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Application of thin layer chromatography in analysis of secnidazole, ornidazole, tinidazole and nimorazole

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Abstract

Secnidazole, ornidazole, tinidazole and nimorazole are the medicines of the group of 5-nitroimidazole derivatives and joint taking these medicines with alcohol leads to the strong intoxication syndrome; therefore they are potential objects of chemical toxicological investigations. The method of thin layer chromatography is widely used in the process of forensic toxicological examinations for screening and confirming investigations. The aim of work is integrated study of visualization conditions of sechnidazole, ornidazole, tinidazole and nimorazole on TLC-plates using standard and particular coloured reagents, and also chromatographic behaviour of the substances using standard mobile phases. To fix the results of visualization of the substances to be analysed some developing modes of TLC-plates with two types of substrate (plastic and glass) and with/without luminophor have been chosen – immediately after processing the substances with a reagent and after drying the plates at the ambient temperature; after heating the plates for 15 minutes at 110°C; in UV-light at two wavelengths – 254 nm and 365 nm – before and after heating. The R_f values of secnidazole, ornidazole, tinidazole and nimorazole in commonly used in forensic toxicological analysis solvents systems using different types of TLC-plates have been set. The individual mobile phases, which ensure optimal separation of the medicines on TLC-plates, have been chosen.

Keywords: secnidazole, ornidazole, tinidazole, nimorazole, thin layer chromatography, colour tests

INTRODUCTION

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 analytes detection and identification respectively [1, 2]. The main focus is the chromatographic behaviour of the substances using standard mobile phases (or solvents systems), as well as the conditions of analytes spots visualization using standard coloured reagents. The data about R_f values in the solvents systems generally accepted in forensic toxicological analysis and spots colouration when developing with generally accepted reagents for medicinal substances, which are potential toxic agents, are published in the well-known guidance «Clarke's...» [1], and this information is regularly updated and supplemented based on the results of researches monitoring. It should be noted that in the latest edition [1] there is only fragmentary information about chromatographic behaviour of the most popular 5-nitroimidazole derivative metronidazole and the results of its visualization on the chromatographic plates using two coloured reagents – methanolic potassium hydroxide solution and acidified potassium permanganate solution. Data about other 5-nitroimidazole derivatives such as sechnidazole, ornidazole, tinidazole and nimorazole are completely absent.

The purpose of our work is integrated study of visualization conditions of sechnidazole, ornidazole, tinidazole and nimorazole on TLC-plates using standard and particular coloured reagents, and also chromatographic behaviour of the substances using standard mobile phases.

MATERIALS AND METHODS

Secnidazole, ornidazole, tinidazole and nimorazole were of pharmacopoeial purity. Chloroform (≥99%, anhydrous, contains 0.5 – 1.0% of ethanol as 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 solutions 1 (1000 μg/mL) were prepared by dissolving 50.0 mg of the respective substance to be analysed (secnidazole, ornidazole, tinidazole or nimorazole) in methanol and the solutions were diluted to 50.0 mL with the same solvent. The reference solutions 2 (100 μg/mL) were prepared by diluting 5.00 mL of the reference solutions 1 to 50.0 mL with methanol.

The rats' blood and urine were extracted using acetonitrile with «salting-out» by ammonium sulphate [3, 4]. Obtained organic extracts were evaporated and dry residues were dissolved in methanol (*blank*-samples) or reference solutions 1 of the substances to be analysed (model samples). All experiments with rats and biological liquids were carried out at Pharmacology Department of National University of Pharmacy.

The colour reagents were prepared according to [1].

Weighing was carried out using digital analytical balance AN100 (AXIS, Ukraine) with d = 0.0001 g.

Glassware satisfied ISO 648:2008 «Laboratory glassware – Single-volume pipettes», ISO 1042:1998 «Laboratory glassware – One-mark volumetric flasks» and calibrated according to ISO 4787:2010 «Laboratory glassware – Volumetric instruments – Methods for testing of capacity and for use» and «Guidelines for calibration in analytical chemistry. Part 2. Multispecies calibration» [5] was used throughout this study.

