | bright yellow (15 min at 110°C)/0.1 |
| | | B | yellow (15 min at 110°C)/0.1 |
| 22 | **+ 5% ferric chloride solution [14, p. 478]** overspray | A | bright yellow (15 min at 110°C)/0.1 |
| | | B | yellow (15 min at 110°C)/0.1 |
| 23 | **acidified iodoplatinate solution [14, p. 2463]** spray | A, B | dark brown (15 min at 110°C)/0.1 |

Notes: * – A – Sorbfil® PTLC-IIH-UV; B – Merck® TLC Silica gel 60G

Processing the plates directly with iodoplatinate solution does not lead to visible results. Acidified iodoplatinate solution after heating causes the formation of dark brown spots.

Developing efavirenz according to the scheme of TLC-screening of the substances of acid and neutral nature leads to the positive results (yellow spots after heating) with Van Urk reagent. Overspraying the plates with 5% ferric chloride solution does not change the previous results.

The chromatographic mobility of efavirenz was studied in 17 solvents systems (Table 2); the systems 1–9 were used as standard mobile phases according to the recommendations of the International Association of Forensic Toxicologists (TIAFT) for TLC-screening of organic compounds of acid, neutral and basic nature [14]; systems 10–12 were used in the general TLC-screening of organic substances in the Ukrainian forensic toxicological laboratories; systems 13–17 were studied with the purpose of choosing the optimal individual solvents systems for research of efavirenz.

**Table 2**

The $R_f$ values for efavirenz

| Mobile phase | | $R_f$ of efavirenz ($n$ = 3) | |
|---|---|---|---|
| | | Sorbfil® PTLC-PH-UV | Merck® TLC Silica gel 60G |
| 1 | chloroform – acetone (8:2) | 0.65 | 0.67 |
| 2 | ethyl acetate | 0.85 | 0.89 |
| 3 | chloroform – methanol (9:1) | 0.76 | 0.71 |
| 3A* | chloroform – methanol (9:1) | 0.70 | 0.72 |
| 4 | ethyl acetate – methanol – 25% $NH_3$ (85:10:5) | 0.18 | 0.11 |
| 5 | methanol | 0.87 | 0.82 |
| 6 | methanol – *n*-butanol (6:4)** | 0.90 | 0.87 |
| 7 | methanol – 25% $NH_3$ (100:1,5) | 0.84 | 0.89 |
| 7A* | methanol – 25% $NH_3$ (100:1,5) | 0.76 | 0.74 |
| 8 | cyclohexane – toluene – diethylamine (75:15:10) | 0.05 | 0.07 |
| 8A* | cyclohexane – toluene – diethylamine (75:15:10) | 0.00 | 0.00 |
| 9 | acetone | 0.84 | 0.85 |
| 9A* | acetone | 0.90 | 0.92 |
| 10 | chloroform – dioxane – acetone – 25% $NH_3$ (47,5:45:5:2,5) | 0.35 | 0.39 |
| 11 | toluene – acetone – ethanol – 25% $NH_3$ (45:45:7,5:2,5) | 0.82 | 0.85 |
| 12 | chloroform – *n*-butanol – 25% $NH_3$ (70:40:5) | 0.90 | 0.93 |
| 13 | chloroform | 0.00 | 0.00 |
| 14 | chloroform – methanol (1:1) | 0.85 | 0.89 |
| 15 | toluene – $CH_3COOH$ conc. (3:1) | 0.10 | 0.15 |
| 16 | chloroform – methanol – $CH_3COOH$ conc. (90:10:1) | 0.25 | 0.29 |
| 17 | toluene – methanol – $CH_3COOH$ conc. (9:1:1) | 0.85 | 0.83 |

Notes: * – 0.1 M KOH in $CH_3OH$, 110°C, 30 min; ** – 0.1 M NaBr

When using the mobile phases 3A, 7A, 8A, 9A the studies were carried out on the plates processed previously with 0.1 mole/L KOH solution in methanol and then dried at 110°C for 30 min. For the mobile phase 6 application the plates were previously processed with 0.1 mole/L NaBr solution.

The results are presented in Table 2.

## Experimental part

Efavirenz was of pharmacopoeial purity. Chloroform (≥99%, anhydrous, contains 0.5–1.0% of ethanol as a stabilizer), ethyl acetate (99.8%, anhydrous), methanol (≥99.8%, puriss. p.a., ACS reagent), ammonium hydroxide solution (≥25% $NH_3$ in $H_2O$, puriss. p.a. plus) were purchased from Sigma-Aldrich Co. LLC (USA). All other reagents were of analytical grade.

The reference solution 1 (1000 μg/mL) was prepared by dissolving 50.0 mg of efavirenz in methanol; the solution was diluted to 50.0 mL with the same solvent. The reference solution 2 (100 μg/mL) was prepared by diluting 5.00 mL of the reference solutions 1 to 50.0 mL with methanol.

The color reagents were prepared according to [14].

The chromatographic plates Sorbfil® PTLC-IIH-UV (silica gel STC-1HP, PETP, luminophor, silica sol, the fraction of 8÷12 μm, the layer thickness of 100 μm) were purchased from IMID LLC (Russia). The chromatographic plates Merck® TLC SILICA GEL 60 (silica gel 60, glass, gypsum, the fraction of 9.5÷11.5 μm, the layer thickness of 140÷160 μm) were purchased from Merck Group (Germany).

The part of plates was previously processed with 0.1 mole/L potassium hydroxide solution in methanol and then dried at 110°C for 30 min. The part of plates was previously processed with 0.1 mole/L sodium bromide solution [14].

To choose the developing color reagents in 10 mL of the reference solution 1 were applied on the plates of both types, and then the reagents were sprayed or poured onto the plates. The results were fixed visually at once and after drying the plate, then the plates were developed in UV-light with the wavelength of 254 nm and 365 nm. At the next stage the plates were heated for 15 min at 110°C (the plates were covered with a glass plate), and after that colors of spots were fixed in visible and UV-light one more time.

To determine sensitivity of the developing color reagents the same experiments were carried out using 1 and 10 mL of the reference solutions 2 of the substances.

Chromatographing was carried out in cells with the volume of 500 mL; 50 mL of the corresponding TLC-systems were placed into them. The cell was saturated for 30 min. In 10 mL of the reference solutions 1 of the substance to be researched were applied on the start line in the distance of 1 cm from the plate edge. The solvent path length was 8 cm. After reaching the finish line by the mobile phase the plate was taken out from the cell, dried at the ambient temperature and developed with the corresponding reagents.

## Conclusions

The behavior of efavirenz when developing with commonly used colored reagents on TLC plates with two types of a substrate (plastic and glass) and with/without a luminophor (or UV-indicator) has been studied. The results of efavirenz development with reagents used for TLC-screening of organic compounds of acid, neutral and basic nature are presented. The $R_f$ values for efavirenz have been determined using different types of TLC-plates for solvent systems used as standard mobile phases according to the recommendations of the International Association of Forensic Toxicologists for TLC-screening of organic compounds of acid, neutral and basic nature, and also in the general TLC-screening of organic substances in the Ukrainian forensic toxicological laboratories.

**Conflict of interests:** authors have no conflict of interests to declare.

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