The chromatographic plates Sorbfil® PTLC-IIH-UV (silica gel STC-1HP, PETP, luminophor, silica sol, 8 ÷ 12 μm fraction, 100 μm layer thickness) were purchased from IMID LLC (Russia). The chromatographic plates Merck® TLC SILICA GEL 60 (silica gel 60, glass, gypsum, 9,5 ÷ 11,5 μm fraction, 140 ÷ 160 μm layer thickness) were purchased from Merck Group (Germany).

The part of plates were 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 were previously processed with 0.1 mole/L sodium bromide solution [1].

To choose the developing colour reagents in 10 μL of reference solutions 1, *blank*-samples and model samples 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 glass plate), and then colours of spots were fixed in visible and UV-light one more time.

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

Chromatographing was carried out in cells with the volume of 500 mL; 50 mL of the respective TLC-systems were placed into them. The cell was saturated for 30 min. In 10 μL of

reference solutions 1 of the substances to be researched were applied on the start line in the distance of 1 cm from the plate edge. The solvents 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 respective reagents.

Results And Discussion

Secnidazole, ornidazole, tinidazole and nimorazole are the medicines of the group of 5-nitroimidazole derivatives and widely used for treatment of infectious diseases [6 – 8]. Administration of 5-nitroimidazole derivatives is accompanied by a number of side effects [9 – 11]. They block the enzymes of alcohol dehydrogenase and acetaldehyde dehydrogenase, therefore when joint taking the medicines with alcohol it is observed the strong intoxication syndrome [12 – 14]. Therefore we can make the conclusion that secnidazole, ornidazole, tinidazole and nimorazole are potential objects of chemical toxicological investigations.

The structural formulae of secnidazole, ornidazole, tinidazole and nimorazole are shown on Figure 1.

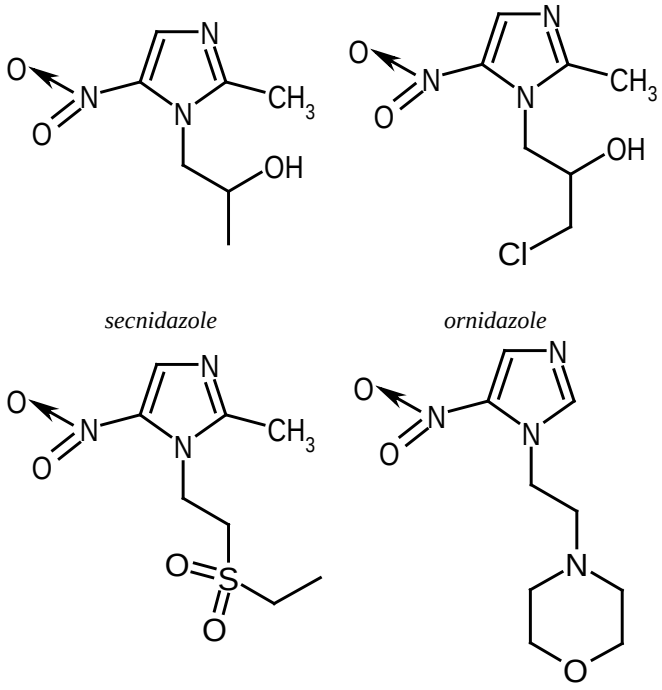

Figure 1. Chemical structures of secnidazole, ornidazole, tinidazole and nimorazole

5-Nitroimidazole derivatives are compounds of amphoteric nature [3, 4, 15] by their properties, therefore when isolating from biological objects they are extracted both in acid and alkaline medium that has been shown experimentally by us [3, 4]. Thus, it is necessary to have information about secnidazole, ornidazole, tinidazole and nimorazole behaviour under TLC-screening conditions used both for substances of basic nature and for substances of acid and neutral nature. And also action of coloured reagents used for making conclusions about the presence/absence of certain pharmacological or chemical groups of substances of acid, neutral and basic nature should be studied.

To fix the results of visualization of the substances to be analysed 4 developing modes of TLC-plates with two types of substrate (plastic and glass) and with/without luminophor (or UV-indicator) have been 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 minutes at 110°C (the plates are covered with glass);

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

The choice of developing modes is due to the following reasons:

- there is no heating of plates after processing with the most of reagents according to the recommendations [1], but in the case of carrying out the researches at high environment temperatures we can see the results of substances visualization, which are not typical for normal conditions; so in our work we have recorded the results of substances colouration under normal conditions and after heating – for all investigated reagents;
- in some cases processing with a reagent may not lead to formation of coloured compounds, but at the same time changes the fluorescence (intensification, change the colouration, quenching) of the substances to be analysed; such signs can become an important differentiating feature; in addition, coloured reaction products may have a characteristic fluorescence that can increase the reliability of detection and identification of the substances to be analysed;
- in preliminary experiments it has been found that in the case of covering the plates with glass when heating the results of spots visualization become more stable, reliable and reproducible;
- the type of substrate of the chromatographic plates can influence the results of the substances visualization, especially when heating; therefore, it is necessary to have information about the visualization of target analytes on both types of widely used chromatographic plates;
- the UV-indicator in the composition of plates stationary phase may also react with the coloured reagents and thereby distort or alter the results of detection of target compounds, therefore, in our work we have recorded the results of processing on plates with and without UV-indicator.

Visualization secnidazole, ornidazole, tinidazole and nimorazole with coloured reagents

It should be noted that all investigated substances fluoresce with violet light at 254 nm on the plates with UV-indicator, and there are brown spots at 365 nm on both types of plates.

A number of studied reagents (Nessler reagent, perchloric acid, phosphoric acid, Erdmann reagent, Froehde reagent, FPN reagent, Liebermann reagent, hydrochloric acid vapour, 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) do not colour secnidazole, ornidazole, tinidazole and nimorazole 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 secnidazole, ornidazole, tinidazole and nimorazole visualization on chromatographic plates are presented in Table 1.

Fuming nitric acid, strong solution of hydrogen peroxide and formaldehyde vapour do not colour the substance on the plates, but change the character of initial fluorescence of analytes or lead to appearance of new fluorescence.

Positive results have been recorded when developing secnidazole, ornidazole, tinidazole and nimorazole with the reagents used in analysis of barbituric acid derivatives – mercuric chloride/diphenylcarbazone reagent and cobalt nitrate/ammonia vapour; the spots of various tints of violet colour appear and they disappear when heating the plates; in UV-light the spots are not visualized.

Table 1 **The results of secnidazole, ornidazole, tinidazole and nimorazole visualization on chromatographic plates**

Reagent	Visualization mode*	Stationary phase**	Substance			
			secnidazole	ornidazole	tinidazole	nimorazole
Forrest reagent [1, p. 478] pour on	1, 2, 4 – 6	A, B	–	–	–	–
	3	A, B	–	–	–	yellow
glacial acetic acid [1, p. 2463] pour on	1 – 3, 5, 6	A, B	–	–	–	–
	4	A	–	–	light brown	brown
		B	–	–	–	yellow
nitric acid, fuming [1, p. 486] pour on	1 – 5	A, B	–	–	–	–
	6	B	–	–	–	–
		A	dark violet	dark violet	violet	light yellow
hydrogen peroxide solution, strong [1, p. 2463] spray	1, 2, 4, 5	A, B	–	–	–	–
	3, 6	A	violet	violet	violet	violet
		B	–	–	–	–
formaldehyde vapour for 5 min. in covered cell	1	A, B	–	–	–	–
	2	A	violet	violet	violet	violet
		B	–	–	–	–
	3	A, B	light brown	light brown	light brown	yellow
mercuric chloride – diphenylcarbazone reagent **[1, p. 2463] spray**	1	A, B	violet	violet	violet	violet
	2 – 6	A, B	–	–	–	–
cobalt nitrate spray, dry + ammonia vapour for 5 min. in covered cell	1	A, B	light violet	light violet	light violet	light violet
	2 – 6	A, B	–	–	–	–
methanolic potassium hydroxide [1, p. 484] spray	1	A, B	pink	light brown	yellow	–
	2	A	light violet	light violet	yellow	light violet
		B	violet	violet	yellow	–
	3	A	yellow	yellow	yellow	–
		B	yellow	yellow	yellow	brown
	4	A	brown	brown	yellow	yellow
		B	brown	brown	yellow	–
	5	A	brown	brown	yellow	brown
		B	light brown	light brown	light brown	–
	6	A	brown	brown	yellow	brown
		B	brown	brown	brown	brown
5 M sodium hydroxide solution pour on	1, 4	A, B	brown	grey brown	yellow	orange
	2, 3, 5, 6	A, B	violet	violet	–	–
sulphuric acid [1, p. 488] pour on	1, 4	A, B	–	–	–	light yellow
	2, 3, 5, 6	A, B	–	–	–	–
Marquis reagent [1, p. 480] pour on	1, 4	A	–	–	–	light yellow
		B	–	–	–	–
	2, 3, 5, 6	A, B	–	–	–	–
formaldehyde/ **sulphuric acid [1, p. 480] pour on**	1, 4	A	–	–	–	light yellow
		B	–	–	–	–
	2, 3, 5, 6	A, B	–	–	–	–
Dragendorff reagent [1, p. 476] spray	1, 4	A	–	–	–	orange
		B	–	–	–	light brown
	2, 3, 5, 6	A, B	–	–	–	–
Iodine vapour for 5 min. in covered cell	1	A, B	light brown	light brown	brown	brown
	2, 3	A, B	–	–	–	–
Mandelin reagent [1, p. 480] pour on	1	A, B	light yellow	light yellow	light yellow	light yellow
	2, 3, 5, 6	A, B	–	–	–	–
formaldehyde vapour for 5 min. in	1	A, B	–	–	–	light yellow

Reagent	Visualization mode*	Stationary phase**	Substance			
			secnidazole	**ornidazole**	**tinidazole**	**nimorazole**
covered cell + Mandelin reagent pour on [p. 612]	2, 3, 5, 6	A, B	–	–	–	–
acidified potassium permanganate solution **[1, p. 478] spray**	1	A, B	light brown	light brown	light brown	light brown
	2, 3, 5, 6	A, B	–	–	–	–
acidified ninhydrin spray **[1, p. 2464] spray**	1, 2	A, B	–	–	–	–
	3	A	–	–	–	–
		B	–	–	–	–
	4	A	–	–	–	crimson, disappear
		B	pink	pink	pink	pink
	5	A, B	–	–	–	–
	6	A	orange	bright orange	orange	–
		B	–	–	–	–
+ FPN reagent [1, p. 478] overspray	1 – 6	A, B	–	–	–	–
+ Dragendorff reagent [1, p. 476] overspray	1, 4	A	–	–	–	orange
		B	–	orange	–	orange
	2, 3, 5, 6	A, B	–	–	–	–
+ acidified iodoplatinate solution [1, p. 2463] overspray	1, 4	A	–	–	–	orange
		B	–	orange	–	orange
	2, 3, 5, 6	A, B	–	–	–	–
0.2% ninhydrin solution in butanol	1	A, B	light pink	light pink	light pink	light yellow
	2	A	brown	brown	–	brown
		B	–	–	–	blue
	3	A	yellow	yellow	orange	yellow
		B	–	–	–	blue
	4	A, B	pink-brown	pink-brown	light pink	red- brown
	5	A, B	–	–	–	–
	6	A	orange	orange	orange	yellow
		B	orange	orange	yellow	yellow
5% ferric chloride solution [1, p. 478] spray	1 – 6	A, B	–	–	–	–
Van Urk reagent (*p*-dimethylaminobenzaldehyde solution in ethanol, acidified) **[1, p. 476] spray**	1 – 3, 5, 6	A, B	–	–	–	–
	4	A, B	–	–	–	pink-orange
+ 5% ferric chloride solution [1, p. 478] overspray	1 – 3, 5, 6	A, B	–	–	–	–
	4	A, B	–	–	–	pink-orange
iodoplatinate solution [1, p. 2463] spray	1, 3 – 6	A, B	–	–	–	–
	2	A	violet	violet	light violet	light violet
		B	–	–	–	–
acidified iodoplatinate solution [1, p. 2463] spray	1 – 3, 5, 6	A, B	–	–	–	–
	4	A	light yellow	light yellow	light yellow	light yellow
		B	dark brown	dark brown	dark brown	dark brown

* 1 – visible, before heating
2 – UV 254 nm, before heating
3 – UV 365 nm, before heating
4 – visible, after heating
5 – UV 254 nm, after heating
6 – UV 365 nm, after heating

** A – Sorbfil® PTLC-PH-UV
B – Merck® TLC Silica gel 60G

Table 2 **R_f values for secnidazole, ornidazole, tinidazole and nimorazole (n = 3)**

№	Mobile phase	Stationary phase*	Substance			
			secnidazole	ornidazole	tinidazole	nimorazole
1	chloroform – acetone (8:2)	A	0.13	0.41	0.18	0.05
		B	0.14	0.44	0.20	0.06
2	ethyl acetate	A	0.23	0.46	0.28	0.09
		B	0.24	0.49	0.33	0.12
3	chloroform – methanol (9:1)	A	0.62	0.67	0.79	0.76
		B	0.64	0.69	0.73	0.72
3A	chloroform – methanol (9:1)	A	0.75	0.75	0.80	0.80
		B	0.72	0.73	0.77	0.78
4	ethyl acetate – methanol – 25% NH_3 (85:10:5)	A	0.80	0.84	0.71	0.70
		B	0.77	0.80	0.68	0.69
5	methanol	A	0.81	0.75	0.79	0.66
		B	0.79	0.73	0.75	0.64
6	methanol – *n*-butanol (6:4)	A	0.75	0.80	0.63	0.55
		B	0.72	0.78	0.64	0.57
7	methanol – 25% NH_3 (100:1,5)	A	0.78	0.80	0.78	0.67
		B	0.75	0.76	0.79	0.64
7A	methanol – 25% NH_3 (100:1,5)	A	0.85	0.85	0.79	0.79
		B	0.82	0.83	0.74	0.76
8	cyclohexane – toluene – diethylamine (75:15:10)	A	0.13	0.13	0.00	0.06
		B	0.11	0.12	0.00	0.01
8A	cyclohexane – toluene – diethylamine (75:15:10)	A	0.13	0.00	0.00	0.06
		B	0.06	0.00	0.00	0.00
9	acetone	A	0.75	0.90	0.78	0.50
		B	0.72	0.92	0.79	0.48
9A	acetone	A	0.84	0.87	0.83	0.74
		B	0.85	0.89	0.86	0.76
10	chloroform – dioxane – acetone – 25% NH_3 (47,5:45:5:2,5)	A	0.65	0.55	0.66	0.53
		B	0.65	0.53	0.68	0.55
11	toluene – acetone – ethanol – 25% NH_3 (45:45:7,5:2,5)	A	0.66	0.66	0.69	0.58
		B	0.68	0.67	0.70	0.55
12	chloroform – *n*-butanol – 25% NH_3 (70:40:5)	A	0.79	0.80	0.84	0.80
		B	0.77	0.81	0.85	0.79
13	chloroform	A	0.00	0.00	0.00	0.00
		B	0.00	0.00	0.00	0.00
14	chloroform – methanol (1:1)	A	0.84	0.79	0.83	0.76
		B	0.81	0.76	0.77	0.72
15	toluene – CH_3COOH conc. (3:1)	A	0.22	0.00	0.21	0.00
		B	0.12	0.00	0.09	0.00
16	chloroform – methanol – CH_3COOH conc. (90:10:1)	A	0.80	0.83	0.78	0.69
		B	0.78	0.81	0.71	0.65
17	toluene – methanol – CH_3COOH conc. (9:1:1)	A	0.31	0.46	0.33	0.10
		B	0.29	0.42	0.30	0.06

* A – Sorbfil® PTLC-PH-UV, B – Merck® TLC Silica gel 60G

The solution of alkali can be used as the specific developing reagent for substances containing nitro group. Detection of 5-nitroimidazole derivatives by processing the plates with alcohol and aqueous solutions of alkalis has been carried out. These developers variably colour the substances to be analysed and change their fluorescence.

The most of reagents used for detection and identification of the substances of basic nature, including so-called "generally alkaloid reagents", allows to visualize on the plates only nimorazole. It is coloured with concentrated sulphuric acid, Marquis reagent and the mixture of formaldehyde and concentrated sulphuric acid, with Dragendorff reagent.

Mandelin reagent colours the spots of all substances in light yellow colour. Modified application of Mandelin reagent – overspray after formaldehyde vapour – does not lead to more distinct results – light yellow colour is fixed only for nimorazole.

The processing of plates with iodine vapour leads to the formation of brown spots for all substances to be analysed.

We also have carried out processing the substance to be analysed according to the scheme of TLC-screening of the substances of basic nature. Developing the plates with acidified potassium permanganate solution leads to formation of light brown spots for all substances; and also after heating the plates the spots are light brown. Application of ninhydrin solution in traditional modification for TLC-screening (acidified ninhydrin spray) results in unstable and unreproducible results. Overspraying the plates with FPN reagent does not lead to visual effects. After overspraying the plates with Dragendorff reagent followed by acidified

iodoplatinate solution only ornidazole and nimorazole coloured in orange colour.

Processing the plates directly with iodoplatinate solution does not lead to visible results. Acidified iodoplatinate solution after heating causes the formation of light yellow spots on plastic plates and dark brown spots on glass plates.

Application of acidified ninhydrin solution in butanol (according the recommendations of Ukrainian forensic toxicological laboratories) leads to formation of clear pink spots immediately after spraying; heating the plates makes spots clear, with brown tint.

Processing the substance to be analysed according to the scheme of TLC-screening of the substances of acid and neutral nature does not give positive results. Spraying the plates with 5% ferric chloride solution does not lead to any visual spots either before or after heating the plates, and also quenches the initial fluorescence at 254 nm and 365 nm. Application of Van Urk reagent (acidified *p*-dimethylaminobenzaldehyde solution in ethanol) colours only nimorazole in pink-orange colour after heating. Overspraying the plates with 5% ferric chloride solution according to the recommendations [1] does not change the results.

Chromatographic mobility of secnidazole, ornidazole, tinidazole and nimorazole

Chromatographic mobility of secnidazole, ornidazole, tinidazole and nimorazole has been studied in 17 solvents systems (Table 2); the systems 1 – 9 are used as standard mobile phases according to TIAFT recommendations for TLC-screening of organic compounds of acid neutral and basic nature [1], systems 10 – 12 are used in the general TLC-screening of organic substances in Ukrainian forensic toxicological laboratories, systems 13 – 17 have been investigated with the purpose of choosing the optimal individual solvents systems for 5-nitroimidazoles study.

When using the mobile phases 3A, 7A, 8A, 9A the investigations 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.

According to the data on R_f values of secnidazole, ornidazole, tinidazole and nimorazole the mobile phases 4 and 6 allow to separate the substances to be analysed.

As non-correlated mobile phases for identification of secnidazole, ornidazole, tinidazole and nimorazole the mobile phase 3 and the mobile phases 4, 6, 9 or 16 can be used; these solvents systems allow to separate the substances to be analysed, and in the solvents system 3 secnidazole has worse mobility than ornidazole, tinidazole and nimorazole, but in the solvents systems 4, 6, 9 and 16 secnidazole has higher R_f values than ornidazole, tinidazole and nimorazole.

CONCLUSIONS

The behaviour of secnidazole, ornidazole, tinidazole and nimorazole when developing on different types of TLC-plates with commonly used coloured reagents has been studied. The R_f values of secnidazole, ornidazole, tinidazole and nimorazole in commonly used in forensic toxicological analysis solvents systems using different types of TLC-plates have been set. The individual mobile phases, which ensure optimal separation of secnidazole, ornidazole, tinidazole and nimorazole on TLC-plates, have been chosen.

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